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Human intestinal *es* nucleoside transporter: molecular characterization and nucleoside inhibitory profiles

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Abstract *Purpose*: To clone and sequence the equilibrative nitrobenzylthioinosine (NBMPR)-sensitive nucleoside transporter (*es*) from the human small intestine and to examine the capacities of nucleosides and nucleoside analogs to inhibit the uptake of uridine by this transporter. *Methods*: Using PCR, *es* was cloned from a cDNA library of the human small intestine. The uptake of ^{3}H-uridine (10 μ*M*) by the recombinant *es*, expressed in *Xenopus* oocytes, was measured in the presence (2 m*M*) and absence of nucleosides and nucleoside analogs. *Results*: The amino acid sequence of this *es* transporter was identical to that of the human placental *es* transporter. Uptake of ^{3}H-uridine by this *es* transporter was inhibitable by 1 μ*M* NBMPR. Removal of the oxygen from the 3′ position or from both the 2′ and 3′ positions, but not from 2′ or 5′ position, resulted in a partial or total loss of the capacity of the nucleosides to inhibit ^{3}H-uridine uptake. No modifications of the adenosine base or of the uridine base (except for 3 and 6 positions on uracil) affected nucleoside inhibitory capacity. *Conclusions*: The *es* transporters of the human intestine and placenta are identical in their amino acid sequences. Moreover, the inhibitory profiles of various nucleoside analogs in inhibiting the uptake of uridine by the intestinal *es* transporter are similar to those obtained with the as-yet-uncloned human erythrocyte *es* transporter. Collectively, these findings suggest that the *es* transporter does not appear to be functionally variant in the human placenta, small intestine or erythrocytes.

Key words *es* nucleoside transporter · Uridine uptake · Human intestine · Nucleoside analogs · Antiviral and anticancer drugs · Inhibitory profiles

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Introduction

Transport of nucleosides, both natural and analogs, across cellular membranes is mediated by both Na^+-dependent and Na^+-independent transporters [9]. The human intestinal epithelial cells are especially dependent on nucleoside transport as they lack de novo nucleoside biosynthetic pathways and must depend on the salvage pathway for their nucleoside needs [15]. Previously, we have found that the equilibrative transporters sensitive (*es*) and insensitive (*ei*) to nitrobenzylthioinosine (NBMPR) as well as the Na^+-dependent concentrative nucleoside transporters (N1 and N2) are expressed in the human intestine [4, 16]. Of these four transporters, the *es* transporter appears to be quantitatively the largest contributor to nucleoside transport in the human jejunum [4]. The equilibrative nucleoside transporters (*es* and *ei*) accept a broad spectrum of purine and pyrimidine nucleosides as substrates while the Na^+-dependent N1 and N2 transporters are narrower in their selectivity, accepting either purines (N1) or pyrimidines (N2) as substrates [9].

The human intestinal nucleoside transporters participate in the absorption of natural nucleosides and may participate in the absorption of orally administered nucleoside drugs used to treat a variety of diseases such as AIDS, hepatitis, and cancer. Moreover, to enhance site-specific delivery of nucleoside drugs to the target (e.g. tumor) cell, the characteristics of nucleoside transporters expressed in non-target cells such as those of the intestine, must be determined. Therefore, we have cloned and sequenced the *es* transporter from the human small intestine. We have compared its sequence to that of the human placental *es* transporter [11] to determine if they

are similar or perhaps tissue-specific isoforms of one another. To date, the human *es* transporter has been cloned from the placenta [11] and from the myeloid leukemic cell line K562 [2]. However, it is the function of the uncloned human erythrocyte *es* transporter that has previously been extensively characterized. To provide insight as to whether the functional characteristics of the human intestinal *es* transporter are similar to those of the human erythrocyte *es* transporter, we compared the inhibitory profiles of various nucleoside analogs in inhibiting the uptake of uridine by the intestinal *es* transporter with comparable data in the literature on the human erythrocyte *es* transporter.

