Membrane Transporters as Targets for the Development of Drugs and Therapeutic Strategies

Molecular Basis of Nucleobase Transport Systems in Mammals

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Nucleobases are water-soluble compounds that need specific transporters to cross biological membranes. Cumulative evidence based on studies using animal tissues and cells indicates that the carrier-mediated transport systems for purine and pyrimidine nucleobases can be classified into the following two types: concentrative transport systems that mediate nucleobase transport depending on the sodium ion concentration gradient; and other systems that mediate facilitated diffusion depending on the concentration gradient of the substrate. Recently, several molecular transporters that are involved in both transport systems have been identified. The function and activity of these transporters could be of pharmacological significance considering the roles that they play not only in nucleotide synthesis and metabolism but also in the pharmacokinetics and delivery of a variety of nucleobase analogues used in anticancer and antiviral drug therapy. The present review provides an overview of the recent advances in our understanding of the molecular basis of nucleobase transport systems, focusing on the transporters that mediate purine nucleobases, and discusses the involvement of intracellular metabolism in purine nucleobase transport and chemotherapy using ganciclovir.

Key words purine nucleobase; transporter; salvage enzyme; equilibrative nucleoside transporter 1 (ENT1)/solute carrier (SLC)29A1; ENT2/SLC29A2; equilibrative nucleobase transporter 1 (ENBT1)/SLC43A3

1. INTRODUCTION

In addition to being the essential building blocks of DNA and RNA, nucleobases are the basic components of nucleosides and nucleotides. In particular, purine nucleosides and their related nucleotides such as ATP, inosine monophosphate (IMP), and cyclic guanosine monophosphate (cGMP), which possess purine nucleobases as the fundamental structures, play an important role in energy storage and signal transduction.\(^1\) The synthesis of purine nucleotides occurs via two distinct pathways. The \textit{de novo} pathway assembles nucleotides from basic materials such as amino acids, bicarbonate, and ribose, and the salvage pathway utilizes purine nucleobases generated from the degradation of nucleosides and nucleotides.\(^2,3\) The latter is advantageous in saving energy and materials for nucleotide production, which many types of cells physiologically adopt. This pathway can salvage not only intracellular purine nucleobases and nucleosides but also extracellular ones from dietary sources and those overproduced in other cells by the \textit{de novo} pathway.\(^4\) Because purine nucleobases are hydrophilic, they cannot permeate biological membranes by simple diffusion. The salvage pathway likely includes a carrier-mediated transport system in the plasma membrane. Although the existence of purine nucleobase transport systems has been previously reported in a variety of mammalian cells and tissues,\(^5,6\) the underlying molecular mechanisms remain to be elucidated. Recently, several solute carrier (SLC) transporters such as sodium-dependent nucleobase transporter 1 (SNBT1)/SLC23A4,\(^7\) equilibrative nucleoside transporter 1 (ENT1)/SLC29A1,\(^8\) ENT2/SLC29A,\(^9\) and equilibrative nucleobase transporter 1 (ENBT1)/SLC43A3\(^10\) have been reported to mediate the transport of purine nucleobases, and their functions have been characterized. Specifically, ENBT1/SLC43A3 is a novel transporter that has been more recently identified as a selective purine nucleobase transporter. These transporters could be promising as pharmacological targets in delivering the structural analogues of nucleobases, such as anticancer and antiviral drugs.

This review summarizes published information on the above-mentioned four SLC transporters, focusing on the functions of nucleobase transport, and discusses their physiological role in purine metabolism. Furthermore, an attempt is made to highlight the involvement of intracellular purine metabolism in the evaluation of transporter activities of purine nucleobases and in chemotherapy involving the use of ganciclovir (GCV).

2. CARRIER-MEDIATED TRANSPORT SYSTEMS FOR PURINE NUCLEOBASES IN MAMMALS

There are multiple transport systems for transporting nucleobases, which can be categorized into two types: the concentrative transport that mediates a unidirectional influx coupled with a \textit{Na}\(^+\) gradient; and the facilitated diffusion that mediates a bidirectional flux across the plasma membrane depending on the substrate concentration gradient. These transport systems could be involved in the disposition of nucleobases and the drugs that mimic them, and it is important to understand their underlying molecular basis. Recent advances in molecular biology techniques have clarified the molecular
basis of purine nucleobase transport systems, which involve a concentrative transporter (sodium-dependent nucleobase transporter 1; SNBT1\(^7\)) and three facilitative transporters (ENT1,\(^8\) ENT2,\(^9\) and ENBT1/SLC43A3\(^10\)). Their substrate specificities as nucleobase transporters are summarized in Table 1.

