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Equilibrative Nucleoside Transporters – A Review

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Abstract

Equilibrative nucleoside transporters (ENTs) are polytopic integral membrane proteins that mediate the transport of nucleosides, nucleobases, and therapeutic analogs. The best-characterized ENTs are the human transporters hENT1 and hENT2. However, non-mammalian eukaryotic ENTs have also been studied (e.g., yeast, parasitic protozoa). ENTs are major pharmaceutical targets responsible for modulating the efficacy of more than 30 approved drugs. However, the molecular mechanisms and chemical determinants of ENT-mediated substrate recognition, binding, inhibition, and transport are poorly understood. This review highlights findings on the characterization of ENTs by surveying studies on genetics, permeant and inhibitor interactions, mutagenesis, and structural models of ENT function.

Nucleosides are important biologically active molecules formed by the amalgamation of a pyrimidine or purine nitrogenous base with either a ribose or 2′-deoxyribose pentose sugar. Pyrimidine and purine nucleosides, and their derivatives, play critical roles in the physiology of prokaryotic and eukaryotic organisms by serving as metabolic precursors in the synthesis of nucleic acids, as major elements of energy metabolism (ATP and GTP), and as ligands for purinergic receptors (adenosine, and inosine) (1, 2). Nucleoside analogs also represent important classes of antineoplastic and antiviral therapeutics (3). Since the activity of many of these hydrophilic compounds relies upon their entry into intracellular metabolic pathways to exert their effectiveness, crossing the cellular membranes is a prerequisite to downstream function.

Two classes of nucleoside transporters mediate physiologic nucleoside transport across cellular membranes: equilibrative nucleoside transporters (ENTs, SLC29 family) and concentrative nucleoside transporters (CNTs, SLC28 family) (4, 5). The CNT and ENT families are structurally unrelated nucleoside transporters with overlapping substrate

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specificities. CNTs are evolutionarily conserved symporters that require an inwardly directed sodium-dependent, or proton-dependent, coupling (reviewed elsewhere (3, 5, 6)). In contrast, ENTs are sodium-independent uniporters with no definitive prokaryotic orthologs. While passive transport is a hallmark of the ENT family, active, proton-linked, equilibrative transporters have been identified in protozoa (7) and activity of the human ENT3 and ENT4 transporters have been shown to be stimulated at lower pH (8). Mammalian ENTs were initially classified into two main groups: the *es* transporters were sensitive to nM concentrations of the inhibitor NBMPR (nitrobenzylthioinosine, NBTI), while the *ei* transporters were either unaffected by NBMPR or inhibited at higher concentrations (μM or higher) (3). Later studies identified 3 archetypical human isoforms (hENT1-3), which display the customary broad substrate selectivity (3). In addition, an evolutionarily divergent transporter (hENT4) was later shown to mediate adenosine transport in a pH-dependent manner with optimal transport occurring at approximately pH 6.0 (9). In spite of this, hENT4 is more commonly known as the plasma membrane monoamine transporter (PMAT) due to its ability to transport organic cations including biogenic amines, cationic therapeutics, and neurotoxins (9, 10). PMAT has substantial substrate overlap and inhibitor specificity with the organic cation transporters OCT1 - 3 in the SLC22 gene family (11–15). Another coinciding feature with OCTs is that PMAT-mediated transport is sensitive to membrane potential and sodium independent (16). Additionally, PMAT-mediated adenosine transport is likely insignificant under normal physiological conditions due to the low affinity and low activity of PMAT towards adenosine and the presence of other adenosine transporters (e.g., ENT1) (10). While PMAT may play a role in adenosine transport in times of ischemia or hypoxia where ENT1 activity is repressed (hypoxia) (17), PMAT will be excluded from this review because functionally it is viewed as a polyspecific organic cation transporter rather than the prototypical ENT.

The ability of ENTs to regulate the flux of nucleosides, nucleobases, and nucleoside-derived therapeutics (Figure 1) has far reaching implications. Adenosine is of particular interest because of its wide-ranging effects on multiple organ systems by interacting with adenosine receptors Adora1, Adora2a, Adora2b, and Adora3 (1, 18, 19) which govern cellular functions via regulation of downstream heterotrimeric G-proteins (20, 21). ENTs also modulate efficacy for a chemically diverse range of therapeutics (>30 FDA/EMA approved drugs) including anticancer (e.g., gemcitabine, cytarabine) (22), antiarrhythmia (e.g., dilazep, dipyridamole) (23, 24), antiviral (e.g., ribavirin, azidothymidine) (25–27), and antihypertensive (e.g., nifedipine) (28, 29) medications (Figure 1). However, medications that exert their effects in the cardiovasuclar (e.g., dilazep, dipyridamole, nifedipine) system are known to have overlapping functions and can affect vasodilation (33–36) as well as platelet activity (37–40). It should be noted that not all studies assessing therapeutic interactions with ENTs have been performed using clinically relevant drug concentrations and this is likely due to the nature of the systems being studied, where external manipulations (e.g., overexpression of an ENT, knockdown of an ENT, expression of a human ENT in a non-human cell line, etc.) and methods utilized require concentrations beyond clinical dosing schemes *in vivo*. In addition, one should consider the complex milieu that exists in cells and tissues. ENTs may have protein or small molecule functional modulators yet to be characterized. hENT1 expression levels have been linked to increased

patient survival for pancreatic cancer patients receiving gemcitabine treatment (41–49). Despite the pharmacological significance of ENT transport and activity, a detailed understanding of how ENTs function at the molecular level remains unknown due to hurdles associated with studying purified transporters (50–53) and native ENT recycling and expression in cells.