Materials and methods

Materials

[5-^{3}H]-uridine (23.6 Ci/mmol and 15 Ci/mmol) was purchased from Moravek Biochemicals (Brea, Calif.). Antiviral agents were kindly supplied by their respective manufacturers: zidovudine (3′-azido-2′,3′-deoxythymidine) and lamivudine (3′-thia-2′,3′-deoxycytidine), Glaxo Wellcome (Research Triangle Park, N.C.); stavudine (2′,3′-didehydro-3′-deoxythymidine) and didanosine (9-[(2-hydroxyethoxy)methyl]guanine), Bristol-Myers Squibb (Princeton, N.J.); zalcitabine (2′,3′-deoxycytidine), Hoffman-La Roche (Nutley, N.J.); ribavirin (1-*b*-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), Schering-Plough Research Institute (Kenilworth, N.J.). Sodium salt of acyclovir (9-[(2-hydroxyethoxy)methyl]guanine) was commercially available (Glaxo Wellcome). Nitrobenzylthioinosine was obtained from RBI (Natick, Mass.). All other nucleosides (natural and analogs) were from Sigma (St. Louis, Mo.). All other chemicals were of the highest analytical grade.

PCR amplification and sequencing of *es* nucleoside transporter from a human intestinal cDNA library

A λZAP cDNA library from human small intestine was constructed for us by Stratagene (La Jolla, Calif.). The library was then mass-excised to yield a plasmid-based cDNA library. This plasmid-based cDNA library was used as a template for the amplification of *es* using the forward primer 5′-AATTTCCGTCCCCCACCAAG-3′, the reverse primer 5′-AGGCAGTCCTTCTGTCCATCC-3′ and the Elongase Enzyme Mix (Gibco BRL, Grand Island, N.Y.) for high fidelity amplification. A PCR product of about 1.5 kb was gel-purified, polished by Pfu polymerase and subcloned into the Srf 1 site of pCR-Script Amp SK(+) (Stratagene) to yield construct pPLH1. This construct was sequenced on both strands by Taq DyeDeoxy Terminator cycle sequencing and was found to contain the complete open reading frame of an *es* nucleoside transporter. Two other independent PCR products were also sequenced in regions where there was a difference in sequence from that of the placental *es* cDNA [11]. Our human intestinal *es* cDNA sequence has been deposited in GenBank (accession no. AF079117).

Xenopus expression and uptake assays

Plasmid pPLH1 was linearized using Not1 and transcribed in vitro using the T7 mMESSAGE mMACHINE kit (Ambion, Tex.). The quality and the size of the in vitro-transcribed mRNA was verified by denaturing agarose gel electrophoresis [8]. *Xenopus* oocytes were treated with 8 mg/ml collagenase type I (Sigma, St. Louis, Mo.) and 1 mg/ml trypsin inhibitor (Sigma) for 60–90 min to remove follicle cells as previously described [4] and allowed to recover overnight at 18 °C in 50% Leibovitz L-15 medium (Gibco BRL, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Bio Whittaker, Walkersville, Md.). Healthy stage-V oocytes were injected with either 10 ng of mRNA or an equal volume of sterile water. Injected oocytes were cultured in 50% Leibovitz L-15 supplemented with 10% fetal bovine serum for 3 days at 18 °C, with daily changes of medium, before conducting the uptake assays.

Assays were performed in 200 µl of transport buffer (100 m*M* choline chloride, 2 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10 m*M* HEPES, pH 7.4) with 8 to 12 oocytes per uptake experiment. Oocytes were rinsed briefly in transport buffer without any radioactive permeant before the uptake assay. Uptake of permeant 10 µ*M* ^{3}H-uridine by oocytes over a 1-min period was measured in the presence or absence of 2 m*M* unlabeled competing nucleosides and nucleobases. Preliminary studies had shown that the uptake of ^{3}H-uridine is linear over at least 1 min. Uptake was stopped by six rapid washes with ice-cold transport buffer, and individual oocytes were lysed in 5% sodium dodecyl sulfate before quantification of radioactivity.

Where necessary, nucleoside analogs were dissolved in solvents (see figure legends). Solvents did not substantially affect uptake of ^{3}H-uridine (< 20% inhibition). When solvents were used, appropriate controls (effect of solvent without nucleoside analog) were always conducted. The data shown in the figures are representative of the results obtained from at least two independent experiments on different batches of oocytes. All uptake data are given as means ± SD for 8 to 12 oocytes. Outliers were identified using Dixon's test for extreme values at $P < 0.05$. Data formatting and statistical analysis of data were carried out using the computer programs Vertica (see Acknowledgements) and SPSS (one-way ANOVA and Student-Newman-Keuls), respectively.