3. CONCENTRATIVE TRANSPORT SYSTEMS

3.1. SNBT1/Slc23a4

Rat SNBT1 (rSNBT1; also known as Slc23a4), which was first cloned from the rat small intestine,\(^7\) is the only transporter identified to date that mediates the specific and concentrative transport of nucleobases in vertebrates. SNBT1 belongs to the SLC23 family comprising four members, including the Na\(^+\)-dependent ascorbate transporters such as sodium-dependent vitamin C 1 (SVCT1)/SLC23A1 and SVCT2/SLC23A2.\(^11\) Although SVCT1 and SVCT2 are highly conserved across species, human SNBT1 demonstrates a pseudogene, which lacks several exons that encode the transmembrane domains (TMDs); SNBT1 orthologues are present in vertebrates other than primates, such as zebrafish, chickens, mice, dogs, and horses.\(^7\) Because similar pseudogenes are found in the genome of higher primates, human SNBT1 seems to have been silenced during the course of evolution.

rSNBT1 consists of 614 amino acids and is 50% identical to the rat homologues of SVCT1 and SVCT2.\(^7\) Based on hydropathic analysis, the rSNBT1 structure was predicted to contain 12 transmembrane domains and two potential N-glycosylation sites. Northern blotting and RT-PCR analyses revealed that rSNBT1 is specifically expressed in the small intestine, with higher levels in the lower region (ileum>jejunum>duodenum).\(^7\) Although the localization of rSNBT1 in the rat gut has not been immunohistologically demonstrated, green fluorescent protein (GFP)-tagged rSNBT1 is localized in the apical membrane of polarized Madin–Darby canine kidney (MDCK) cells, which have been commonly used to examine the cellular distribution of membrane proteins.\(^7\) This suggests that rSNBT1 is located on the luminal side (the brush border membrane side) of intestinal epithelial cells.

rSNBT1 mediates the Na\(^+\)-dependent active transport of purine and pyrimidine nucleobases and nucleobase-like drugs. In a heterologous expression system using HEK293 cells, rSNBT1 was reported to mediate the transport of purine nucleobases (guanine, hypoxanthine, and xanthine, but not adenine), pyrimidine nucleobases (thymine and uracil, but not cytosine), and uridine; thymidine and ascorbate were not found to be transported.\(^7\) This transport was driven by the Na\(^+\) concentration gradient across the plasma membrane, with a Na\(^+\)/substrate stoichiometry of 1:1. The uracil uptake mediated by rSNBT1 can be significantly inhibited by dipyrindamole but not by nitrobenzylthioinosine (NBMPR), papaverine, or phlorizin.\(^7\) Kinetic analysis and inhibition studies revealed that the \(K_m\) values and IC\(_{50}\) values of uracil, guanine, hypoxanthine, and thymine are in the range of 10–100 \(\mu\)M (Table 1). rSNBT1 can be significantly inhibited by 5-fluorouracil (5-FU), oxypurinol, and 6-mercaptopurine,\(^7\) suggesting that it can recognize nucleobase derivatives.

The functional characteristics of rSNBT1 are in agreement with those of the carrier-mediated transport systems of uracil and hypoxanthine in the animal gut. Studies using the everted gut sacs of rats revealed that the uptake of uracil was abolished in the absence of Na\(^+\) and was saturable with an apparent \(K_m\) value of 40.3 \(\mu\)M.\(^7\) Similarly, the Na\(^+\)-dependent transport system for 5-FU was studied using rat jejunal tissue rings (with an apparent \(K_m\) value of 74 \(\mu\)M) and rabbit ileum mounted using Ussing chambers.\(^7\) Theisinger et al. demonstrated the Na\(^+\)-dependent uptake of hypoxanthine into the brush border membrane vesicles using calf proximal jejenum, with a reported \(K_m\) value of 165 \(\mu\)M, and the uptake was not inhibited by adenine and nucleosides.\(^7\) These suggest that SNBT1 plays a key role in the intestinal absorption of nucleobases from ingested food in vertebrates other than higher primates.

