Cancer Chemother Pharmacol (2000) 46: 394–402

ORIGINAL ARTICLE

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Structure-inhibitory profiles of nucleosides for the human intestinal N1 and N2 Na^+-nucleoside transporters

Received: 16 August 1999 / Accepted: 24 May 2000

Abstract *Purpose*: To determine the structure-inhibitory profiles of nucleosides for the N1 and N2 Na^+-nucleoside transporters of the human intestine. *Methods*: The uptake of ^{3}H-labeled prototypic substrates of the N1 (inosine) and N2 (thymidine) transporters into human intestinal brush border membrane vesicles was measured by a rapid filtration technique in the presence and absence of various uridine and adenosine analogs and antiviral and anticancer nucleoside drugs (100 and 1000 μM). *Results*: In the ribose ring, the 3′-oxygen is required for inhibition of uptake of nucleosides by both the N1 and N2 transporters. The structural requirements for such inhibition differ with respect to modifications on the 5′ position of the sugar ring or on the base. The N2 transporter is more tolerant to these substitutions than is the N1 transporter. The 6 position on uracil and the 8 position on adenine are critical for inhibition of uptake of nucleosides by both the N1 and N2 nucleoside transporters. *Conclusions*: These data are the first evidence that the binding site(s) of the human N1 and N2 transporters differ in their interaction with analogs of their common substrates, uridine and adenosine. Such studies can provide insight into the critical structural determinants of the substrate necessary for recognition by the Na^+-nucleoside transporters of the human intestine.

Key words Human intestine · hCNT1 and hCNT2-Na^+-nucleoside transporters · N1 (*cif*) and N2 (*cit*) Na^+-nucleoside transporters · Nucleoside drugs · Antiviral drugs · Anticancer drugs · Drug absorption

Introduction

Nucleoside permeation across cell membranes is a complex process mediated by multiple transporters. These transporters can be classified into two broad categories: the Na^+-dependent concentrative (energy-requiring) transporters and the Na^+-independent equilibrative (or facilitative) transporters. Five principal Na^+-dependent nucleoside transporters have been identified through differential inhibition characteristics and substrate selectivity of uptake by whole cells and by membrane vesicles isolated from various organs [6]. The N1 (*cif*) transporter is generally purine specific; inosine or guanosine serve as model substrates. The N2 (*cit*) transporter is pyrimidine specific; thymidine serves as a model substrate. Both the N1 and N2 transporters transport uridine and adenosine [13]. The N3 (*cib*) transporter has broad specificity and accepts both purines and pyrimidines, including uridine and adenosine [14]. The N4 transporter, expressed in the human kidney, is identical to the N2 transporter in terms of substrate specificity, except that the purine guanosine is also a substrate [7]. The guanosine-specific transporter (*csg*), expressed in acute promyelocytic leukemia cells, differs from other Na^+-dependent transporters in that it is sensitive to inhibition by nitrobenzylthioinosine [5].

We have shown that both N1 and N2 Na^+-dependent nucleoside transporters are present in the brush border membrane of the human jejunum [3, 9] and along the entire length of the small intestine [8]. The highest nucleoside transporter activity in the intestine is usually found in the jejunum [9], a primary site of absorption of most drugs administered orally. Since these transporters are likely to be important in the absorption of nucleoside

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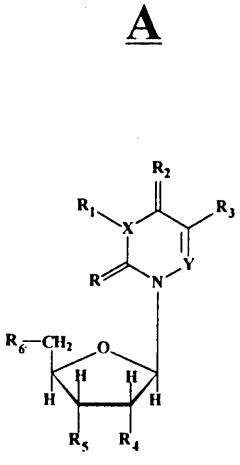
drugs, their simultaneous expression in the brush border membrane of the human intestine provides us with a unique opportunity to study the structural requirements of nucleosides necessary for high-affinity interaction with these transporters. To accomplish this goal, the uptake affinity (K_m) of these transporters for a large array of radiolabeled nucleosides, or the inhibitory capacities (K_i) of these nucleosides toward these transporters must be determined. However, the vast array of nucleoside molecules needed to be studied and the lack of availability of radiolabeled nucleosides limit this approach. To overcome these shortcomings, we have chosen to gain insight into the critical structural determinants required for high affinity nucleoside-transporter interaction by using an indirect method, namely the determination of structure-inhibitory profiles. In this manuscript, we report the structure-inhibitory profiles of a large number of nucleoside (uridine and adenosine) analogs, each representing a substitution on the base, the sugar, or both (Fig 1), for the human intestinal N1 and N2 Na^+-nucleoside transporters.