Results

Sequence analysis of the human intestinal *es* showed that there were three sites where the sequence was different from the placental *es* sequence [11]. The two differences in the open reading frame, at position 600 (A to a G) and 1191 (C to a T), did not result in any changes in the amino acid sequence. The other difference, found in the 5′ untranslated region was the insertion of a triplet (GCA) at position minus-53 from the start of the open reading frame. These differences were not due to random DNA polymerase infidelity since two other independently obtained PCR products of human intestinal *es* also showed these differences. These differences most probably reflect normal polymorphism in the human population. Uptake of ^{3}H-uridine in cRNA-injected oocytes ranged from 0.27 ± 0.18 to 0.79 ± 0.22 pmol/oocyte per min, with an average of 0.60 ± 0.15 pmol/oocyte per min. This wide range in uptake data is not surprising as different batches of oocytes tend to demonstrate variable levels of expression of heterologous proteins. Uridine uptake by the recombinant human intestinal *es* in *Xenopus* oocytes was on average 86.6% ($n = 3$) inhibitable by 1 µ*M* NBMPR, confirming that we had cloned a functional *es* transporter.

The inhibitory profiles of nucleosides and their analogs (2 m*M*) on uptake of 10 µ*M* ^{3}H-uridine by the recombinant *es* transporter in *Xenopus* oocytes are presented in Figs. 1–3.

Discussion

Although the substrate selectivity of the *es* transporter has been extensively characterized, most of these