3.2. SNBT1-Like Transport Systems

Na\(^+\)-dependent nucleobase transport systems have been observed not only in the gut but also in several cell lines and membrane vesicles derived from nonintestinal tissues, and the transport systems have been found to include unique inhibitor/substrate specificities distinct from those of SNBT1. Such SNBT1-like transport systems are present in LLC-PK1\(^15–17\) and OK cells.\(^8\)

Table 1. Kinetic Properties of Concentrative and Equilibrative Transporters for Nucleobases

<table>
<thead>
<tr>
<th>Nucleobase</th>
<th>Concentrative</th>
<th>Facilitative</th>
<th>Average level in plasma or serum (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m) or IC(_{50}) value (mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rSNBT1/Slc23a4(^7)</td>
<td>hENT1/SLC29A1(^8)</td>
<td>hENT2/SLC29A2(^8)</td>
</tr>
<tr>
<td>Adenine</td>
<td>N.D.</td>
<td>3.2</td>
<td>1.8, 1.1</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>0.0174*</td>
<td>6.0</td>
<td>1.5, 0.7</td>
</tr>
<tr>
<td>Guanine</td>
<td>0.0807*</td>
<td>P.N.D.</td>
<td>P.N.D.</td>
</tr>
<tr>
<td>Thymine</td>
<td>0.0127*</td>
<td>6.3</td>
<td>6.0, 1.7</td>
</tr>
<tr>
<td>Uracil</td>
<td>0.0212</td>
<td>P.N.D.</td>
<td>2.6</td>
</tr>
<tr>
<td>Cytosine</td>
<td>N.D.</td>
<td>P.N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>0.0690*</td>
<td>2.3</td>
<td>2.6, &gt;5</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>&gt;0.1*</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Nucleoside</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uridine</td>
<td>&gt;0.1*</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Ganciclovir</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

*IC\(_{50}\) value. N.D., specific uptake was not detected. P.N.D., parameters were not determined. N.A., data not available.
derived from the renal proximal tubules of the pig and opossum, respectively. These transport systems recognize purine and pyrimidine nucleobases, with the exception of adenine, with Na\textsuperscript+-substrate stoichiometry of 1:1.\textsuperscript{15} The functional localization of hypoxanthine uptake was on the apical side of LLC-PK1 monolayers.\textsuperscript{15} Although these characteristics seem to be comparable to those of rSNBT1, the substrate affinities were much higher, as indicated by lower $K_m$ values of 0.79\textsuperscript{15} and 0.78 $\mu$M\textsuperscript{15} for hypoxanthine in LLC-PK1 and OK cells, respectively; these values are more than 20-fold lower than the IC\textsubscript{50} value of rSNBT1 (17.4 $\mu$M).\textsuperscript{7} Furthermore, dipyridamole, a moderate rSNBT1 inhibitor (IC\textsubscript{50}>0.1 mM), was reported to reduce the transport of hypoxanthine in LLC-PK1 and OK cells markedly ($K_i$ values; 4.6\textsuperscript{15} and 0.9 $\mu$M\textsuperscript{18}, respectively). Likewise, such high-affinity Na\textsuperscript+-dependent nucleobase transport systems were observed in brush border membrane vesicles derived from the guinea pig renal cortex.\textsuperscript{19} Furthermore, other types of SNBT1-like transport systems have been reported in the guinea pig placenta\textsuperscript{20} and the rabbit choroid plexus,\textsuperscript{21} albeit with some differences regarding the substrate specificity and Na\textsuperscript+-substrate stoichiometry from those observed in LLC-PK1 and OK cells, as well as in rSNBT1. This evidence strongly implies the existence of transporters other than SNBT1 in the Na\textsuperscript+-dependent nucleobase transport systems. However, to date, the molecular basis of these nucleobase transport systems remains unclear. More recently, the group of Hediger and colleagues has suggested the possibility that SLC23A3, which is an orphan transporter belonging to the SLC23 family expressed in the yolk sac, kidney, and placenta in mice, and only in the kidney in humans, may function as a nucleobase transporter.\textsuperscript{22} Immunohistochemical analysis indicated that mouse Scl23a3 localizes at the apical membrane of renal proximal tubular cells,\textsuperscript{22} highlighting its physiological role in the renal reabsorption of any compound. However, electrophysiological and uptake assays in the expression systems of *Xenopus laevis* oocytes and HEK293 cells failed to demonstrate nucleobase transport activities, which prompted the authors to speculate on the involvement of a regulatory subunit in modulating the transport.\textsuperscript{22} Further research is required to elucidate the molecular mechanisms underlying Na\textsuperscript+-dependent nucleobase transport systems.