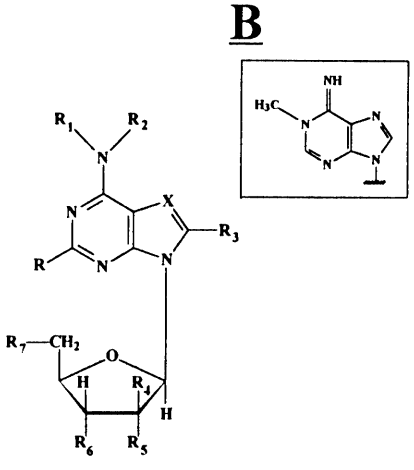
Materials and methods

Materials

[Methyl-^{3}H]-thymidine (20 Ci/mmol), [2,8-^{3}H]-inosine (40 Ci/mmol), [6-^{3}H]-2′-deoxyuridine, [6-^{3}H]-5-fluorouridine, [6-^{3}H]-idoxuridine, [6-^{3}H]-floxuridine, and [8-^{3}H]-2-Cl-adenosine were purchased from Moravek Biochemicals (Brea, Calif., USA). Antiviral agents were kindly supplied by their respective manufacturers: zidovudine (AZT; 3′-azido-3′-deoxythymidine) and lamivudine (3TC; 3′-thia-2′,3′-dideoxycytidine), Glaxo Wellcome (Research Triangle Park, N.C., USA); didanosine (ddI; 2′,3′-dideoxyinosine) and stavudine (d4T; 2′,3′-dideoxydidehydrothymidine), Bristol-Myers Squibb (Princeton, N.J., USA); zalcitabine (ddC; 2′,3′-dideoxycytidine), Hoffman-La Roche (Nutley, N.J., USA); ribavirin (1-β-D-ribofuranosyl-1 H-1,2,4-triazole-3-carboxamide), Schering-Plough Research Institute (Kenilworth, N.J., USA). The sodium salt of acyclovir (9[(2-hydroxyethoxy)methyl]guanine; Zovirax sterile powder; Glaxo Wellcome) was available commercially. All other nucleoside analogs, valinomycin and phloridzin were purchased from Sigma Chemicals (St. Louis, Mo., USA). All other chemicals were of the highest analytical grade available.

Fig. 1 Analogs of uridine (**A**) and adenosine (**B**) modified on the base, sugar, or both, used in the experiments depicted in Figs. 2, 3, 4





Compound	X	Y	R	R_1	R_2	R_3	R_4	R_5	R_6
Uridine	N	C	O	H	O	H	OH	OH	OH
Base modification									
3-Methyluridine	N	C	O	CH_3	O	H	OH	OH	OH
3-Deazauridine	C	C	O	H	O	H	OH	OH	OH
4-Thiouridine	N	C	O	H	S	H	OH	OH	OH
5-Fluorouridine	N	C	O	H	O	F	OH	OH	OH
5-Bromouridine	N	C	O	H	O	Br	OH	OH	OH
5-Iodouridine	N	C	O	H	O	I	OH	OH	OH
6-Azauridine	N	N	O	H	O	H	OH	OH	OH
Sugar modification									
2'-Deoxyuridine	N	C	O	H	O	H	H	OH	OH
3'-Deoxyuridine	N	C	O	H	O	H	OH	H	OH
2',3'-Dideoxyuridine	N	C	O	H	O	H	H	H	OH
Base and sugar modification									
5-Fluoro-2'-deoxyuridine [Floxuridine]	N	C	O	H	O	F	H	OH	OH
5-Fluoro-5'-deoxyuridine [5'dFUrd]	N	C	O	H	O	F	OH	OH	H
5-Iodo-2'-deoxyuridine [Idoxuridine]	N	C	O	H	O	I	H	OH	OH
5-(Trifluoromethyl)-2'-deoxyuridine [Trifluridine]	N	C	O	H	O	CF_3	H	OH	OH
5-(2-Bromovinyl)-2'-deoxyuridine [Brivudin]	N	C	O	H	O	CH=CHBr	H	OH	OH

Compound	X	R	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Adenosine	N	H	H	H	H	H	OH	OH	OH
Base modification									
1-Methyladenosine	(see inset)								
2-Chloroadenosine	N	Cl	H	H	H	H	OH	OH	OH
N_6-Methyladenosine	N	H	CH_3	H	H	H	OH	OH	OH
N_6, N_6-Dimethyladenosine	N	H	CH_3	CH_3	H	H	OH	OH	OH
7-Deazaadenosine	C	H	H	H	H	H	OH	OH	OH
8-Bromoadenosine	N	H	H	H	Br	H	OH	OH	OH
Sugar modification									
5'-deoxyadenosine	N	H	H	H	H	H	OH	OH	H
9-β-D-Arabinofuranosyladenine [Vidarabine]	N	H	H	H	H	OH	H	OH	OH
Base and sugar modification									
2-Chloro-2'-deoxyadenosine [Cladribine]	N	Cl	H	H	H	H	H	OH	OH
2-Fluoro-9-β-D-arabinofuranosyladenine [Fludarabine]	N	F	H	H	H	OH	H	OH	OH

Preparation of brush border membrane vesicles (BBMV) and uptake studies

Human small intestines (ligament of Treitz to the cecum) were obtained from breathing adult organ donors (victims of vehicular or cerebrovascular accidents in otherwise good health) of both sexes and BBMV from the small intestine were prepared as described previously [9]. Vesicles were suspended in 50 mM HEPES-TRIS buffer (pH 7.4), 0.1 mM $MgSO_4$, and 225 mM KCl. The purity of membrane vesicles was routinely monitored (alkaline phosphatase activity of the vesicles was enriched 15-fold and Na^+-K^+-ATPase activity was diminished ca. 50% with respect to the starting homogenate). Preliminary studies established that membrane vesicles were free of contamination by functional basolateral membranes; glucose uptake was completely inhibited by phloridzin, a specific inhibitor of the brush border Na^+-glucose transport activity. In addition, the stability of the BBMV was evaluated by measuring the initial rates of 4 μM D-glucose uptake every 2 h over a 6-h period. The initial rate of D-glucose transport remained constant for at least 6 h following their preparation. All experiments were performed within this time frame.

