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Low Specificity of the Nucleoside Transport Mechanism of RPMI 6410 Cells

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SUMMARY

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The toxic effects of various nucleoside antimetabolites toward RPMI 6410 cells in culture were eliminated or reduced when the growth medium contained 5 μM nitrobenzylthioinosine (NBMPR), a potent inhibitor of nucleoside transport. When cells were "protected" in this way from the growth inhibitory effects of trifluorothymidine, arabinosylcytosine or 6-azauridine, the cellular content of these agents was much reduced relative to that of cells cultured in the absence of NBMPR, indicating that NBMPR impairment of nucleoside transport was the basis of the protective effect. It is implicit in these results that the toxic nucleosides against which protection is afforded by NBMPR are "substrates" for the nucleoside transporter. The cytotoxic effects of a number of nucleosides of diverse structure were ameliorated by NBMPR and these compounds were judged to be substrates for the transporter. Because compounds as diverse as showdomycin, 5-azacytidine, sangivamycin and nebularine were permeants by this criterion, it was concluded that the specificity of the nucleoside transport mechanism of RPMI 6410 cells is low with respect to the base portion of nucleosides.

INTRODUCTION

In animal cells, nucleoside-specific transport[1] elements of the plasma membrane mediate the movement of nucleosides across the plasma membrane; simple diffusion may also contribute to some extent to nucleoside fluxes. The existence of "high" K_m, facilitated diffusion transport processes with low specificity toward nucleoside permeants has been demonstrated in several cell types (1–5). For example, Plagemann and co-workers have shown the existence in cultured Novikoff hepatoma cells of a facilitated diffusion mechanism that mediated the permeation of uridine, thymidine and deoxycytidine; K_m values for these permeants at 24° ranged between 70 and 400 μM (5–7). Characterization of such transport processes has depended upon (i) rapid sampling technology that enabled measurement of initial rates of nucleoside uptake, and (ii) the use of cells incapable of ana-

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[1] The passage of nucleosides across the plasma membrane of animal cells is mediated by nucleoside specific transport elements ("transporters") of the plasma membrane; there may be more than one type of transport (transporter) mechanism. The term "transport" refers to the transporter-mediated movement of nucleosides across the plasma membrane and does not include the subsequent metabolic events which befall influent nucleoside molecules. The term "uptake" includes both transport and metabolic events.

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bolizing permeant (erythrocytes, kinase-deficient variants or ATP-depleted cells). The existence of high K_m nucleoside transport processes in animal cells has also been demonstrated by others (8–11). It is recognized that initial rates of nucleoside uptake[1] measure transport rates in cells that metabolize nucleoside permeants (3, 5, 8), a concept that has been demonstrated experimentally (5).

Using transport methods based on measurement of initial rates of permeant uptake, the existence of "low" K_m (about 10 μM and less) mechanisms for the transport of adenosine (10, 12–14) and of uridine (15) have been shown. For example, in HeLa cells the influx of adenosine and uridine is initiated by NBMPR[2]-sensitive (see below) transport processes for which K_m values at 20° were 2.5 and 4.2 μM, respectively (13, 15). Strauss *et al.* (10) have shown the existence in lymphocytes of both high and low K_m mechanisms for adenosine transport, and Kolassa *et al.* (12) have reported a similar finding with erythrocytes. Low K_m values for adenosine transport have also been reported in other studies employing rapid sampling technology (16, 17). The low K_m transport processes have not been characterized as facilitated diffusion processes, nor have specificities been adequately defined. In view of the low concentrations of nucleosides in blood plasma (for example, see 18–20), the low K_m nucleoside transport mechanisms would appear to be of physiological importance. Relationships between the high K_m and low K_m transport processes have not been explored, although in this connection it may be noted that both are profoundly inhibited by NBMPR (2, 6, 13, 15). Thus, more than one type of nucleoside transport mechanism may exist, or the mechanism may exist in more than one form (perhaps kinase-coupled or uncoupled). The term "transport mechanism" is used in this report in the general sense, recognizing that more than one type or form of nucleoside transporter may exist.