Functional Characterization of Mammalian ENT Proteins

To date, there are three archetypical human ENTs (hENT1-3) (3), and one putative ENT (ENT4), and each transporter has a varied but overlapping substrate transport profile (Table 1). Human ENT1-3 are broadly expressed, however, they are localized with more abundance in certain tissues. Early characterization of ENTs was done in erythrocytes (54, 55), followed by mammalian tumor cell lines (56, 57), then *Xenopus laevis* oocytes (3), and this eventually lead to attempts of characterizing purified transporters (50–53, 58) – but the ENTs proved to be resistant to characterization in detergent solubilized, purified form. Only recently have reports been made that ENTs are amenable to heterologous expression, purification, and functional characterization (59, 60).

Tissue Localization

While all ENTs are considered to be ubiquitously expressed across most tissue types, they each have specific tissues that have an overall higher abundance of expressed protein or mRNA. According to the Human Protein Atlas (http://www.proteinatlas.org (61)), hENT1 protein expression is highest in the adrenal gland, ovary, stomach, duodenum, small intestine, and colon while mRNA was most abundant in the adrenal gland. hENT2 protein expression was highest across a broader range of tissue types including various neurological tissues, segments of the gastrointestinal tract, skin, placenta, parathyroid gland, appendix, testis, urinary bladder, heart muscle, nasopharynx, pancreas, and gallbladder with mRNA being most abundant in skeletal muscle. Protein expression of hENT3 predominates in cerebral cortex, lateral ventricle, ovary, adrenal gland, and testis with higher levels of mRNA expression in placenta, urinary bladder, and ovary. ENT1 and ENT2 are primarily found in the plasma membrane while ENT3 contains an N-terminal dileucine motif (DE)XXXL(LI) (65) (characteristic motif for endosomal/lysosomal targeting) in the hydrophilic region of the sequence that precedes the first transmembrane domain (TMD) leading to an enrichment of ENT3 in the intracellular membranes of the endosome/lysosome and mitochondria (66). It should be noted that mutation of the dileucine motif causes the protein to be targeted to the plasma membrane (65).

SNPs, splice variants, and knockout models

Unlike other transporter families, the SLC29 genes show infrequent genetic variation (67–69) suggesting that the SLC29A1-2 genes are under substantial selective pressure, with nonsynonymous mutations being selected against. While few variations have been identified in hENT1, hENT1 polymorphisms have been associated with patients that are non-responsive to gemcitabine treatment, a nucleoside analog chemotherapeutic (70). There are currently no reported splice variants of hENT1, however, a variant of a mouse homolog (mENT1) has been reported as a product of alternative splicing at the end of exon 7

(mENT1.2) and is widely distributed (71). Choi *et al.* (72) developed the first reported ENT1-null mouse and demonstrated that it maintained normal reproductive behavior, had no gross anatomical abnormalities, and survival rates were similar to wild-type mice, although, bodyweight was significantly less than wild-type littermates (72–74). Several studies have utilized ENT1 null mice (20, 72–77) and found that these mice possess elevated circulating adenosine and thymidine levels in the plasma, reduced cellular uptake of adenosine (20, 73, 75), aberrant bone density (73, 74), dysregulation of the calcification of soft tissues associated with the enthesis regions of the vertebral column and sternum (73), increased resistance to oxidative stress (76), deficit in locomotor activity and motor coordination (74, 77), increased voluntary ethanol self-seeking behaviors associated with increased resistance to acute ethanol intoxication and reduced aversive effects of ethanol (72, 77), and that the absence of ENT1 is associated with reduced anxiety-like behavior in mice (78).

Two deletion variants have been found in the coding region of hENT2 (67, 69). The first is a splice variant that results in a frameshift encoding a 326-residue protein (nHNP36) that lacks the first three TMDs of hENT2 (64, 67, 79). HNP36 in both humans and mice has been shown to be nonfunctional as a transporter (64, 67), and HNP36 is associated with growth factor-induced delayed early response genes, but the function of HNP36 remains elusive (64, 79). The second variant results in a two-amino acid deletion and a nonsynonymous substitution of a third residue (67, 69). ENT2 knockout mice show increased levels of adenosine in bronchoalveolar fluid and alveolar space (80, 81), and that knockdown of ENT2 provided protection to acute lung injury (80, 81).