Uptake of ^{3}H-labeled inosine (0.5 μM), thymidine (1 μM), 2′-deoxyuridine (0.5 μM), 5-fluorouridine (0.5 μM), idoxuridine (0.5 μM), floxuridine (0.5 μM), or 2-Cl-adenosine (0.5 μM) into human intestinal BBMV were measured in the presence of a Na^+-gradient (150 mM, out > in) at room temperature as described before [9]. Briefly, incubation was initiated by mixing 10 μl BBMV with 40 μl incubation medium which contained (final concentration) various nucleosides or their analogs (100 or 1000 μM), 50 mM HEPES-TRIS buffer (pH 7.4), 0.1 mM $MgSO_4$, and 150 mM NaCl plus 75 mM KCl. All experiments were conducted under voltage-clamped conditions using 3 μM valinomycin. Termination of uptake assay was carried out at 10 s by the rapid filtration technique. The resulting uptake values in the presence of the various nucleoside inhibitors were expressed as a percent of the uptake obtained in the absence of these inhibitors. Where solvents were used to dissolve the nucleosides (maximal final concentration in the incubation media of 10 mM HCl, 10 mM NaOH, 10 mM NH_4OH, or 0.5% DMSO), the uptake values were expressed as percent of those observed in the presence of the solvents but in the absence of the inhibitor. None of the solvents used inhibited the uptake of the tracer nucleoside by more than 25%. When passive diffusion of ^{3}H-labeled nucleoside into the intestinal BBMV was evaluated, the uptake of the tracer was measured by substituting 150 mM NaCl with 150 mM KCl in the incubation media. Experiments were conducted with pooled membrane vesicles isolated from at least three different intestines. The data in each figure are expressed as the mean ± SD of triplicate measurements and are representative of experiments conducted on two different batches of pooled vesicles. To determine if a nucleoside analog significantly ($P < 0.05$) inhibited the uptake of either ^{3}H-inosine or ^{3}H-thymidine, the data were analyzed using an analysis of variance (ANOVA). If a significant F value was found, the Student's unpaired t-test was used to compare the differences in inhibition of the uptake of tracer nucleosides by the various nucleosides or their analogs.

Results

The inhibition of the uptake of radiolabeled inosine (0.5 μM) and thymidine (1 μM) by intestinal BBMV in the presence of uridine or adenosine analogs, antiviral drugs, or anticancer drugs are shown in Figs. 2, 3, 4. Except for 2-chloroadenosine (1 μM), the uptake of tritiated 2′-deoxyuridine (1 μM), 5-fluorouridine (1 μM), floxuridine (1 μM), and idoxuridine (1 μM) by the intestinal BBMV showed a transient overshoot phenomenon only in the presence of a 150-mM Na^+-gradient (out > in). For brevity, the time-courses of uptake of only idoxuridine and 2-chloroadenosine are shown in Fig 5. The inhibition by 100 μM thymidine or inosine of the uptake of these radiolabeled nucleoside analogs by intestinal BBMV is presented in Table 1.

Discussion

Due to the large array of nucleoside analogs investigated and since most of the analogs studied are not available

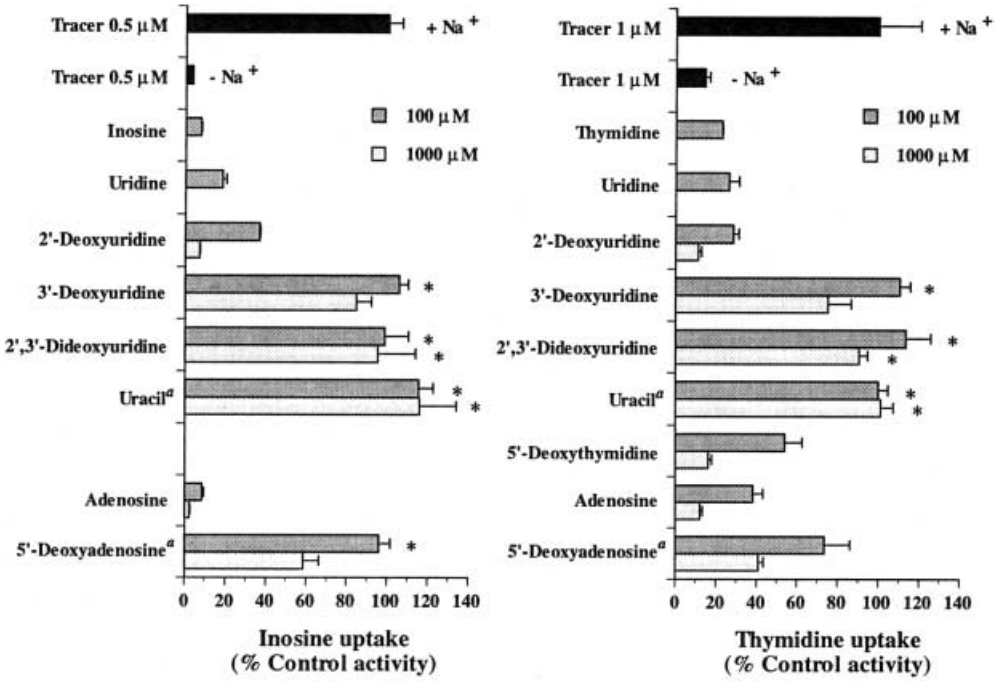


Fig. 2 Na^+-nucleoside uptake of ^{3}H-inosine (0.5 μM) and ^{3}H-thymidine (1 μM) by the intestinal brush border membrane vesicles (BBMV) in the absence and presence of 100 and 1000 μM of various sugar-modified nucleoside analogs. Except for those marked with an *asterisk*, all analogs significantly ($P < 0.05$) affected the uptake of the tracer nucleosides. The following solvents were used to solubilize the nucleoside analogs (1000 μM): *a* NaOH 2 mM. Solvent concentrations were ten times lower when 100 μM of the nucleoside analogs was used