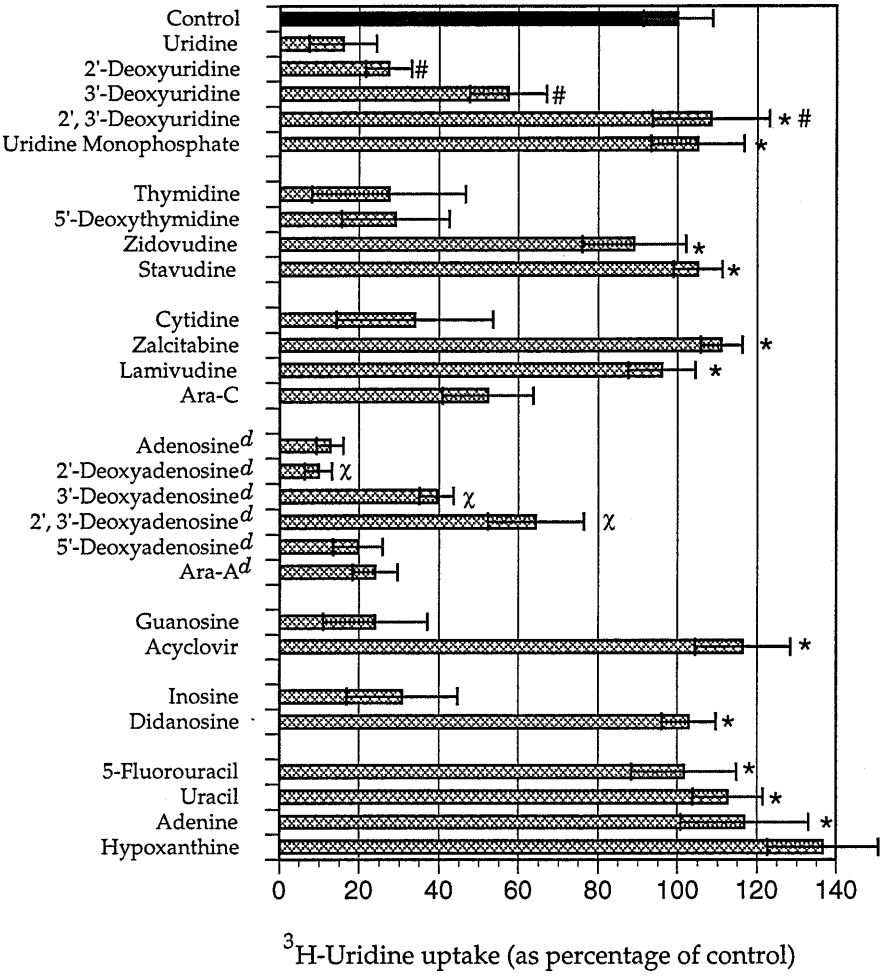

Fig. 1 Inhibitory profiles of sugar-modified nucleoside analogs on ^{3}H-uridine uptake by *es*. *Xenopus* oocytes expressing the recombinant human intestinal *es* transporter were incubated with 10 μM of ^{3}H-uridine in the absence (control, *solid bar*) and in the presence of 2 mM of competing unlabeled nucleosides and their analogs (*hatched bars*). The data shown are representative of results obtained from at least two independent experiments. All uptake data are given as means ± SD for 8 to 12 oocytes. Uptake of ^{3}H-uridine in cRNA-injected oocytes ranged from 0.27 ± 0.18 to 0.79 ± 0.22 pmol/oocyte per min. Uptake of ^{3}H-uridine in water-injected oocytes ranged from 0.3% to 3% of the corresponding cRNA-injected oocytes within the same batch. Zidovudine(3′-azido-2′,3′-deoxythymidine), stavudine (2′,3′-didehydro-3′-deoxythymidine), zalcitabine (2′,3′-deoxycytidine), lamivudine (3′-thia-2′,3′-deoxycytidine), ara-C (9-β-D-arabinofuranosyl-cytosine), ara-A (9-β-D-arabinofuranosyl-adenine), acyclovir (9-[(2-hydroxyethoxy)methyl]guanine), didanosine (9-[(2-hydroxyethoxy)methyl]guanine). *not significantly different from the control ($P > 0.05$). #, χ, significantly different from each other ($P < 0.05$). *d*, 1% DMSO as solvent, final concentration

studies have been carried out in cells obtained or derived from a variety of species, primarily human and rodent [3, 6, 7, 10, 13, 19]. Unfortunately, none of the studies using human cells included a comprehensive array of nucleoside analogs where systematic substitutions on the sugar or the base or both were included. Because of known structural differences in the *es* transporter across species, possible contamination from the *ei* transporter, and the possibility of the existence of tissue-specific isoforms [17, 20], the data obtained from these previous studies are difficult to combine to obtain a comprehensive picture of the critical structural determinants of the nucleoside molecule necessary for potent inhibition of the *es* transporter. To overcome these problems, and to determine if the *es* transporter is polymorphically expressed, we cloned and sequenced the human intestinal *es* transporter. Then we evaluated the capacities of various nucleosides and their analogs to inhibit uridine uptake by this transporter in the expression system *Xenopus laevis* oocytes. The use of a recombinant cDNA clone in a single expression system with little or no endogenous nucleoside transporter activity avoids any potential cross-contamination from *ei* and cross-species differences in *es*. Recently, the *es* transporter from the human placenta has been cloned by Griffiths et al., and these authors have also carried out a study on the capacity of a limited number of drugs such as cladribine and gemcitabine to inhibit uridine uptake by the *es* transporter [11]. In the present study we extended and greatly enlarged the body of information produced by Griffiths et al. [11] by studying the inhibitory capacity of a wide array of nucleoside analogs, each representing a different substitution on either the sugar, the base or both.