NBMPR and various related compounds have been identified as potent and specific inhibitors of uridine permeation in human erythrocytes (2, 21); inhibition of this facilitated diffusion process which, in erythrocytes, proceeds in the absence of permeant metabolism (1), demonstrated clearly inhibition of nucleoside transport by NBMPR and congeners. NBMPR and related compounds are also potent inhibitors of nucleoside transport in various other cell types (13, 16, 22, 23). For example, in HeLa cells, the mediated component of adenosine entry was eliminated in the presence of 5 μM NBMPR, revealing a non-saturable component which might represent simple diffusion (13). HeLa cells possess sites to which NBMPR binds reversibly, but with high affinity (K_{dissoc} about 0.1 nM); NBMPR occupancy of these sites resulted in inhibition of the uptake of various nucleosides (24).

We have previously reported that NBMPR and related compounds protected mouse lymphoblastoid cells in culture from the antiproliferative effects of several cytotoxic nucleoside drugs (25). Such protection implied that the cytotoxic nucleoside entered the cells by the nucleoside transport mechanism. In the present study, we have demonstrated that RPMI 6410 cells, a cultured line of human lymphoblastoid cells (26), were protected by NBMPR from toxic nucleosides of very diverse structure (Fig. 1) and have concluded that these cells possess a NBMPR-inhibited nucleoside transport mechanism of low specificity.

MATERIALS AND METHODS

RPMI 6410 cells were cultured in RPMI 1640 medium supplemented with 10% dialysed fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml); the 25 ml cultures, in loosely capped 50 ml bottles, were incubated at 37° in humidified, 5% CO_2-air. Cell concentrations, initially about 1×10^5 cells/ml, were kept below 5×10^5 cells by dilution with fresh medium, with and without drugs. Cell concentrations were determined with an electronic particle counter.

[2] Abbreviations: NBMPR (nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; NBTGR, 2-amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; HEPES, 2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid. Structural formulae for other compounds are given in Fig. 1.

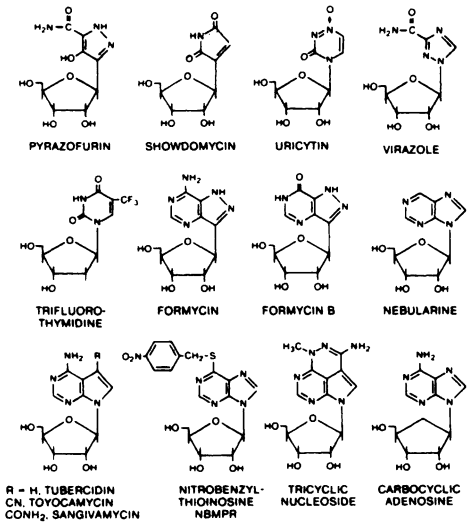

FIG. 1. *Structural formulae*

Cell culture materials were purchased from Grand Island Biological (Calgary, Alberta). Dr. S. R. Naik of this laboratory prepared NBMPR and NBTGR (27) from 6-thioinosine and 6-thioguanosine generously provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Isotopically labeled nucleosides were purchased from Moravek Biochemicals (City of Industry, Calif.).

The time course of nebularine uptake by RPMI 6410 cells was measured by the following procedure[3] which employed NBMPR to end intervals of uptake. Cells from exponentially proliferating cultures were resuspended in serum-free "transport medium" consisting of RPMI 6410 medium which lacked bicarbonate but was supplemented with 20 mM HEPES (pH 7.4). Replicate incubation mixtures were prepared and intervals of uptake were started by the rapid mixing of 0.75 ml of cell suspension (4.5×10^6 cells/ml) with 0.75 ml of transport medium containing 20 μM [G-^{3}H]nebularine (10 μCi/ml); both components were at 4°. To end uptake intervals, a 0.75 ml portion of 15 μM NBMPR in transport medium at 22° was added to each assay mixture and 0.5 ml samples were immediately transferred to 1.5 ml plastic centrifuge tubes containing 0.15 ml of oil[4] and spun for 30 sec in an Eppendorf Model 3200 microcentrifuge to pellet cells under the oil layer. The medium portions of the incubation mixtures were removed by suction and tube portions above the oil were rinsed with 1.0 ml water. For assay of cellular radioactivity by liquid scintillation counting, 0.75 ml of 0.5 M KOH was added with mixing to each centrifuge tube (cells plus oil) and after dissolution of cells, tube contents were transferred to counting vials with rinsing by scintillant (28).