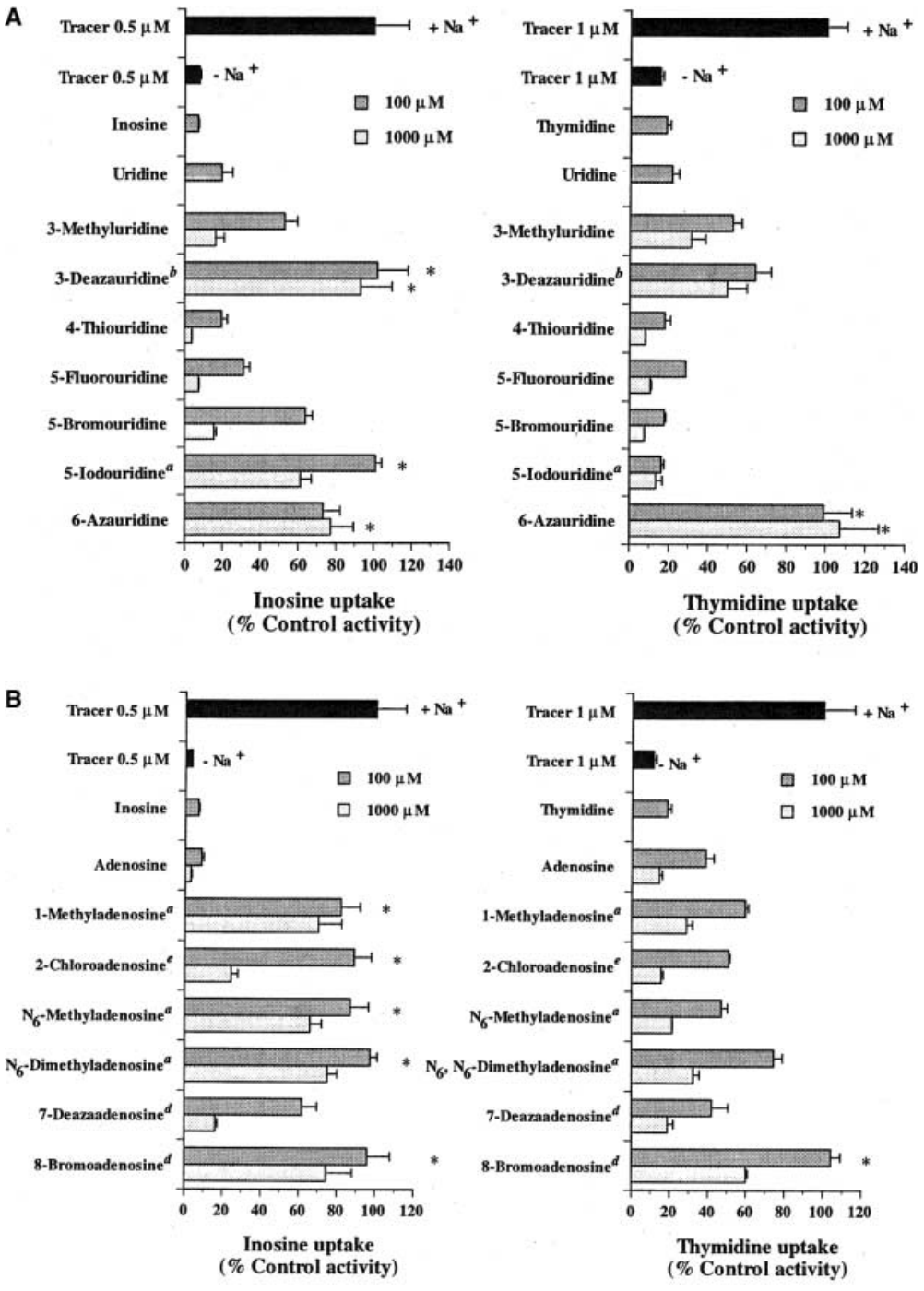


Fig. 3 Na^+-nucleoside uptake of ^{3}H-inosine (0.5 μM) and ^{3}H-thymidine (1 μM) by the intestinal BBMV in the absence and presence of 100 and 1000 μM of various base-modified uridine (**A**) and adenosine (**B**) analogs. Except for those marked with an *asterisk*, all analogs significantly ($P < 0.05$) affected the uptake of the tracer nucleosides. The following solvents were used to solubilize the nucleoside analogs (1000 μM): *a* NaOH 2 mM, *b* NaOH 10 mM, *d* HCl 10 mM, *e* DMSO 0.2%. Solvent concentrations were ten times lower when 100 μM of the nucleoside analogs was used

in the radiolabeled form, it was not feasible to determine the inhibitory potency (K_i) or the Michaelis-Menten constant (K_m) of their uptake. Instead, to gain insight into the similarities and differences in the nucleoside bindings sites of the N1 and N2 transporters, we adopted a method to determine the inhibitory profiles of uridine and adenosine analogs (Fig. 1) on the uptake of tracer substrates (thymidine or inosine) of the N1 and N2 transporters [9]. The analogs chosen represented systematic modifications on either the ribose moiety, the nucleobase moiety, or both. In addition, we also investigated the inhibitory profiles of various purine and pyrimidine anticancer and antiviral drugs for the intestinal N1 and N2 nucleoside transporters. The inhibition at both a low (100 μM) and a high concentration (1000 μM) of each nucleoside analog was studied. The former concentration is approximately ten times greater than the affinity constant (K_m) for transport of either uridine or adenosine while the latter is likely representative of the nucleoside concentration in the small intestine if the analog were to be administered orally. Thus, if an analog has an affinity to the transporter comparable to that of uridine or adenosine, the degree of inhibition of uptake of these tracer substrates by