Notably, there are no reports on the saturable and Na\textsuperscript+-dependent nucleobase transport system in tissues and cell lines derived from humans. This may imply the unique handling of nucleobases in the human body, supporting the evidence that SNBT1 is genetically deficient in higher primates. Interestingly, a genetic deficiency in uricase, which is an enzyme that converts purine nucleobases to the final metabolite of urate, is also known in higher primates as a case of genetic silencing during the course of evolution.\textsuperscript{23,24} The physiological relationship between the two genes is currently unclear. Therefore, the possibility should be considered that the metabolism and disposition of exogenous nucleobases and nucleobase analogues in humans may differ from those in experimental animals such as the mouse, rat, and dog, which are commonly used in preclinical studies in drug development and discovery.

4. FACILITATIVE TRANSPORT SYSTEMS

4.1. **ENT1/SLC29A1** ENT1 was first cloned from human placenta as a NBMPR-sensitive nucleoside transporter.\textsuperscript{25} ENT1 is a member of the SLC29 family comprising four members in humans: ENT1; ENT2; ENT3; and ENT4.\textsuperscript{26} These ENT-type transporters have been found in many higher eukaryotes, and their mammalian homologues are highly conserved across species. All ENTs are predicted to contain 11 transmembrane domains with a cytoplasmic N-terminus, an extracellular C-terminus, and a large extracellular loop between the first and second transmembrane domains.\textsuperscript{27} The amino acid homology between rat and human ENT1 is more than 80%.\textsuperscript{28} ENT1 cloned from rats and humans facilitates the diffusion of a broad range of purine and pyrimidine nucleosides across the plasma membrane, with $K_m$ values in the range of 0.05–0.68 mM, which indicate relatively high affinity. The most notable characteristic of ENT1-mediated transport is that it can be inhibited by extremely low concentrations of NBMPR and dipyridamole; for example, their respective $K_i$ values for human ENT1 (hENT1) are 6 and 48 nM, respectively.\textsuperscript{29} (Detailed information on the physiology and function of ENTs as nucleoside transporters can be found elsewhere.\textsuperscript{30,31})

Recently, Yao et al. have demonstrated that, in the *Xenopus laevis* oocyte expression system, ENT1 has the capability to transport not only nucleosides but also nucleobases such as adenine, hypoxanthine, guanine, thymine, and uracil\textsuperscript{19} (Table 1). Although the affinity and efficiency values of ENT1 for nucleobases are much lower than those for nucleosides\textsuperscript{3,26} the transport activities are obvious. The uptake of adenine mediated by hENT1 can be strongly inhibited by NBMPR, with an IC\textsubscript{50} value of 4.01 nM, in addition to being competitively inhibited by uridine, a nucleoside substrate of ENT1. More recently, Huang et al. have assessed the physicochemical interactions between purified recombinant hENT1 and its nucleobase substrates using isothermal titration calorimetry and found adenine-specific binding with a dissociation constant ($K_d$) of 5 mM.\textsuperscript{30} Those results suggest that ENT1 recognizes nucleobases at the substrate binding site of nucleosides.

The physiological role and contribution of ENT1 in nucleobase transport remains unclear. ENT1 is ubiquitously expressed in rodents and humans and found abundantly in the frontal and parietal lobes of the cerebral cortex and renal tubular epithelial cells. The cellular localization of ENT1 primarily occurs at the basolateral membrane of polarized cells such as intestinal epithelial cells.\textsuperscript{30} Thus, ENT1 is principally involved in the flux of its substrates between the blood and cells. However, the nucleobase transport specific to ENT1 is not observed in the tissues and primary cells of animals that show high ENT1-mediated nucleoside transport activity. Hypoxanthine uptake is neither inhibited by dipyridamole in mouse primary microvascular endothelial cells, which exhibit considerable transport activity of 2-chloroadenosine by Ent1, nor significantly altered in cells from Ent1-deficient mice.\textsuperscript{34} However, one study reported the involvement of ENT1 in hypoxanthine transport in a murine T-lymphoma cell line, S49, which was mostly inhibited by 10 nM of NBMPR.\textsuperscript{35}

