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Glycine 154 of the equilibrative nucleoside transporter, hENT1, is important for nucleoside transport and for conferring sensitivity to the inhibitors nitrobenzylthioinosine, dipyridamole, and dilazep

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Abstract

hENT1 and hENT2 are members of the human equilibrative nucleoside transporter family. hENT1 is ubiquitously expressed and plays an important role in the disposition and pharmacological activity of nucleoside drugs and nucleosides, such as adenosine. hENT2 is expressed in only a few tissues (e.g. muscle). hENT1 and hENT2 differ in their affinity for nucleoside substrates and in their sensitivity to inhibitors, such as nitrobenzylthioinosine (NBMPR). hENT1 has higher (or equal) affinity to hENT2 for all natural nucleosides except inosine. hENT1 is also more sensitive to NBMPR inhibition ($IC_{50} \sim 0.4$–8 nM) when compared with hENT2 ($IC_{50} \sim 2.8$ μM). This difference in inhibition potency is substantially dependent on the difference in amino acid at position 154 in hENT1 (glycine) and hENT2 (serine). Since NBMPR competitively inhibits nucleoside transporter activity, we hypothesized that G154 may also play a role in the transport of natural nucleosides and in the inhibition by other hENT1 inhibitors, dipyridamole (DP), and dilazep (DZ). Our results, using a yeast expression system, demonstrate that substituting glycine 154 of hENT1 with serine of hENT2 converts hENT1 to a transporter that exhibits partial characteristics of hENT2. For example, this conversion reduces sensitivity of hENT1 to the inhibitors NBMPR, DP, and DZ and reduces its transport affinity for the natural nucleosides cytidine and adenosine. However, this conversion renders hENT1 less sensitive to inhibition by anti-HIV drugs azidothymidine, dideoxyinosine, and the nucleobase, hypoxanthine. Collectively, these results suggest that glycine 154 plays an important role in the transport of nucleosides and in sensitivity to the inhibitors NBMPR, DP, and DZ.

Keywords: Nucleoside; Transporter; Nitrobenzylthioinosine; Dipyridamole; Dilazep; ddI

1. Introduction

Nucleoside transporters play an important role in physiology by regulating the extracellular concentration of adenosine and by salvaging nucleosides [1]. These transporters are also important in the disposition and pharmacological activity of nucleoside drugs important in the treatment of life-threatening diseases, such as cancer and viral diseases (e.g. hepatitis C) [2,3]. Nucleoside transporters can be divided into two broad classes [4], equilibrative and concentrative. The equilibrative or facilitative transporters are sodium-independent and transport nucleosides down a concentration gradient. In humans, four different equilibrative transporter genes (hENT1–4) have been identified of which two (hENT1 and hENT2) have been functionally characterized [5]. While hENT1 appears to be ubiquitously expressed, hENT2 is expressed primarily in the skeletal muscle, pancreas, prostate, thymus, heart, and brain [6]. Both hENT1 and hENT2 transport purines and pyrimidines but with different affinities [5]. hENT1 transports cytidine, guanosine, thymidine, and adenosine with a higher affinity (∼0.6, 0.14, 0.3, and 0.04 mM) than hENT2 (∼5.6, 2.7, 0.7, and 0.14 mM),

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Abbreviations: NBMPR, nitrobenzylthioinosine; ENT, equilibrative nucleoside transporter; DP, dipyridamole; DZ, dilazep; h, human; r, rat; G, glycine; AZT, 3′-azido-3′-deoxythymidine or azidothymidine; ddC, dideoxycytidine; ddI, dideoxyinosine; pCMBS, *p*-chloromercuribenzenesulfonic acid; HX, hypoxanthine.

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while hENT2 transports inosine with a higher affinity than hENT1 (~0.05 mM vs. 0.17 mM). Moreover, unlike hENT1, hENT2 can transport nucleobases [7]. The two transporters also differ strikingly in their sensitivity to inhibition by the inosine analog, NBMPR [1,5]. NBMPR is a potent inhibitor of hENT1 ($IC_{50} \sim 0.4$–8 nM) while it is a moderate inhibitor of hENT2 ($IC_{50} \sim 2.8$ μM) [5,8]. Likewise, DP and DZ inhibit hENT1 more potently ($K_i \sim 48$ and 19 nM) than hENT2 ($K_i \sim 6.2$ and 134 μM) [9].