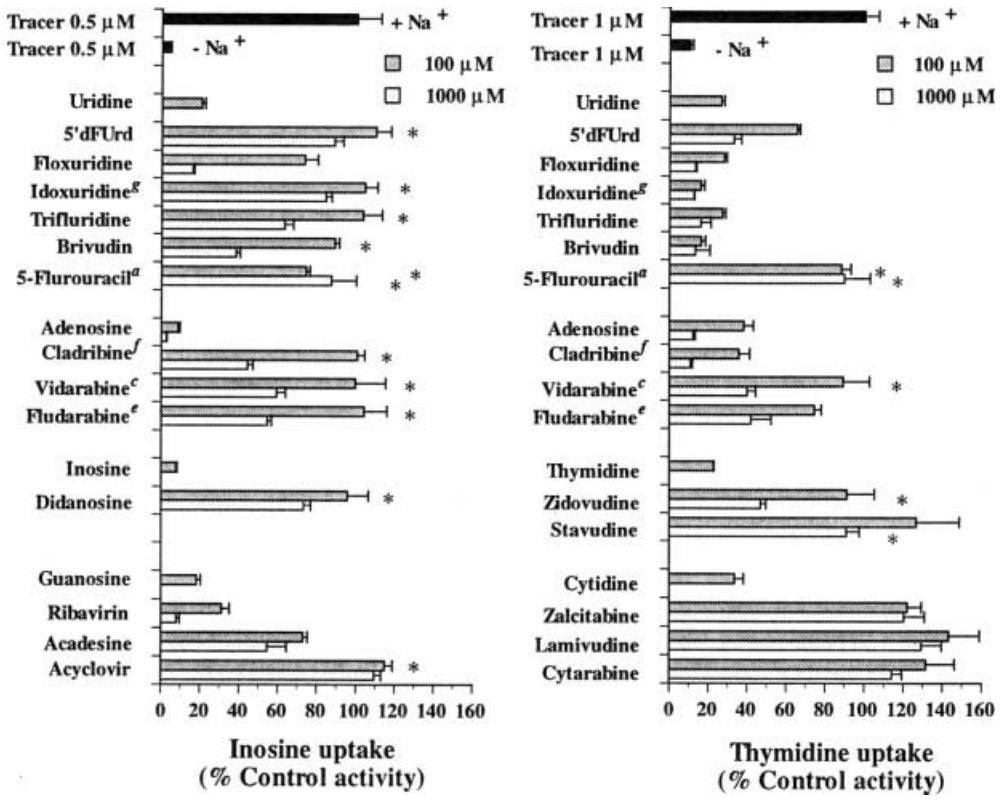


Fig. 4 Na^+-nucleoside uptake of ^3H-inosine (0.5 µM) and ^3H-thymidine (1 µM) by the intestinal BBMV in the absence and presence of 100 and 1000 µM of various antiviral and anticancer nucleoside and nucleobase drugs. Except for those marked with an *asterisk*, all analogs significantly ($P < 0.05$) affected the uptake of the tracer nucleosides. The following solvents were used to solubilize the nucleoside analogs (1000 µM): *a* NaOH 2 mM, *c* HCl 2 mM, *e* DMSO 0.2%, *f* DMSO 0.5%, *g* NH_4OH 10 mM. Solvent concentrations were ten times lower when 100 µM of the nucleoside analogs was used

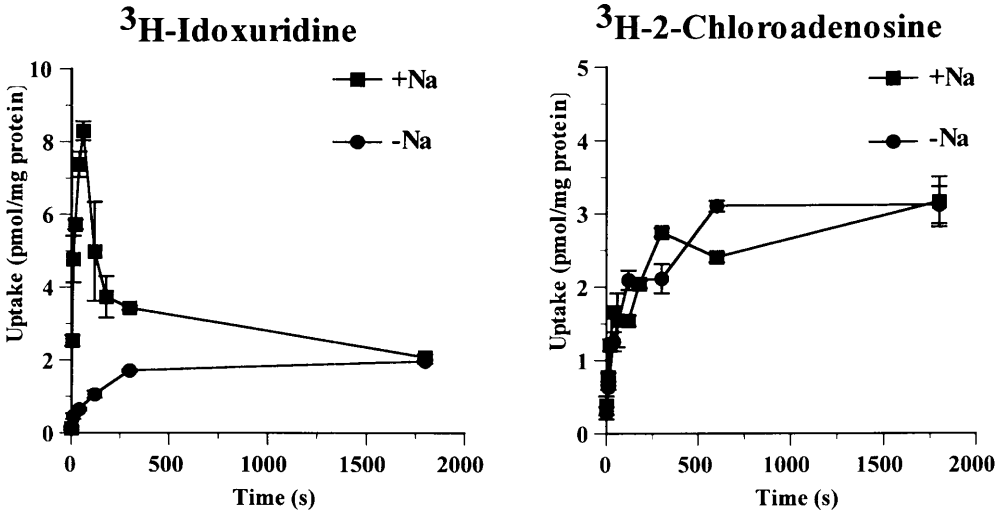


Fig. 5 The time-courses of uptake of ^3H-idoxuridine (1 µM) and ^3H-2-chloroadenosine (1 µM) by intestinal BBMV in the presence and absence of a 150-mM Na^+-gradient (out > in)