RESULTS AND DISCUSSION

In the present study, the ability of NBMPR to protect proliferating RPMI 6410 cells against cytotoxic nucleosides was used to explore the permeant specificity of the NBMPR-sensitive nucleoside transport mechanism. The following rationale was employed: (i) cytotoxicity would signal that cellular uptake of the toxic agent had taken place, (ii) NBMPR protection against that toxicity would indicate that transport (the initial step of the uptake process) of the agent was inhibited, and (iii) protection would imply that the agent was a substrate for the transport mechanism. This rationale depends upon the sensitivity of nucleoside transport in RPMI 6410 cells to inhibition by NBMPR[5]. The inhibition of nucleoside transport by NBMPR has been demonstrated in a variety of cell types and, accordingly, the generality of the inhibition is not at issue. Previous reports have demonstrated that NBMPR occupancy of specific, high-affinity binding sites for NBMPR on cells resulted in inhibition of nucleoside transport (6, 24, 30). The experiments of Fig. 2 and 3 demonstrated, respectively, that (i) RPMI 6410 cells possess such high affinity binding sites for NBMPR and (ii) NBMPR inhibited transport of the toxic nucleoside nebularine by RPMI 6410 cells.

[3] E. R. Harley, E. Y. Lau, S. Yang, C. E. Cass and A. R. P. Paterson, unpublished results.

[4] Dow Corning 550 silicone oil (84 volumes) plus light paraffin oil (Fisher 0-119, 16 volumes); final specific gravity, 1.03 g/ml at 22°.

[5] Another possibility, that NBMPR protection effects might derive from blockade of kinase activities which initiate anabolism of the toxic nucleoside, is unlikely in view of other reports (15, 16, 29).

The experiment of Fig. 2 measured the site-specific binding of [G-^{3}H]NBMPR by RPMI 6410 cells at 37° using a previously established method (24). Mass law analysis of the binding data by the method of Scatchard (31) indicated that RPMI 6410 cells possess about 3.3 × 10^5 binding sites per cell and that the NBMPR dissociation constant at these sites was 0.48 nM (Fig. 1B). The characteristics of high affinity NBMPR binding by RPMI 6410 cells and HeLa cells (24) are similar.

In the experiment of Fig. 3, the uptake of [G-^{3}H]nebularine by RPMI 6410 cells during intervals of a few seconds was measured by a method employing (i) NBMPR as a rapid stopping agent and (ii) rapid separation of cells from permeant-containing medium by centrifugal pelleting under an oil layer. It is seen that the time course of nebularine uptake was linear and intersected at the ordinate with the time course for uptake by cells pretreated with the transport inhibitor. It is apparent that the method measured the initial rate of nebularine uptake and that NBMPR virtually eliminated nebularine uptake.

These results (Figs. 2 and 3), taken together with the established role of NBMPR as a potent, tightly bound inhibitor of nucleoside transport, indicate that NBMPR inhibited nucleoside transport in RPMI 6410 cells.

Protection of RPMI 6410 cells by NBMPR against toyocamycin toxicity is illustrated in Fig. 4; in cultures containing 15 nM toyocamycin, cell numbers diminished, but in the presence of 5 μM NBMPR and 15 nM toyocamycin, the cell proliferation rate was 90% of that in the absence of additives. The cell proliferation rate was not changed by the presence of 20 μM NBMPR in the growth medium (data not shown). Because of the foregoing arguments, inhibition of toyocamycin transport by NBMPR was the apparent basis of the protection demonstrated. This conclusion was supported by the data of Table 1 which are from an experiment in which RPMI 6410 cells were cultured in medium containing toxic concentrations of ^{3}H-labeled nucleoside drugs (trifluorothymidine, 6-azauridine and arabinosylcytosine) with and without 5 μM NBMPR. Cell proliferation in the presence of NBMPR was associated

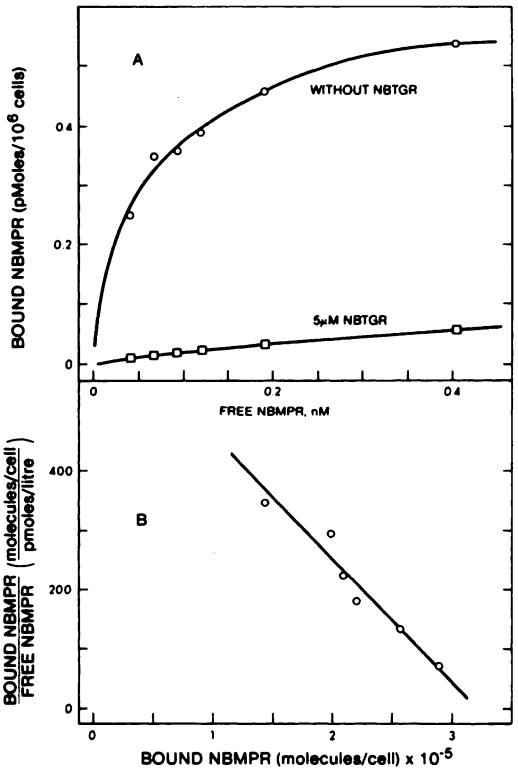

FIG. 2. *The presence of high affinity binding sites for NBMPR on RPMI 6410 cells*