The amino acid residues of hENT1 and hENT2 that confer their difference in the affinity for the nucleosides and in sensitivity to inhibitors, have recently begun to be ascertained. For example, expression of rat/human ENT1 chimeras in *Xenopus* oocytes have revealed that transmembrane domains (TMD) 3–6 are important for the differences in sensitivity of hENT1 and rENT1 to DP [10]. Likewise, rENT1/rENT2 chimeras have also implicated TMD 5 and 6 of rENT2 in nucleobase translocation [7] and TMD 3–6 of rENT1 for binding to NBMPR [11]. Within TMD 3–6, through random mutagenesis and functional complementation in yeast, our laboratory has shown that glycine 179 is critical for nucleoside transport function. Mutations at this position reduce sensitivity of hENT1 to NBMPR but do not change the affinity of nucleosides for the transporter [8]. Point mutations outside the domains 3–6 have also been shown to alter sensitivity of hENT1 to inhibitors. For example, using a yeast-based random mutagenesis strategy, Visser *et al*. have identified two critical amino acids, Met33 [9] and Asn338 [12], important in sensitivity to inhibitors. Mutation of Met33 reduces sensitivity of hENT1 to DP and DZ inhibition, while mutation of Asn338 reduces sensitivity of hENT1 to the inhibitors draflazine, soluflazine, DP, and DZ. However, none of these amino acids completely account for the above listed differences between hENT1 and hENT2 in nucleoside affinity and inhibitor sensitivity. In the present work, we have focused on glycine 154 of hENT1 which has been previously implicated in hENT1 sensitivity to NBMPR [13]. However, in that report, Hyde *et al*. did not provide a detailed characterization of this observation nor the importance of this residue, if any, on the affinity for the natural nucleosides. In this report, we provide the first detailed characterization of the contribution of this residue to sensitivity of hENT1 to NBMPR, DP, and DZ and to the difference in affinity of hENT1 and hENT2 for the natural nucleosides.

2. Materials and methods

2.1. Construction of plasmids

Plasmid p426GPD-hENT1 was prepared by cloning only the coding region of hENT1 cDNA, including the stop codon between *Eco*RI and *Hin*dIII site in the plasmid p426GPD [14]. Briefly, hENT1 was amplified using primers 5′-AACTAGTGGATCCCCCGGGCTGCAGGAATTGGACCATGACAACCAGTCAC-3′ and 5′-ATGACTCGAGGTCGACGGTATCGATAAGCTGTCACACAATTGCCCG-3′ and the resulting fragment, with overhangs homologous to the sequences at the end of p426GPD cut with *Hin*dIII and *Eco*RI, was co-transformed with vector p426GPD cut with *Hin*dIII and *Eco*RI into the YPL1 strain [8]. These subclonings were conducted using the gap repair procedures described earlier [8]. The point mutation G154S was created by a modified gap repair method also described in SenGupta *et al*. [8]. The presence of the G154S mutation was confirmed by sequencing the full coding sequence.

2.2. Nucleoside transport and inhibition studies

Nucleoside transport assays were conducted as described earlier [8], except that the yeast cells (YPL1 strain) expressing hENT1 or G154S variant were grown in glucose-containing media and cells in log phase were used for the transport assays. The transport of all radiolabeled nucleosides used (adenosine, cytidine, and uridine) was linear up to 1 hr. Therefore, all transport experiments were conducted for 20 min. All nucleoside stock solutions were made in 100% DMSO. Final concentration of DMSO in transport buffer was 1%. Final concentration of natural nucleosides used for inhibition experiments, as indicated in the figure legends, varied depending on their reported K_m or IC_{50} values. An estimate of non-mediated uptake of the nucleosides was obtained by conducting transport experiments in the presence of 2 mM adenosine.

For IC_{50} determinations, graded concentrations of unlabeled inhibitors (0–10 mM) were added to transport reactions containing [^{3}H]adenosine (1 μM). IC_{50} values were estimated by fitting the following model to the data by nonlinear regression (WinNONLIN®):

$$E = E_{max} - (E_{max} - E_0)\left(\frac{C}{C + ic_{50}}\right)$$

where E_{max} is the uptake in absence of any inhibitor, E_0 is the uptake not attributed to the transporter, and C is the concentration of the inhibitor.