100 µM of the analog or the natural nucleoside (uridine or adenosine) will be quantitatively similar. If the inhibition differed substantially and significantly [i.e., little (< 20%) or no inhibition by the analog and virtually complete inhibition by uridine or adenosine], this was interpreted as loss of high-affinity interaction of the nucleoside with the nucleoside transporters. We note here that in such cases the analog may still be inhibitory at 1000 µM, however such a result signifies a much lower affinity (≫100 µM) of the analog for the nucleoside transporter when compared with their natural substrates. To determine if the nucleoside analogs that were inhibitors were also substrates of the transporters, we selected to study the uptake of one or two of these analogs representing each class of inhibitors: sugar-modified, base-modified, or sugar and base modified.

Interaction of sugar-modified nucleosides with the N1 and N2 Na^+-nucleoside transporters of the human intestine

At 100 µM, adenosine and uridine significantly inhibited the uptake of both inosine and thymidine by the

Table 1 Inhibition by 100 μM thymidine or inosine of uptake of tritiated nucleoside analogs by intestinal brush border membrane vesicles in the presence of a 150-mM Na^+-gradient. All values except for those marked with an *asterisk* were significantly different from the uptake values obtained in the absence of the inhibitor. Experiments 1 and 2 were conducted on two separate batches of vesicles, each pooled from three or four intestines. The magnitude of inhibition varies for the two batches most likely due to differing levels of expression of the two transporters

^{3}H-nucleoside analog	Experiment	Percent uptake in the presence of:	
		Thymidine 100 μM	Inosine 100 μM
2′-Deoxyuridine	1	46 ± 3	58 ± 1
2′-Deoxyuridine	2	59 ± 2	28 ± 2
5-Fluorouridine	1	33 ± 3	74 ± 1
5-Fluorouridine	2	51 ± 4	45 ± 2
Idoxuridine	1	17 ± 1	98 ± 5*
Idoxuridine	2	28 ± 2	89 ± 6*
Floxuridine	1	25 ± 1	64 ± 2
Floxuridine	2	40 ± 3	58 ± 2
2-Chloroadenosine	1	105 ± 5*	117 ± 5*
2-Chloroadenosine	2	103 ± 5*	103 ± 9*

intestinal BBMV confirming that these nucleosides are substrates of both the N1 and the N2 transporters [9] (Fig. 2). Adenosine was less inhibitory ($P < 0.05$) toward the uptake of thymidine than toward the uptake of inosine, indicating that adenosine has a poorer affinity for the pyrimidine transporter (N2) than for the purine transporter (N1). Irrespective of the concentration, the natural nucleosides, uridine and 2′-deoxyuridine (2′dUrd), significantly and substantially (>60%) inhibited inosine and thymidine transport by the N1 and the N2 transporters, respectively, suggesting that both the ribose and deoxyribose nucleosides have high affinity for the two transporters. In contrast, 3′deoxyuridine (3′dUrd) and 2′,3′-dideoxyuridine (ddUrd) had no significant inhibitory effects at 100 μM and showed either no inhibitory effect or only a modest one ($P < 0.05$) at the high concentration of 1000 μM. This finding suggests that retention of the 3′-oxygen (but not of the 2′-oxygen) on the sugar ring is a structural requirement for a nucleoside analog to interact with high affinity (that is comparable to that of uridine) with the N1 and the N2 transporters. This finding was supported further when the interaction of nucleoside drugs with substituents on the 3′ position or 2′ and 3′ positions of the sugar ring were examined (Fig. 4). Such substitutions, as in the anti-HIV drugs AZT, d4T, ddC, or ddI, abolished the capacity (at 100 μM) of these nucleoside drugs to inhibit the uptake of their corresponding natural pyrimidine or purine nucleoside.

In contrast to the 2′ and 3′ substitutions, removal of oxygen from the 5′ position differentially affected the inhibitory capacity of the nucleoside analog toward the N1 and N2 nucleoside transporters. While the removal of 5′-oxygen (as in 5′-deoxyadenosine) reduced the inhibitory effect of 100 μM adenosine (from ca. 90% to ca. 0%) on inosine uptake, the same substitution (at 100 μM) only modestly affected (from ca. 40% to ca. 25%) the ability of adenosine to inhibit the uptake of thymidine ($P < 0.05$). This finding was supported when the inhibitory effect of 100 μM 5′-deoxythymidine was examined. The inhibitory effect of this analog was reduced only modestly when compared with that of thymidine. Thus, while the presence of 5′-oxygen was required for inhibition of N1 nucleoside transporter activity at 100 μM, this was not the case for the N2 nucleoside transporter. Besides substitutions of oxygen on the 2′, 3′, or 5′ positions, modifying the ribose ring of adenosine and cytidine to the corresponding arabinoside (vidarabine or ara-A, cytarabine or ara-C) ring also abolished the inhibitory capacity of the nucleoside analogs toward the nucleoside transporters (Fig. 4). The base, uracil (100 or 1000 μM), did not inhibit the uptake of either thymidine or inosine. That is, the ribose ring is essential for inhibition of the two nucleoside transporters.