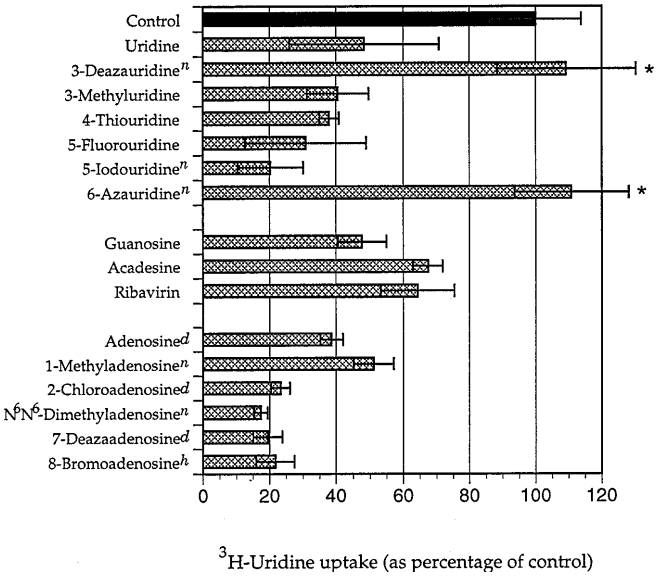

Fig. 2 Inhibitory profiles of base-modified nucleoside analogs on ^{3}H-uridine uptake by *es*. *Xenopus* oocytes expressing the recombinant human intestinal *es* transporter were incubated with 10 μ*M* of ^{3}H-uridine in the absence (control, *solid bar*) and in the presence of 2 m*M* of competing cold nucleosides and their analogs (*hatched bars*). Data shown are representative of the results obtained from at least two independent experiments. All uptake data are given as means ± SD for 8 to 12 oocytes. Acadesine (5-amino-4-carboxamide-1-β-D-ribofuranoside), ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide). *not significantly different from the control ($P > 0.05$). *d*, 1% DMSO; *n*, 0.004 *N* NaOH; and *h*, 0.004 *N* HCl as solvents, final concentrations

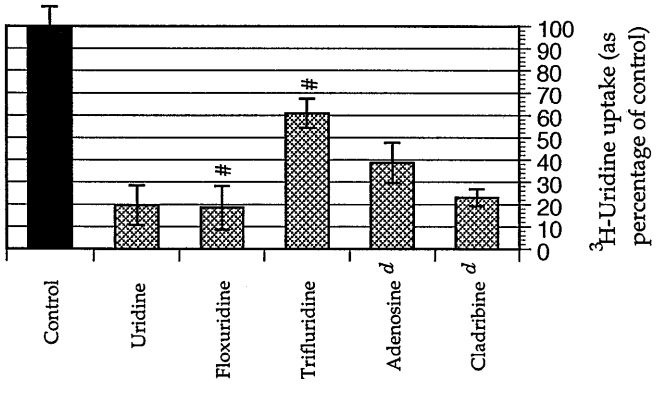

Fig. 3 Inhibitory profiles of sugar plus base-modified nucleoside analogs on ^{3}H-uridine uptake by *es*. *Xenopus* oocytes expressing the recombinant human intestinal *es* transporter were incubated with 10 μ*M* of ^{3}H-uridine in the absence (control, *solid bar*) and in the presence of 2 m*M* of competing cold nucleosides and their analogs (*hatched bars*). Data shown are representative of the results obtained from at least two independent experiments. All uptake data are given as means ± SD for 8 to 12 oocytes. Cladribine (2-chloro-2′-deoxyadenosine), floxuridine (5-fluoro-2′-deoxyuridine), trifluridine (5-[trifluoromethyl]-2′- deoxyuridine). #significantly different from each other ($P < 0.05$). *d*, 1% DMSO as solvent, final concentration

Sequence analysis of human intestinal *es*

The cloning and sequencing of the human intestinal *es* transporter revealed for the first time that the *es* transporters of the human placenta [11], human myeloid leukemic (K562) cells [2], and the human small intestine are identical in their amino acid sequences. These identical sequences in three different human cell types are suggestive of a lack of tissue-specific polymorphism in the expression of the human *es* transporter.

Interaction of nucleosides and nucleoside analogs with the human intestinal *es* transporter

Because *es* is known to transport both purines and pyrimidines, we chose uridine, thymidine and cytidine and their respective modified analogs as representative of pyrimidine inhibitors and adenosine, guanosine, and inosine and their respective analogs as representative of purine inhibitors. Among the selected modified purine and pyrimidine inhibitors were antiviral and antitumor drugs such as zidovudine and 5-fluorouridine.

We chose to study the inhibitory profiles of a large array of compounds in inhibiting the uridine uptake by the human intestinal *es* because the data generated will be the basis for future studies to map the binding site(s) of the transporter and to allow comparison with similar data on the human erythrocyte *es* transporter. At this point, we chose not to determine the inhibitory potency (K_i) or the Michaelis-Menten constant (K_m) of the uptake of the 44 analogs tested due to the large number of compounds used and also because not all of these compounds exist in their radiolabeled forms.