The K_m for cytidine and adenosine transport was determined by measuring [^{3}H]cytidine or [^{3}H]adenosine transport into yeast cells in the presence of increasing concentrations of unlabeled cytidine (0–5 mM) or adenosine (0–2 mM), respectively. The K_m of transport was determined by nonlinear regression analysis (WinNONLIN®) of tracer displacement curves as described before [15].

3. Results

We first confirmed that glycine 154 of hENT1, when mutated to the corresponding residue in hENT2, serine, rendered the transporter insensitive to NBMPR (Fig. 1). The determination of IC_{50} value of NBMPR for the G154S

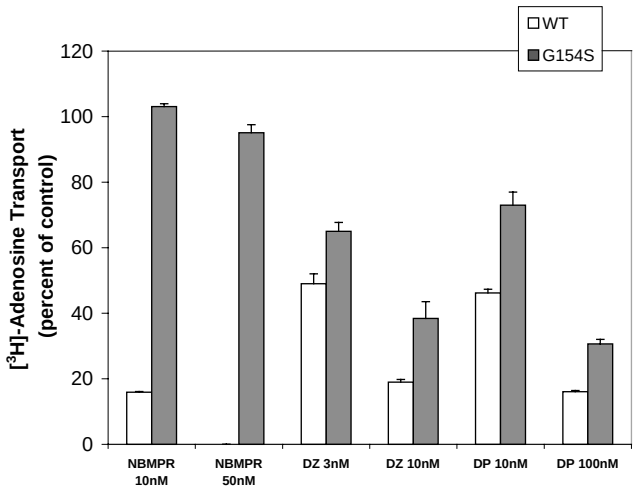

Fig. 1. Inhibition of [^{3}H]adenosine (1 μM) transport by hENT1 (open bars) or G154S (filled bars) mutant, corrected for non-mediated transport, in the absence (control) or the presence of nitrobenzylthioinosine (NBMPR), dilazep (DZ), or dipyridamole (DP). G154S was significantly more resistant to inhibition by the three inhibitors when compared with the wild-type transporter ($P < 0.05$). Each bar represents the mean ± SD of three independent experiments. Note, hENT1-mediated adenosine transport in the presence of 50 nM NBMPR was almost zero and, therefore, not visible in the figure.

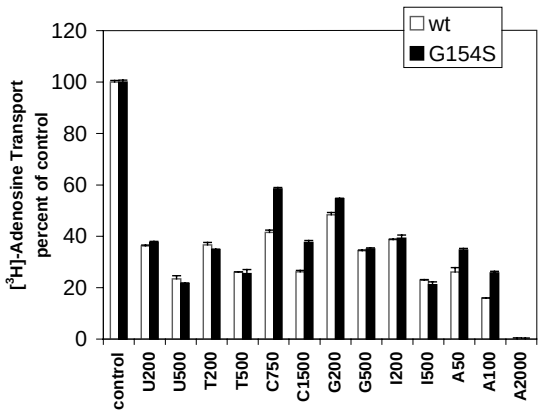

Fig. 2. Inhibition of [^{3}H]adenosine (1 μM) transport by hENT1 (open bars) or G154S mutant (filled bars), corrected for non-mediated transport, in the absence (control) or in the presence of various natural nucleosides. The nucleosides are denoted by a single letter code followed by the concentration (μM) used. Letters U, T, C, G, I, and A denote uridine, thymidine, cytidine, guanosine, inosine, and adenosine, respectively. Only cytidine, guanosine (at 200 μM), and adenosine exhibited significantly reduced ($P < 0.05$) inhibition of the G154S mutant when compared with the wild-type. Each bar represents the mean ± SD of at least three independent experiments.

transporter confirmed this observation (Table 1). This led us to ask if this mutation also alters the sensitivity of the transporter to other hENT1 inhibitors. At fixed concentrations of DP and DZ, significant differences were detected in their capacity to inhibit wild-type hENT1 and the G154S mutant (Fig. 1). The IC_{50} values of DP and DZ for these two transporters revealed a 7- and 3-fold reduced sensitivity of the G154S transporter to these two inhibitors (Table 1).