Collectively, the above findings indicate that at 100 μM, the 3′ position is an absolute requirement for inhibition of the N1 and N2 Na^+-nucleoside transporters. For the ribose nucleosides, the 2′ hydroxyl must be in the *cis* position to the 3′ hydroxyl to inhibit both the N1 and the N2 nucleoside transporters. In addition, although the 5′-oxygen is required for inhibition of the N1 transporter at 100 μM, this is not the case for the N2 transporter.

Interactions of base-modified nucleosides with the N1 and N2 Na^+-nucleoside transporters of the human intestine

The structure-inhibitory profiles of the N1 and N2 Na^+-nucleoside transporters were further investigated by measuring the effect of base-modified uridine and adenosine analogs on the uptake of thymidine and inosine by intestinal BBMV (Fig. 3A,B). As seen above for sugar modifications, the N1 transporter was found to be much more sensitive to modifications on the base of both uridine and adenosine. Increasing the size of the substituent at the 5 position from fluorine to iodine significantly diminished the affinity of the nucleoside for the N1 transporter while the N2 transporter was insensitive to such substitutions (Fig. 3A). This suggests that either this position is not critical to binding of the nucleoside to the N2 binding site or it is tolerant to changes in the size or electrophilicity of the substituent. For both the N1 and the N2 transporters, retention of the carbon at the 6 position (see 6-azauridine) appears to be critical for substantial inhibition of the transporters.

It should be noted here that the lack of inhibition of nucleoside uptake by 3-deazauridine and 6-azauridine may, in part, be explained by significant ionization of these compounds at physiological pH. Both 3-deazauridine and 6-azauridine have pKa values (6.5 and 6.7, respectively) close to the physiological pH and are, therefore, substantially ionized (>80%) at the pH of the incubation medium (pH 7.4). Previous data on the

uptake of these compounds by the equilibrative NBMPR-sensitive nucleoside transporter (*es*) suggest that only the unionized species can be transported by the *es* transporter [10, 11]. On examining the pKa of other nucleoside analogs (where available), none were found to have a value which would result in substantial ionization (>80%) of that analog.

When base-modified adenosine analogs were examined, the findings were similar to those obtained with base-modified uridine analogs (Fig. 3B). Except for the 8 position, the N1 transporter was much more sensitive to substitutions on the adenine rings than was the N2 transporter. Collectively, these data suggest that the N2 transporter is much more permissive to substitutions on the nucleoside base than is the N1 transporter and the 8 position appears to be critical for the nucleosides to inhibit both transporters.

Interaction of nucleoside and nucleobase drugs with the Na^+-nucleoside transporters of the human intestine

Since the intestinal nucleoside transporters may contribute significantly to the absorption of nucleoside drugs [10], we investigated the effect of selected nucleoside drugs used in the treatment of viral diseases (for example, AIDS) and cancer on the uptake of thymidine and inosine. As expected from the data presented above, modification of the sugar ring on both the 2′ and 3′ positions as in the anti-HIV dideoxynucleosides, AZT, d4T, ddC, 3TC, and ddI, abolished the ability of these drugs to inhibit the nucleoside transporters (Fig. 4). In fact, except for ddI and AZT, the remaining dideoxynucleosides were incapable of inhibiting the uptake of either inosine or thymidine even at 1000 μM. Surprisingly, these compounds (d4T, 3TC, ddC) induced a slight but statistically significant ($P < 0.05$) stimulation of thymidine uptake. No definitive explanation can be offered for these observations. At first glance our AZT data appear to be at variance from those obtained by Ritzel et al. [11] with recombinant human N2 transporter (hCNT1) expressed in *Xenopus* oocytes. In their expression system, AZT was found to be a substrate of hCNT1 and, at 5 mM concentration, significantly inhibited hCNT1-mediated uptake of tritiated uridine [11]. However, at this extremely high concentration, both AZT and ddC also inhibited the uptake of thymidine (68% and 41% inhibition, respectively) in our vesicle studies. This result underscores our observation that both of these dideoxynucleosides are poor inhibitors of thymidine transport by the N2 transporter. This lack of inhibition by dideoxynucleosides of the human intestinal nucleoside transporters is consistent with the data obtained in the clinic where the absorption of these drugs appears to be dose independent [2, 4]. Additionally, no interaction at the level of absorption has been documented when these drugs are coadministered. Similarly, modification of the ribose nucleoside to the arabinoside nucleoside, as in the adenosine analog vidarabine and the cytidine analog cytarabine, abolished the inhibition of the nucleoside (at 100 μM) toward the N1 and the N2 transporter. In addition, as seen above with uracil, the presence of an intact ribose moiety is required for inhibition of the nucleoside transporters. Consequently, 5-fluorouracil and acyclovir do not inhibit the uptake of nucleosides by these transporters (Fig. 4).