4.2. **ENT2/SLC29A2** Human ENT2 (hENT2) was identified as a NBMPR-insensitive nucleoside transporter by Crawford et al.\textsuperscript{36} and Griffiths et al.\textsuperscript{37} The amino acid sequence of hENT2 is 46% identical to that of hENT1. hENT2 is ubiquitously expressed, abundant in the skeletal muscle, and localized at the basolateral membrane in polarized cells. In humans and rats, ENT2 transports purine and pyrimidine nucleosides with lower affinities than those of ENT1, with the
exception of inosine. However, ENT2 can transport purine and pyrimidine nucleobases with higher activities than those of ENT1. Yao et al. demonstrated that uptake clearances (the values of $V_{\text{max}}/K_m$) of hENT2 for nucleobases are higher than those for nucleosides in the expression system of *Xenopus laevis* oocytes; however, the nucleobase $K_m$ values are much higher than those for nucleosides. This high efficiency of ENT2-mediated nucleobase transport implies its involvement in the salvage pathways of nucleobases. NBMPR-insensitive and nucleoside-inhibitable nucleobase transport has been reported in CHO and Novikoff cells (strain NISI-67), MCFT-7, a human umbilical vein endothelial cell line (ECV304), and a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB2 cells).

There are limited reports on ENT2 deficiency in mice. Eckle et al. found that Ent2-/- mice showed attenuated pulmonary edema and improved gas exchange following acute lung injury, and their survival rates were significantly higher than those of the wild-type mice. Given that extracellular adenosine plays a physiological role as a signaling molecule, the deletion of Ent2 in mice caused elevated adenosine levels in the bronchoalveolar fluid, thereby facilitating a beneficial effect. This raises the possibility that ENT2 expressed in the lung plays a role in the regulation of extracellular adenosine levels, rather than salvaging the intracellular purine. Genomic analysis revealed that single-nucleotide polymorphism (SNP) frequency in ENT2 is extremely low, suggesting that it plays a crucial role in nucleotide synthesis and metabolism. Further studies are needed to elucidate the role of ENT2 as a nucleobase transporter.

### 4.3. ENBT1/SLC43A3

SLC43A3 has recently been identified as a facilitative purine-selective nucleobase transporter. Based on its molecular and functional characteristics, the SLC43A3 protein was designated "equilibrative nucleobase transporter 1 (ENBT1)." However, because the term "ENBT1" was previously used to denote a putative transporter and a system that mediates dipyridamole-insensitive hypoxanthine transport in cells such as primary human cardiac microvascular endothelial cells, the use of "ENT2" as an alias for SLC43A3 would cause confusion. The present review advocates the use of either SLC43A3 or the same alongside ENBT1.

SLC43A3 is a member of the SLC43A family comprising two other members of Na+-independent neutral L-amino acid transporters, LAT3/SLC43A1 and LAT4/SLC43A2, both of which mediate electroneutral and facilitative transport of neutral amino acids such as L-leucine, L-valine, and L-phenylalanine. The transmembrane topology of SLC43A3 is comparable to that of LAT3 and LAT4, which have 12 TMDs, an extracellular loop between TMD1 and TMD2 with a putative N-glycosylation site, and large intracellular loops between TMD6 and TMD7 with weak amino acid homologies between them (approximately 27% identical). This structural similarity suggests that facilitated diffusion may be common to members of the SLC43A family.

The function of SLC43A3 as a transporter remained unclear for a long time. Mouse Scl43a3 was first identified in 2001 as an orphan transporter, which was found to be upregulated during tubulogenesis in a cell culture model of kidney development. This transporter is highly expressed in several embryonic epithelial tissues such as lung and liver; therefore, it was named embryonic epithelia gene 1 (EEG1). Although subsequent functional identification of LAT3 and LAT4 suggested that EEG1 acted as an amino acid transporter, functional screening using several types of amino acids could not confirm its role as a transporter in *Xenopus laevis* oocytes expressing human SLC43A3. This issue was resolved by a study attempting to identify the cDNA of a purine nucleobase transporter using comprehensive functional screening, in which human SLC43A3 was found to stimulate the accumulation of [3H]adenine in HEK293 cells. Currently, SLC43A3 is considered to be a molecular entity of the facilitative purine-selective nucleobase transport system involved in the uptake of adenosine and hypoxanthine in many types of cells.