Since NBMPR competitively inhibits transport of nucleosides by the ENTs, we hypothesized that the G154 residue is also important in the binding and/or translocation of natural nucleosides. We therefore compared the ability of various natural nucleosides to inhibit adenosine transport by the wild-type and G154S mutant at fixed concentrations. These concentrations were chosen to produce approximately 50 and ⩾50% inhibition of the wild-type transporter. If the wild-type or the G154S mutant differ in their sensitivity to the inhibitors, the ability to detect this difference is maximum when the inhibitior concentration used produces 50 or <50% inhibition. Two nucleosides, cytidine and adenosine, exhibited significantly lower ability (in the order listed) to inhibit the G154S mutant compared to the wild-type at both the low and high concentrations. Guanosine showed a modest difference in inhibition at 200 μM but not at 500 μM. This is because, as indicated above, when the inhibitory capacity is only modestly different, the ability to detect a difference in inhibition decreases as the concentration of inhibitor increases. In contrast, uridine, thymidine and inosine did not show such a difference at any concentration tested (Fig. 2). Since cytidine and adenosine showed the maximum difference in their capacity to inhibit G154S and hENT1, we sought to ascertain if this difference translated into a difference in the ability of the two transporters to transport these nucleosides. The K_m value for cytidine or adenosine transport indicated an approximately 3-fold lower affinity of G154S to transport cytidine or adenosine when compared to the wild-type transporter (Table 2). The

Table 1
As demonstrated by higher IC_{50} values, [^{3}H]adenosine (1 μM) transport by the G154S mutant is significantly ($P < 0.05$) more resistant to inhibition by NBMPR, dilazep, or dipyridamole when compared to the wild-type

	hENT1 (nM)	hENT1-G154S (nM)	Ratio of IC_{50} values	
			hENT1-G154S:hENT1	hENT2:hENT1
NBMPR	2.9 ± 0.4	8624 ± 856*	∼2500	∼7000[a]
Dilazep	3.14 ± 0.87	9.0 ± 1.27*	∼3	∼10000[b]
Dipyridamole	9.4 ± 1.2	66.4 ±14*	∼7	∼71[a]

The data are mean ± SD of three independent experiments.
[a]Ref. [5].
[b]Ref. [9], ratio calculated on the basis of K_i values.
*$P < 0.05$.

Table 2
The apparent affinity (K_m) of hENT1-G154S for cytidine and adenosine is significantly ($P < 0.05$) lower than that for the wild-type but that of uridine is not different (mean ± SD; N = 3)

	hENT1		hENT1-G154S		Ratio of K_m values	
	K_m (μM)	V_{max} (pmol/10^7 cells/20 min)	K_m (μM)	V_{max} (pmol/10^7 cells/20 min)	hENT1-G154S:hENT1 ratio	hENT2:hENT1 ratio
Cytidine	207 ± 47	120 ± 23	613 ± 154*	148 ± 46	~3	7.7[a]
Adenosine	11.3 ± 1.2	100 ± 9	32.3 ± 2.6*	101.3 ± 4.6	~3	2.8[a]
Uridine	43 ± 10	89 ± 10	67 ± 22	94 ± 16	~1.5	~1.0[a]

[a]Ref. [5].
*$P < 0.05$.

G154S mutation, however, did not significantly affect the V_{max} of these substrates. Furthermore, as expected from our fixed concentration experiments (Fig. 2), the K_m and V_{max} for uridine transport did not differ between wild-type and G154S. Interestingly, the inhibition of adenosine transport by uridine (Fig. 2) is lower than that expected from the K_m of uridine. This may be due to inhibition by uridine of adenosine transport that is not a simple classical competitive inhibition.

Since hENT2 is more sensitive than hENT1 to inhibition by nucleobases and dideoxynucleosides [5,16], we tested the effect of the G154S mutation on sensitivity to inhibition of [^{3}H]adenosine transport by the anti-HIV dideoxynucleosides, 3′-azido-3′-deoxythymidine or azidothymidine (AZT), dideoxycytidine (ddC), dideoxyinosine (ddI), and by the nucleobase, hypoxanthine (HX) (Fig. 3). Our results show that the G154S mutant is less sensitive to inhibition by AZT (3 mM), ddI (3 mM), and HX (2.5 mM) than the wild-type hENT1 while ddC exhibited no significant difference in inhibition between the two transporters.