Interaction of sugar-modified nucleoside analogs with the human intestinal *es* transporter

The broad specificity of the *es* transporter is demonstrated by significant inhibition (>60%) of ^{3}H-uridine uptake by thymidine, cytidine, adenosine, guanosine, and inosine (Fig. 1). Removal of the 2′-oxygen from either uridine or adenosine did not significantly affect their inhibitory capacity (Fig. 1). Consistent with this observation, modifying the sugar ring of adenosine or cytidine at the 2′-position to the corresponding arabinoside (ara-A, 9-β-D-arabinofuranosyl-adenine; ara-C, 9-β-D-arabinofuranosyl-cytosine) also did not affect their inhibitory capacities. In contrast, removal of the 3′ oxygen or of both the 2′ and 3′ oxygens resulted in a partial (about 50%) or total loss of inhibition of uridine uptake, the loss being larger when both the 2′ and 3′ oxygens were removed (Fig. 1). Modifications at both the 2′ and the 3′ positions resulted in the total loss of inhibitory capacity for both purine and pyrimidine nucleoside drugs, as shown by the loss of inhibition by zidovudine, didanosine, stavudine, and lamivudine. This result is in contrast to that found by us with the human

intestinal Na^+-dependent N1 and N2 transporters, where the removal of the 3′ oxygen resulted in a complete loss of inhibition of uptake of ^{3}H-inosine and ^{3}H-thymidine by the N1 and N2 transporters, respectively [16]. In addition, the removal of oxygen at the 5′ position had no effect on the inhibitory capacity, but the addition of a bulky and charged phosphate group to that same position (nucleotides) resulted in a complete loss of the capacity of that analog to inhibit uridine uptake (Fig. 1). Other researchers have also found that the *es* transporter in human erythrocytes does not transport nucleotides [3]. Interestingly, both the N1 and N2 transporters were sensitive to removal of oxygen from the 5′ position, with N2 (about 30–50% inhibition) being more tolerant to the change than N1 (0% inhibition) [16]. The above data suggest that the Na^+-independent *es* transporter is less sensitive to changes at the 5′ position when compared to the Na^+-dependent N1 or N2 transporters.

Consistent with the above findings on modifications of the sugar ring, opening of the ribose ring as in acyclovir, or removal of the sugar ring as in the nucleobases adenine, uracil, 5-fluorouracil, and hypoxanthine abolished the inhibitory capacity of the modified analogs (Fig. 1). This could have been due either to the loss of the 2′ and 3′ oxygens or to the loss of rigidity in the sugar moiety in the case of acyclovir.

Except for substitution at the 5′ position, these findings are in general agreement with those reported previously with mammalian cells and cell lines [3, 6, 7, 10, 13, 19]. In contrast to our data which show that 5′-deoxyadenosine and 5′-deoxythymidine inhibit uridine uptake with a capacity similar to adenosine and thymidine, respectively, experiments with the murine leukemia L1210 cells [13] have found that such modified analogs show diminished affinities for the *es* nucleoside transporter. Since experiments with human erythrocytes have also shown only a modest decrease (about twofold) in the affinity of 5′-deoxythymidine versus thymidine for the *es* transporter [6], the murine leukemia L1210 cell experiments likely reflect species differences. Indeed, the ability of dipyridamole to inhibit the *es* transporters differs considerably between humans and rodents [21].

Interaction of base-modified nucleoside analogs with the human intestinal *es* transporter

In contrast to the effects of changes in the ribose moiety, Fig. 2 shows that most changes in the base moiety did not disrupt the capacity of the nucleoside to inhibit uridine uptake. Except for the removal of nitrogen at the 3 position (3-deazauridine) or exchange of carbon at the 6 position with a nitrogen (6-azauridine) in uracil, all other substitutions or modifications in the base moiety had minimal impact on uridine uptake by the *es* transporter. The two analogs concerned, 3-deazauridine (pKa 6.5) and 6-azauridine (pKa 6.7), are significantly ionized ($>80\%$) at pH 7.4 and above (as in our uptake assay conditions). The transport of 3-deazauridine and 6-azauridine (presumably via a NBMPR-sensitive transporter) in human lymphoblastoid cells is pH-dependent at pH 7.4 or above [1, 5]. These authors suggested that charged molecules may be poor substrates of the human lymphoblastoid cell *es* transporter although this conclusion cannot be generalized as the interaction of pyrazofurin (pKa 6.7) with the transporter is not pH sensitive [1]. Our results confirm that indeed these substrates, when charged, lose their capacities to inhibit uridine uptake. It is possible that the charges disrupt the bonding necessary for interaction.