Consistent with the data presented above for uridine and adenosine analogs, structural modifications of the sugar ring at the 5′ position or of the nucleoside base differentially affected the inhibitory capacity of the nucleoside drugs toward the N1 and the N2 nucleoside transporters. For example, 5-fluoro-5′-deoxyfluoridine (5′dFUrd; 100 μM) significantly inhibited thymidine uptake by the N2 transporter, but this analog did not inhibit inosine uptake at all. Likewise, substitutions on the base of the 2′-deoxynucleosides, as in trifluridine, cladribine, and idoxuridine, considerably diminished the ability of the nucleoside to inhibit the N1 transporter but not the N2 transporter. Based on the above findings, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a guanosine analog modified only on the base, would not be expected to inhibit the N1 transporter or be transported by the N1 nucleoside transporter. However, ribavirin is transported by the N1 nucleoside transporter with high affinity (ca. 8 μM) [10]. Likewise, based on our inhibition data, acadesine (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside), a guanosine analog modified only on the base, appears to be a substrate of the N1 transporter. Both ribavirin and acadesine have the bicyclic guanine ring modified to a monocyclic triazole or imidazole ring. Thus, modifications other than simple substitutions on the nucleoside base may have differing inhibitory capacity toward the N1 transporter (Fig. 4).

The data obtained above differ from those obtained by Brett et al. [1] with the N4 nucleoside transporter expressed in the human kidney. In their study, the ribose moiety was not an absolute requirement for transport since uracil was found to be an inhibitor of uridine transport by the N4 nucleoside transporter. In addition, in a separate study, 2′,3′-dideoxyuridine was found to be a substrate of the N4 transporter [7]. These differences suggest that the N1 and N2 nucleoside transporters have different nucleoside structure-inhibitory profiles than the N4 transporter. The structure-inhibitory profiles of the human N1 and N2 transporters also appear to differ from those of the corresponding rat transporters. At 1000 μM concentration, acyclovir was found to significantly inhibit the Na^+-dependent uptake of ^{3}H-inosine in HeLa cells expressing the rat N1 transporter (SPNT-int) [12]. This result differs from ours where acyclovir was incapable of inhibiting the uptake of tracer inosine at 1000 μM concentration. Likewise, 1 mM ddC was found to be a significant inhibitor of uridine uptake by the recombinant rat N2 transporter (rCNT1) expressed in *Xenopus* oocytes [15] while no inhibition was observed at the same concentration in our study. Such differences

may be due to species variations or may be an artifact of the heterologous expression system used in the cited studies. In this regard, it is important to note that the study reported here is the first investigation of the structure-inhibitory profiles of nucleosides for the human N1 and N2 transporters when they are present in their natural milieu, the small intestinal epithelial membrane.

The results of the above study provide data on the critical structural determinants of nucleosides necessary for inhibition of the N1 and N2 transporters of the human intestine. However, in order to extrapolate these data to define the critical structural determinants of nucleosides for substrate recognition by the transporters, these inhibitors must be shown to be substrates of the nucleoside transporters. This can be accomplished by demonstrating either Na^+-dependent uptake of each of the inhibitors or that the inhibitor is a competitive inhibitor of the transporters. Because conducting uptake studies on each inhibitor would be impossible, both logistically and because not all inhibitors are available in the radiolabeled form, we have determined the intestinal BBMV uptake of at least one representative of each group of nucleoside inhibitors; sugar-modified (2′-deoxyuridine), base-modified (5-fluorouridine, 2-chloroadenosine), or sugar and base modified (idoxuridine, floxuridine). Except for 2-chloroadenosine, all four showed a transient overshoot behavior in the presence of a 150-mM Na^+-gradient (see Fig. 5 for representative curves) indicating sodium-dependent transport of these nucleosides. In addition, the uptake of tritiated (0.5 μM) 2′-deoxyuridine, 5-fluorouridine, and floxuridine by intestinal BBMV was found to be significantly inhibited by 100 μM of thymidine or inosine. In contrast, the uptake of idoxuridine was significantly inhibited by only 100 μM thymidine and not by 100 μM inosine while the uptake of 2-choloradenosine was not inhibited by either nucleosides (Table 1). Except for 2-chloroadenosine, these data are consistent with the inhibitory capacities of these nucleoside analogs as shown in Figs. 2, 3, 4. At 100 μM, 2′-deoxyuridine, 5-fluorouridine, or floxuridine significantly inhibited the uptake of tracer thymidine and inosine by intestinal BBMV confirming that these analogs are substrates of both the N1 and the N2 transporters. Idoxuridine (100 μM) inhibited the uptake of only tracer thymidine confirming that this analog is predominantly a substrate of the N2 transporter. Although 2-chloroadenosine is not taken up by intestinal BBMV by a Na^+-nucleoside transporter (Fig. 5), at 100 μM it was found to significantly inhibit the uptake of tracer thymidine (Fig. 3B). Collectively, these data suggest that, except for 2-chloroadenosine, the remaining four nucleoside analogs are indeed substrates of either the N1 or the N2 transporter or both. The lack of Na^+-dependent uptake of 2-chloroadenosine underscores the observation that a nucleoside can be an inhibitor without being a substrate.

Collectively, our data suggest that the ribose or 2′-deoxyribose moiety is a requirement for high-affinity interaction of nucleosides with the human intestinal N1 and N2 nucleoside transporters. However, the structural requirement for such high-affinity interaction differs with respect to modifications on the 5′ position of the sugar ring or on the base of the nucleoside. The N2 transporter is more tolerant to these substitutions than is the N1 transporter. To our knowledge, this is the first time that a comprehensive study of the structure-inhibitory profiles of nucleosides for the N1 and the N2 transporters has been conducted. Moreover, this study was conducted with the transporters present in their natural milieu, namely the human intestinal epithelial membrane, thus avoiding potential artifacts introduced by studying these transporters in an expression system. These findings provide insight into differences between the nucleoside binding sites of the two transporters and can guide future experiments to map the binding sites of these two intestinal transporters.

Acknowledgements This study was supported by a grant from the National Institutes of Health GM54447.

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