Preliminary experiments established that the time course of [G-^{3}H]NBMPR binding to RPMI 6410 cells at the lowest initial NBMPR concentration to be employed (0.5 nM) was complete in 10 min; thus, cell-bound NBMPR was in equilibrium with free NBMPR in the medium after this interval. Incubation mixtures contained 1.5 × 10^6 cells in 6.0 ml of serum-free RPMI 1640 medium with or without 5 μM NBTGR; these mixtures, prepared in duplicate and containing graded concentrations of [G-^{3}H]NBMPR, were incubated at 37° for 10 min. Cell pellets from the incubation mixtures were dissolved in alkali and assayed for [G-^{3}H]NBMPR content by liquid scintillation counting (28); the medium fraction of each incubation mixture was assayed under the same conditions for [G-^{3}H]NBMPR content. Specifically-bound NBMPR was determined as the difference between the cellular content of [G-^{3}H]NBMPR in the presence and absence of 5 μM NBTGR (Panel A) and mass law analysis of these data by the method of Scatchard (31) (Panel B) gave these constants: number of sites per cell, 3.3 × 10^5; K_{dissoc}, 0.48 nM.

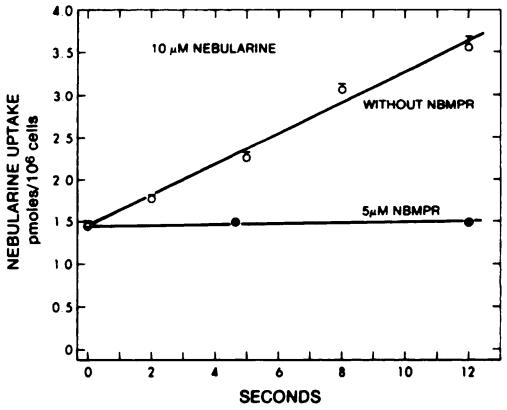

FIG. 3. *NBMPR inhibition of nebularine transport by RPMI 6410 cells*

Cellular uptake of [G-^{3}H]NBMPR during the intervals indicated was determined as described in detail in MATERIALS AND METHODS. Intervals of uptake were started by the rapid mixing of equal volumes of cell suspension and 10 μM [G-^{3}H]-nebularine in transport medium, both at 4°. Intervals of uptake were ended by the rapid addition of NBMPR (final concentration 5 μM). Cells from triplicate samples of the incubation mixture were immediately pelleted under oil and assayed for ^{3}H content. In some incubation mixtures (●), cells were exposed to 5 μM NBMPR for 5 min prior to the uptake assay. Error bars indicate average deviation from the mean.

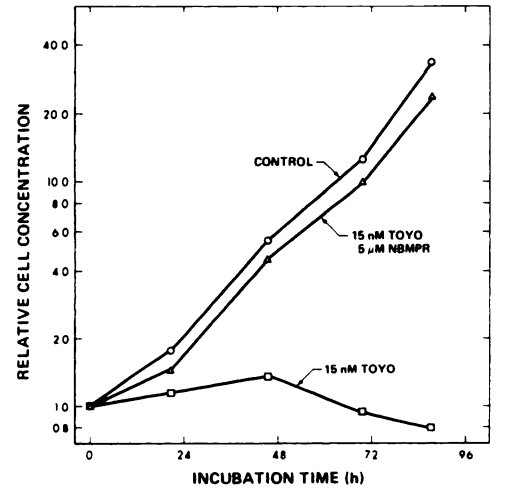

FIG. 4. *Protection of RPMI 6410 cells against toyocamycin toxicity*

RPMI 6410 cells were cultured in the presence of 15 nM toyocamycin (Toyo) with and without 5 μM NBMPR, or without additives (control).

with low cellular concentrations of the toxic drugs throughout the period of culture. Conversely, without NBMPR protection, cell proliferation was inhibited and cellular concentrations of the toxic drugs were several times higher than in the presence of NBMPR. Similar results with nebularine have been reported (32).