The substrate specificity of ENBT1/SLC43A3 is similar to that of Na+-independent purine nucleobase transport systems that are different from those of ENT1 and ENT2, which include carrier-mediated transport systems characterized in many types of cells, including human erythrocytes, COR-L239, LLC-PK1, PK15NTD, rat cortical neurons, mouse primary microvascular endothelial cells, and rabbit cornea epithelial cells. ENBT1/SLC43A3 selectively mediates the transport of purine nucleobases such as guanine, hypoxanthine, xanthine, and adenosine, but not that of nucleosides. Uracil, a pyrimidine nucleobase, is recognized by ENBT1/SLC43A3 as a substrate, albeit weakly, whereas thymine, a methylated derivative of uracil, is not. Purine analogues such as 6-thioguanine, 6-mercaptopurine, acyclovir, and GCV inhibit the [3H]adenine uptake induced by ENBT1/SLC43A3 introduced in MDCK cells, but 5-FU, a uracil analogue, shows no such effect. The IC50 value of GCV was found to be 1.67 µM. Therefore, only purine derivatives seem to have an affinity for ENBT1/SLC43A3. The order of affinity of purine nucleobases for ENBT1/SLC43A3 with IC50 values are as follows: adenosine (13 µM) > guanine (70 µM) > hypoxanthine (350 µM). This indicates that ENBT1/SLC43A3 has a higher affinity for purine nucleobases than ENT1 and ENT2 (Table 1). Dipyridamole, a potent inhibitor of both ENT1 and ENT2, has no effect on cellular [3H]adenine uptake. NBMPR, a potent inhibitor of ENT1, inhibits the uptake partially at 200 µM. Decynium-22, which is a known inhibitor of organic cation transporters such as OCT3/SLC22A3 and PMAT/SLC29A4, is sufficiently potent to inhibit cellular [3H]adenine uptake almost completely at 10 µM. It was reported that decynium-22 inhibited [3H]adenine and [3H]guanine uptake in PK15NTD cells with IC50 values of 0.35 and 0.58 µM, respectively.
Human ENBT1/SLC43A3 is expressed abundantly in the liver, lung, and heart, and ubiquitously in the pancreas, testis, thymus, placenta, and kidney.\textsuperscript{10,48} Immunohistochemical studies showed that ENBT1/SLC43A3 is localized on the sinusoidal membrane of human hepatocytes\textsuperscript{49} and on the basolateral membrane of epithelial cells of the proximal convoluted tubule in the human kidney.\textsuperscript{48} Consistent with these results, GFP-tagged ENBT1/SLC43A3 is localized on the basolateral membrane of polarized MDCKII cells.\textsuperscript{50} However, the regulatory mechanism of intracellular trafficking of ENBT1/SLC43A3 has not yet been clarified.

Considering its functional characteristics and cellular localization, ENBT1/SLC43A3 possibly regulates the influx or efflux of purine nucleobases, subject to their concentration gradients, across the membrane between blood and tissues. In the liver, ENBT1/SLC43A3 may be involved in the efflux of purine nucleobases resulting from excessive de novo purine nucleotide synthesis. This was supported by a single-pass rat liver perfusion study, which reported that inosine and nucleobases such as adenine, hypoxanthine, and xanthine were released from the liver into the perfusate at a concentration ranging from 0.2 to 0.4 \textmu M, while adenosine was undetectable.\textsuperscript{51} Purine nucleobases could be effluxed from the liver to the systemic circulation and subsequently taken up by the extrahepatic tissues expressing ENBT1/SLC43A3, such as the lung and heart. Therefore, the transport of purine nucleobases between the liver and lung could be of physiological relevance considering that the lung receives blood from the liver before being distributed to other organs through the systemic arteries.