From the nucleoside inhibitory profile data presented here and that of Patil and Unadkat [16], it is interesting to note that the human intestinal N1 is most sensitive to changes in the nucleoside structure, followed by N2 and then by *es*. In addition, some inhibitory profiles of base-modified nucleoside analogs in the inhibition of uridine uptake by *es* are more similar to that of the human intestinal N2 transporter than to that of N1 of the human intestine [16].

Interaction of sugar plus base-modified nucleoside analogs with the human intestinal *es* transporter

Consistent with the individual effects of base and sugar modifications described above, substitution of a hydrogen with a halogen on the 5 position of the base and the removal of oxygen from the 2′ position of the sugar, as in floxuridine (5-fluoro-2′-deoxyuridine) and cladribine (2-chloro-2′-deoxyadenosine), resulted in no change in inhibitory profile of these analogs (Fig. 3) from those of their corresponding individual modifications (see Figs. 1 and 2). However, the addition of a much bulkier trifluoromethyl group to the 5 position on uracil and the removal of oxygen from the 2′ position of the sugar (trifluridine; 5-[trifluoromethyl]-2′-deoxyuridine) resulted in a significant decrease ($P < 0.05$) in inhibitory capacity (Fig. 3). These data suggest that the size of the substituent on the 5 position of uracil may affect the interaction of the nucleoside to the *es* transporter.

Our data show that several clinically important drugs such as ribavirin, ara-A and ara-C are possible permeants of the human intestinal *es* transporter. Although inhibition of ^{3}H-nucleosides by their corresponding analogs can occur even if they are not themselves permeants, data in the literature (where available) indicate that the analogs, which were inhibitors in our assay, are indeed high affinity permeants of the *es* transporter. For example, ribavirin uptake by human erythrocytes involves the *es* transporter [12] and ara-C influx into thymocytes is mediated by a NBMPR-sensitive transporter [18].

In conclusion, the *es* transporter of the human intestine is identical in amino acid sequence to the *es* transporter of the human placenta and human myeloid leukemic cells. In addition, the inhibitory profiles of the nucleosides for the *es* intestinal transporter are virtually identical, where available, to that of the human

erythrocyte *es* transporter. These findings suggest that these two transporters are functionally similar. In addition, the results of this systematic inhibitory profile study permit us to draw several important conclusions concerning possible structural determinants required for interaction with the recombinant human intestinal *es*. Firstly, these results suggest that the ribose moiety is the primary (but not exclusive) critical determinant in maintaining the inhibitory capacity of a nucleoside. Secondly, the 2′ and the 3′ oxygens of ribose synergistically impart inhibitory characteristics to the nucleosides. Thirdly, modifications of the nucleoside base do not affect the capacity of the nucleoside to inhibit uridine uptake by the *es* transporter except for those that result in a charged molecule, such as the modifications in 3-deazauridine and 6-azauridine. These critical structural determinants predict that gemcitabine (2′,2′-difluorodeoxycytidine) should be an excellent permeant of the human *es* transporter. Indeed, as predicted, gemcitabine is a high-affinity substrate of the *es* transporter and this transporter activity is required for manifestation of its toxicity in vitro [14].

Collectively, our findings on the *es* transporter are both similar and different from those previously obtained by us for the Na^+-dependent intestinal N1 and N2 transporters [16]. To retain high inhibitory capacity, the presence of the 3′ oxygen is critical to the nucleosides for both the N1 and the N2 transporters. With respect to substitutions on the base, the N1 transporter is much less tolerant than the N2 transporter. In this respect, the N2 transporter is more similar to the *es* transporter described here than is the N1 transporter. Using these data, mapping studies of the critical structural determinants of the binding sites on these transporters are in progress in our laboratory.

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