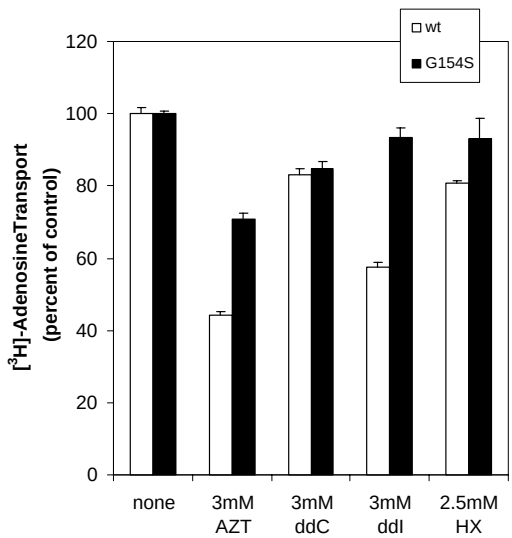

Fig. 3. Inhibition of [^{3}H]adenosine transport (1 μM), corrected for non-mediated transport, by wild-type hENT1 (open bars) or G154S mutant (filled bars) in the absence (control) or presence of various anti-HIV dideoxynucleosides or hypoxanthine (HX). [^{3}H]Adenosine transport by wild-type (open bar) or G154S (filled bar) mutant was measured in the absence of any inhibitors (100%) and in the presence of 3 mM AZT, ddC, ddI, or 2.5 mM HX. G154S mutant was significantly ($P < 0.05$) less sensitive to inhibition by AZT, ddI, or HX when compared with the wild-type. Each bar represents the mean ± SD of three independent experiments.

4. Discussion

Chimera studies with rat ENTs have revealed that TMD 3–6 of ENT2 are important for the lower NBMR sensitivity of rENT2 when compared with rENT1 [11]. Modification by pCMBS of cysteine 140 within TMD 4 of rENT2 resulted in impaired transporter function. Therefore, this amino acid is solvent accessible and probably constitutes part of the substrate binding pocket [17]. The amino acids glycine 154 and serine 141 in hENT1 and hENT2, respectively, are analogous to cysteine 140 of rENT2. Topology models of hENT1, which have been verified experimentally, have predicted that glycine 154 of hENT1 is located on the exofacial aspect of TMD 4 [18]. Thus, as previously reported, we first confirmed that mutation of G154 in hENT1 to the corresponding residue in hENT2, serine, renders the transporter drastically less sensitive to NBMPR inhibition. The sensitivity of G154S to NBMPR inhibition is 2500-fold lower than wild-type hENT1 (Table 1). The IC_{50} value of NBMPR for G154S is comparable to those published for hENT2 (~2.8 μM) [5], suggesting that mutation of glycine 154 of hENT1 to serine of hENT2 renders the mutant as insensitive to NBMPR as wild-type hENT2. Interestingly, our studies have revealed that G154 is also important in determining sensitivity of hENT1 to inhibition by DP and DZ (Table 1). While the G154S mutation reduced the sensitivity of hENT1 to DZ approximately 3-fold, it reduced the sensitivity to DP by approximately 7-fold. These ratios, however, are smaller than the ratios of IC_{50} values of hENT2 and hENT1, indicating that G154S is only partially responsible for the higher sensitivity of hENT1 towards inhibition by DP and DZ.

Besides being important for the difference in sensitivity of hENT1/2 to the inhibitors NBMPR, DP, or DZ, glycine 154 also plays a role in the transport of natural nucleosides. Inhibition studies revealed that the affinities of cytidine and adenosine for hENT1 are likely to be significantly affected when glycine 154 of hENT1 is mutated to serine (Fig. 2).