The data of Table 2 also support the conclusion that NBMPR protection of cultured cells against cytotoxic nucleosides derived from inhibition by NBMPR of the transport of these agents. NBMPR protected RPMI 6410 cells against toxic concentrations of several nucleosides, but not against toxic concentrations of the corresponding aglycones. Table 2 presents data for only a single concentration of each

TABLE 1

Correlation between NBMPR inhibition of drug uptake by RPMI 6410 cells and protection against drug cytotoxicity

Cells were cultured in medium containing the specified concentrations of [G-^{3}H]trifluorothymidine, [5-^{3}H]6-azauridine or [5,6-^{3}H]arabinosylcytosine, with or without 5 μM NBMPR. At the times specified, samples were taken for determination of cell concentration and known numbers of cells were collected on 0.45 μ nitrocellulose filters. The filters were washed with cold 0.15 M NaCl containing non-isotopic drug at 10 × the concentrations listed, and then were assayed for ^{3}H content by a combustion-liquid scintillation procedure employing a Packard Model 306 Sample Oxidizer.

Drug	Hours of culture	Drug uptake (pmoles/10^6 cells) in NBMPR at these concentrations:		Relative cell number in NBMPR at these concentrations:	
		0 μM	5 μM	0 μM	5 μM
Trifluorothymidine	0	8	8	1.0	1.0
(1 μM)	22	154	44	0.98	1.30
	28	255	36	0.95	1.90
	46	369	25	1.04	3.80
	51	352	18	1.01	5.46
6-Azauridine (3 μM)	0	4	4	1.0	1.0
	22	21	12	1.15	1.22
	28	22	8	1.20	1.91
	46	17	9	1.28	3.76
	51	22	8	1.35	4.96
Arabinosylcytosine	0	1	2	1.0	1.0
(1 μM)	22	11	6	0.91	1.14
	28	15	6	0.95	1.49
	46	25	6	0.93	2.61
	51	23	6	1.06	3.42

TABLE 2

NBMPR failure to protect RPMI 6410 cells against cytoxic nucleobases

RPMI 6410 cells were cultured in medium containing the specified concentrations of the agents listed, with and without 5 μM NBMPR. Cell concentrations were determined daily and proliferation rates (population doublings in 72 hr) are expressed as percentages of those in control cultures (no additives); control rates were between 3.25 and 3.95 doublings in 72 hr.

Cytotoxic agent			Cell proliferation rate (% of control)	
Conc.	Name	Source[a]	−NBMPR	+NBMPR
μM				
50[b]	2,6-Diaminopurine	A	−14	−24
100	2,6-Diaminopurine riboside	B	29	65
100	2,6-Diaminopurine deoxyriboside	A	51	78
100[b]	Purine	A	42	44
1	Purine riboside (nebularine)	A	9	88
10[b]	6-Methylpurine	A	14	17
0.2	6-Methylpurine riboside	B	−13	97
2[b]	5-Fluorouracil	A	−24	−21
0.02	5-Fluorouridine	A	62	95
0.005	5-Fluorodeoxyuridine	A	29	74

[a] Sources: A, commercial; B, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.

[b] Tested with and without 5 μM NBMPR at various concentrations that produced graded inhibitions of cell proliferation over a range that included 50% of control rates; inhibition was not diminished in the presence of NBMPR.

agent; however, in each instance, a range of concentrations was tested (data not shown). Since these concentrations were not selected to maximize differences between cell proliferation in the presence and absence of NBMPR, the apparent differences between the NBMPR protection against different agents (e.g., nebularine and 5-fluorouridine, Table 2) may pertain only to the experimental conditions specified. Such differences would be diminished by (a) diffusional entry of the toxic nucleoside into cells and (b) cleavage of the toxic nucleoside during the long interval of culture with release into the medium of a toxic aglycone against which NBMPR would not afford protection. Such possibilities might account for the lesser protection by NBMPR against the nucleosides of 2,6-diaminopurine and 5-fluorouracil relative to that against nebularine and 6-methylpurine riboside (Table 2).