The expression of SLC43A3 and its alteration have been discussed in studies on comprehensive transcriptome analyses with the use of several tissues and cells. SLC43A3 was identified as one of the 58 endothelial-specific markers expressed in different mammalian tissues, including brain endothelial cells.\textsuperscript{52} The hypoxanthine uptake mediated by the Na\textsuperscript{+}-independent dipyridamole-insensitive transport system that is inhibited by adenine, which is comparable to that mediated by ENBT1/SLC43A3, occurs in microvascular endothelial cells isolated from the human heart and mouse skeletal muscle.\textsuperscript{34,66} The induced SLC43A3 expression may be implicated in inflammation and carcinogenesis. A study on endotoxemia showed that SLC43A3 is one of the six genes that are significantly upregulated in circulating monocytes isolated from healthy volunteers infused with lipopolysaccharide.\textsuperscript{59} In addition, interferon-\gamma induces Slc43a3 expression in DC2.4 cells, which is a mouse dendritic cell line.\textsuperscript{60} Furthermore, a study on radiation carcinogenesis in humans found that SLC43A3 expression in cancerous thyroid tissue is higher than normal and occurs in a radiation dose-dependent manner.\textsuperscript{61} Saare et al. reported that epigenetic regulation of SLC43A3 occurs in the human endometrium, which is a unique organ that possesses a dynamic DNA methylation pattern depending on the menstrual cycle phase to regulate its own breakdown and regeneration.\textsuperscript{62} DNA methyleome analysis of the endometrium revealed that SLC43A3 is one of the hypermethylated genes during the mid-secretory phase when compared with those during the late phase. A CpG site in the SL&C43A3 promoter region was found to be differentially methylated in patients with endometriosis. In a report highlighting the pathophysiological role of SLC43A3, a novel fusion oncogene was identified following transcriptome analysis of cells derived from angiosarcoma patients.\textsuperscript{63} This oncogene was found to encode an in-frame fusion protein of SLC43A3 and NUP160, a constituent of the nuclear pore complex, which lacks the first portion of SLC43A3 amino acids including the TMDs from TMD1 to TMD5, implying its dysfunction as a membrane transporter. Transfection of the fusion gene in normal microvascular endothelial cells altered the expression of angiogenesis-related genes, in a fashion similar to that observed in angiosarcoma cells, by an unknown mechanism. Taken together, it is possible that SLC43A3 is involved in cellular proliferation, development, angiogenesis, inflammation, and carcinogenesis.

Posttranscriptional regulation of SLC43A3 has not been elucidated to date. However, differences in the glycosylation status of ENBT1/SLC43A3, which depends on the host cells, were reported. Bodoy et al. demonstrated that mouse ENBT1/SLC43A3, which is transiently expressed in HeLa cells, is glycosylated, while that intrinsically expressed in the liver and kidney is not.\textsuperscript{40} This feature has not been reported for LAT3 and LAT4. Western blot analysis confirmed that intrinsic ENBT1/SLC43A3 in HeLa cells is present as a primary form around the predicted molecular mass and as a secondary form in the higher-molecular weight range,\textsuperscript{56} suggesting posttranscriptional modification. It is unclear whether such differences in the glycosylation of ENBT1/SLC43A3 can alter its transport function.

It is evident that ENBT1/SLC43A3 is involved in purine salvage under physiological conditions; however, the role of ENBT1/SLC43A3 in nucleotide homeostasis remains unclear. Mice harboring a nonsense mutation in Slc43a3 are phenotypically normal and do not show embryonic lethality,\textsuperscript{48} suggesting that the function of Enbt1/Slc43a3 can be compensated for by other transport systems such as those of Ent1 and Ent2. Alternatively, the de novo synthesis of purine nucleotides replaces the salvage pathway that involves Embt1/Slc43a3. Although urinary excretion and plasma levels of purine nucleobases and their metabolites have not been determined, those of 20 amino acids remained relatively unchanged.\textsuperscript{48}

As described earlier, the uptake of purine nucleobases mediated by ENBT1/SLC43A3 involves a functional interaction with salvage enzymes in mammalian cells. Purine nucleobases that are transported into cells are salvaged to corresponding nucleotides by specific enzymes such as adenine phosphoribosyl transferase (APRT; for adenine) and hypoxanthine phosphoribosyl transferase 1 (HPRT1; for guanine and hypoxanthine).\textsuperscript{53} This mechanism allows ENBT1/SLC43A3 to mediate apparent concentrative transport when radioactive substrates such as [\textsuperscript{3}H]adenine are used, as illustrated in Fig. 1. The transport in HeLa cells is inhibited by the silencing of constitutive ENBT1/SLC43A3 or APRT by RNA interference.\textsuperscript{10} suggesting that APRT mediates the acceleration and enhancement of [\textsuperscript{3}H]adenine uptake by metabolic channeling in cooperation with ENBT1/SLC43A3. It is notable that enzymes involved in de novo purine biosynthesis form a reversible, transient multienzyme complex, known as the purinosome, under purine-depleted conditions.\textsuperscript{64} This finding may raise the possibility of forming a multiprotein complex of ENBT1/SLC43A3 and salvage enzymes, as a transport metabolon,\textsuperscript{65} which is a functional and structural complex to maximize the coupled transport/catalytic flux, as described for that of SLC26A6 and carbonic anhydrases.\textsuperscript{66} Further studies are nec-
necessary to determine whether ENBT1/SLC43A3 can directly interact with salvage enzymes.