Determination of kinetics of transport of cytidine and adenosine revealed that the K_m for transport of these nucleosides was 3-fold greater for G154S mutant than that for the wild-type hENT1. This compares favorably with the difference in affinity of hENT1 and hENT2 for these nucleosides (Table 2). When compared with hENT1, hENT2 has approximately 7- and 3-fold higher K_m for cytidine and adenosine transport. However, this mutation does not render hENT1 like hENT2 with respect to affinity for inosine or thymidine as evidenced by a lack of difference in the ability of these natural nucleosides to inhibit adenosine transport by G154S and wild-type hENT1. hENT1 has lower affinity (K_m) for inosine (0.17 mM) [5], than hENT2 (0.05 mM) while it has a higher affinity to transport thymidine (0.30 mM vs. 0.71 mM) [5]. Thus, other amino acid residues within hENT1/2 are likely important in determining the difference in affinity between the two transporters for these natural nucleosides. In addition, because the K_m for uridine transport did not change due to G154S mutation (Table 2), this suggests that the G154S mutation does not produce a general structural change in hENT1 that reduces affinity for all substrates.

Recently, hENT2 has been shown to transport anti-HIV drugs, such as AZT, ddC, and ddI, more efficiently than does hENT1 [19]. These data are in agreement with the observation that hENT2 can transport nucleobases, such as adenine and HX [7]. That is, unlike hENT1, the sugar ring is not required for a compound to be a substrate of hENT2. Moreover, we and others have shown that, even when the nucleoside has an intact sugar ring, the nucleoside must possess a 3′-hydroxyl group to be a recognized [16,20]. If it is substituted, as is the case in dideoxynucleosides (including anti-HIV dideoxynucleosides), such a substitution renders the nucleoside a poor substrate/inhibitor of hENT1. Therefore, we asked if the G154S mutation reduces the dependency of hENT1 on the presence of the ribose ring and converts it into an hENT2-like transporter sensitive to inhibition by nucleobases or dideoxynucleosides. Surprisingly, we found that the G154S mutation rendered the transporter less sensitive to inhibition by the dideoxynucleosides, AZT and ddI, and the nucleobase, HX. ddC, which, amongst the three anti-HIV dideoxynucleosides tested, exhibited the least inhibition of wild-type hENT1, did not show a significant difference in inhibition between the G154S mutant and the wild-type hENT1. These data suggest that amino acid residues other than G154 likely play a role in the binding of these anti-HIV dideoxynucleosides to hENT1.

Single amino acid residues within hENT1 have previously been shown to have multiple effects on the hENT1 protein. For example, glycine 179 of hENT1 (which is conserved in hENT2) has been shown to be important for binding to both NBMPR and DP [8]. Another residue of hENT1 implicated in the binding of DZ and DP is methionine 33 [9]. Changing this residue to isoleucine (present in hENT2) increases the IC_{50} of hENT1 for DP and DZ. Surprisingly, none of these residues, when mutated, seem to affect the affinity of hENT1 for natural nucleosides. Since NBMPR, DP, and DZ are competitive inhibitors of hENT1, these data suggest that multiple amino acid residues differentially contribute to the binding of hENT1 to various inhibitors and substrates. That is, the natural nucleosides and the inhibitors have overlapping binding sites. Our studies, reported here, suggest that glycine 154 is one such overlapping site. This residue is not only important for the binding and/or translocation of natural nucleosides, cytidine, adenosine, and guanosine (but not thymidine or inosine), but also plays a role in conferring sensitivity to NBMPR, DP, and DZ. Alternatively, the effect of G154S mutation on substrate and inhibitor binding could be due to alteration in tertiary structure of the transporter. However, such alteration is unlikely, as this would be expected to have an effect on the binding of all substrates and inhibitors rather than the selective ones observed here. This is illustrated by the lack of significant difference in K_m and V_{max} of uridine transport between wild-type and G154S mutant. Nevertheless, to demonstrate that glycine 154 residue is close to the substrate translocation channel, we substituted glycine 154 with cysteine to assess solvent accessibility of this residue with a sulfahydryl reactive reagent. However, these studies could not be completed as this substitution rendered the transporter nonfunctional.

In conclusion, our studies have revealed for the first time an amino acid residue of hENT1, G154, which selectively affects the affinity of hENT1 for the nucleosides, cytidine and adenosine, and plays a role in conferring sensitivity to the transporter to inhibition by NBMPR, DP, and DZ. Mutating this residue to an analogous residue in hENT2, serine, renders the transporter hENT2-like with respect to transport affinity for cytidine, adenosine, and sensitivity to inhibition by NBMPR. However, it does not render the transporter hENT2-like with respect to sensitivity to inhibition by dideoxynucleosides and nucleobases.

Acknowledgments

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