Of the many nucleoside analogues that have been synthesized and tested as potential therapeutic agents, a number are toxic to cultured cells and animals. The structural diversity of the toxic nucleoside analogues has afforded a means of examining the permeant specificity of the nucleoside transport mechanism. To this end, we have applied the criterion, discussed above, that NBMPR protection of proliferating cells from otherwise toxic concentrations of a particular nucleoside implies that entry of that nucleoside is mediated by the nucleoside transport mechanism. Table 3 summarizes experiments in which a variety of toxic nucleosides were tested in this manner. A generalization which emerges from these data is that the NBMPR-sensitive nucleoside transport mechanism of RPMI 6410 cells is of low specificity with respect to the aglycone portion of its substrates: structures as diverse as showdomycin, 5-azacytidine, sangivamycin, and Townsend's "tricyclic nucleoside" (33) (Fig. 1) are substrates for the nucleoside transport mechanism by the "NBMPR protection" criterion. This apparent low specificity of the transport mechanism for the nucleoside base supports an earlier conclusion by Taube and Berlin (14) that flexibility of the adenosine transport mechanism of rabbit polymorphonuclear leukocytes enabled a variety of purine and pyrimidine nucleosides to be accommodated as permeants. The several examples in Table 3 of the absence of NBMPR protection against toxic nucleosides are noteworthy. Entry of 8-azainosine, 3-deazacytidine and 5-iododeoxycytidine into the cultured cells would not appear to be mediated by the NBMPR-sensitive transport mechanism.

Our interpretation of NBMPR protection

TABLE 3

NBMPR protection of RPMI 6410 cells against various cytotoxic nucleosides

RPMI 6410 cells were cultured in medium containing various concentrations of the agents listed in the presence and absence of 5 μM NBMPR. Cell concentrations were determined daily and proliferation rates (population doublings in 72 hr) are expressed as percentages of those in control cultures (no additives). Data selected to illustrate NBMPR protection against toxicity are presented.

Cytotoxic agent			Cell proliferation rate (% of control[a])	
Conc.	Name	Source[b]	−NBMPR	+NBMPR
μM				
1	2-Azaadenosine	C	−13	98
3	8-Azaadenosine	C	21	91
12	5-Azacytidine	A	1	62
100	8-Azainosine	C	6	6
3	6-Azauridine	A	−5	58
5	Carbocyclic adenosine	D	−10	84
100	3-Deazacytidine	E	73	75
100	5-Iododexycytidine	A	73	87
0.5	2-Fluoroadenosine	C	30	85
20	2′-Fluoro-2′-deoxycytidine	F	40	59
6	Formycin	G	−18	98
100	Formycin[b]	G	−14	31
0.15	Pyrazofurin	H	52	84
0.01	Sangivamycin	I	−13	87
75	Showdomycin	A	−25	97
0.015	Toyocamycin	I	3.5	90
0.01	Tricyclic nucleoside	B	−1	98
0.1	Tubercidin	A	−27	88
80	Uricytin	J	24	70
100	Ribavirin	K	37	92
100	Xylosyladenine	F	69	92

[a] Proliferation rates in the absence of additives (control) were between 3.62 and 3.97 cell population doublings in 72 hr.

[b] Sources: A, commercial; B, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.; C, J. A. Montgomery, Southern Research Institute, Birmingham, Ala.; D, Y. F. Shealy, Southern Research Institute, Birmingham, Ala.; E, A. Bloch, Roswell Park Memorial Institute, Buffalo, N. Y.; F, G. A. LePage, University of Alberta, Edmonton, Alberta; G, H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan; H, M. J. Sweeney, Eli Lilly and Co., Indianapolis, Ind., I, L. B. Townsend, University of Utah, Salt Lake City, Utah; J, T. A. Khwaja, LAC/USC Cancer Center, Los Angeles, Calif.; K, ICN Pharmaceuticals, Inc., Irvine, Calif.

of the cultured cells against toxic nucleosides is that NBMPR impairment of the nucleoside transport mechanism prevented manifestation of cytotoxicity through reduction in rates of cellular uptake of the toxic agents. Although blockade by NBMPR of the transport of a variety of nucleosides, both natural and synthetic, does not necessarily imply that a single type of transport mechanism mediates the entry of all, it is apparent that transportability of a nucleoside analogue, that is, acceptability as a substrate of the nucleoside transporter, is a determinant of cytotoxicity. Protection of mice by NBMPR against otherwise lethal doses of several individual toxic nucleosides, (nebularine, tubercidin and toyocamycin (32)), and against either component of a 3-deazauridine-arabinosylcytosine combination (34) has also been demonstrated in this laboratory, suggesting that NBMPR and related inhibitors of nucleoside transport may afford a means of altering the disposition in the body of nucleoside analogues employed as therapeutic agents and, perhaps as well, that of endogenous nucleosides.

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