Based on the cooperation of ENBT1/SLC43A3 and salvage enzymes, the $K_m$ values calculated from the apparent uptake of radiolabeled nucleobases may indicate cumulative affinity of the cooperative processes that involve ENBT1/SLC43A3 and salvage enzymes. A kinetic analysis study showed that the $K_m$ value (1.32 $\mu M$) of the specific uptake of [3H]hypoxanthine was markedly different from the hypoxanthine IC$_{50}$ value (350 $\mu M$) of [3H]adenine uptake mediated by ENBT1/SLC43A3.\textsuperscript{10} This inconsistency in the affinities has been attributed to the rate-limiting step in salvage enzyme-dependent metabolism, suggesting that the $V_{\text{max}}/K_m$ of ENBT1/SLC43A3 is higher than that of salvage enzymes in mammalian cells. The apparent $K_m$ value (1.32 $\mu M$) of [3H]hypoxanthine uptake is comparable to that of HPRT1-mediated metabolism for hypoxanthine (3 $\mu M$).\textsuperscript{38} In this case, the IC$_{50}$ value may represent the affinity of ENBT1/SLC43A3. Thus, the cooperative function of a nucleobase transporter and salvage enzymes should be taken into consideration when radiolabeled nucleobase uptake studies are conducted to evaluate the affinity for the transporter in cells expressing salvage enzymes.

The metabolic channeling in cooperation with ENBT1/SLC43A3 plays a role in chemotherapy with GCV, an antiviral drug used to treat and prevent cytomegalovirus infections. GCV is converted to its monophosphate form by exogenous viral kinases, such as cytomegalovirus UL97 phosphotransferase and herpes simplex virus thymidine kinase (HSV-TK), and is subsequently phosphorylated by host cell kinases to generate a cytotoxic GCV triphosphate; the activation of GCV does not occur in the absence of viral kinases.\textsuperscript{67} It was also demonstrated that the uptake of [3H]GCV by ENBT1/SLC43A3 increases in cells expressing HSV-TK, leading to a high cytotoxic effect.\textsuperscript{56} This suggests that HSV-TK facilitates GCV uptake by ENBT1. Kinetic analysis showed that the apparent $K_m$ value of GCV (96.7 $\mu M$) for ENBT1-mediated uptake was markedly lower in cells expressing HSV-TK than in normal cells (2.75 mM), indicating the cooperative function of ENBT1/SLC43A3 with HSV-TK in GCV uptake. Because the former $K_m$ value is comparable to those of the phosphorylation of GCV by HSV-TK ($47.6$ and $69$ $\mu M$),\textsuperscript{68,69} it is possible that GCV uptake mediated by SLC43A3/ENBT1 is rate limited by the metabolic process, which is similar to the purine nucleobase uptake cooperatively mediated by SLC43A3/ENBT1 and salvage enzymes. This cooperative function may have clinical relevance for adenovirus-mediated gene therapy exploiting the HSV-TK/GCV system.\textsuperscript{70,71}

5. CONCLUSION AND FUTURE PERSPECTIVES

Although there have been many studies on nucleoside transport, little attention has been paid to nucleobase transport. The molecular mechanisms being intensively studied of late have led to the identification of the major transporters. These transporters could play a significant role in the regulation of purine salvage and intracellular pool of purine nucleotide in biological systems. Considering the distribution of nucleobases, ENBT1 is promising as a target of nucleobase-based drugs and their delivery for treating cancer and infectious diseases. However, for effective therapy using these drugs, it
is important to elucidate the role of each transporter in purine salvage, in addition to documenting the alterations in their expression and functionality under different physiological and pathological conditions. Because the expression of ENT1 can be used as an effective biomarker for the diagnosis and prognosis of diseases such as cancer and the prediction of drug efficacy,\(^2\) it could be applicable to diseases and chemotherapies involving nucleobase transporters. The molecular basis of pyrimidine nucleobase transport systems remains unclear, and its elucidation is expected in the future. Understanding the molecular mechanism of purine and pyrimidine nucleobase transport in humans would be important and helpful for drug discovery and development and for exploring therapeutic strategies.

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**Conflict of Interest** The author declares no conflict of interest.

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