In marked contrast to hENT1-2, mutations in hENT3 have been linked to multiple disease states (82–85). SLC29A3 (hENT3) germline mutations are generally associated with autosomal recessive disorders such as H syndrome (83), pigmented hypertrichosis with insulin-dependent diabetes (PHID) syndrome, (82, 83), Faisalabad histiocytosis (83), and sinus histiocytosis with massive lymphadenopathy (SHML, Rosai Dorfman disease) (83). Mutations in hENT3 have also been associated with depression (85), osteoporosis (84), and increased survival of non-small-cell lung cancer patients treated with gemcitabine (86). Several of these disease causing variants were studied (G247S, G437R, M116R, and T449R) and all were shown to alter nucleoside transport with the C-terminal mutations resulting in a partial reduction of transport (8). Residue G427 was also shown to be critical for transporter function (8). In addition to nonsynomyous mutations, two deletion mutations resulting in frameshifts with early C-terminal truncations at residues 404 and 444 significantly reduced transport due to changes in protein stability (8). The nonsense mutation, E444X, leads to truncation at residue 444 and retains a higher level of function (8). ENT3 null mice developed spontaneous and progressive macrophage-dominated histiocytosis, altered macrophage function, lysosomal nucleoside buildup, and elevated intralysosomal pH (87). Mice lacking ENT3 also developed spontaneous splenomegaly and lymphadenopathy by eight weeks of age, and had a significanlty shorter life span relative to wild-type littermates (87). These studies suggest that defects in ENT3 have strong involvement in lysosomal storage disorders associated with histiocytosis.

Permeant Interactions

ENTs regulate the plasmalemmal flux of purine and pyrimidine nucleosides and nucleobases. A summary of currently known substrate selectivities for various human, yeast, parasitic protozoa, and plants can be found in Table 1. In addition to endogenous ligands, ENTs modulate efficacy for a variety of FDA/EMA approved therapeutics, and ENTs are known biomarkers for drug efficacy in the treatment of certain human cancers (41, 88). hENTs 1–3 have demonstrated varying levels of nucleoside, nucleobase, and nucleoside-analog transport (3, 65, 89). However, unlike hENT1-2, hENT3 transport appears to be proton coupled (8). Generally, ENTs have K_m values for the transport of nucleosides in the high micromolar range (~ 100 – 800 μM) (3, 90). While ENT1 and ENT3 are capable of transporting nucleobases, hENT2 has a slightly higher affinity for nucleobases compared to hENT1 (3.2 mM – 6.3 mM vs. 1.5 mM – 6.0 mM) for transporters assessed in *Xenopus* oocytes (3). ENT3 has a greater sensitivity to antivirals, and is stimulated at acidic pH (3, 8, 66). ENTs are known for their inability to transport nucleotides, however it has been reported that ENT3 is capable of transporting ATP and other therapeutics with triphosphate modifications (66). Therefore, understanding the chemical basis of permeant interactions is pivotal to engineering the next generation of antiviral and antineoplastic therapeutics. Understanding the chemical and structural requirements of the permeants on an atomic level has remained elusive, as no atomic resolution structure of an ENT from any species has been obtained.

Chemically, nucleosides are the consolidation of a nitrogenous base (purine or pyrimidine) with a pentose sugar. The pentose sugar has been repeatedly shown to be the primary determinant for ENT transport (91–96). ENT1 is selective for ribose or arabinose moieties (92), and sensitive to modifications at the C(2′) and C(5′) positions with the C(3′) hydroxyl being essential for substrate binding (93–95, 97, 98). The C(3′) position is also essential for permeants of ENT2 (94, 95), and ENT2 is also sensitive to modification at the C(5′) position (94, 95). Furthermore, chemical based studies have revealed the following: 1) ENTs have a weak preference for permeants adopting the C(2′)-endo/C(3′)-exo (South) sugar pucker conformation (96), 2) modifications at the C(3′)-position, a lack of conformational flexibility, and loss of a portion of the sugar ring are factors capable of decreasing the ability of some nucleosides to function as transportable substrates (93), 3) the pyrimidine moiety is the essential base component (97), and 4) there is a nitrogen to carbon bond specificity between the nitrogenous base and the sugar (97). It is currently unclear if ENT-mediated transport is: 1) affected by regions of electronegativity, 2) sensitive to the orientation of the purine/pyrimidine ring about the glycosidic linkage (*anti* vs. *syn*), 3) facilitated by hydrophobic interactions, and 4) what role hydrogen bonding plays in the transport mechanism. Therefore, a detailed chemometric understanding of ENT transportable ligands remains unresolved.

Functional characterization of transporter-permeant interactions is preferable using purified protein in a defined environment to exclude any overlap from the presence of other endogenous transporters, pumps, and/or metabolic activity. Given the difficulties associated with obtaining purified, active ENTs (50–53, 58), the use of other informative flux assays have been used including: 1) recombinant proteins produced in NT-deficient cells such as *S.*

cerevisiae cells (knockout strains that lack nucleoside transporter activity), *X. laevis* oocytes (no endogenous nucleoside transport activity) (3), human or porcine cell lines (mutated to have null nucleoside transport activity (e.g., CCRF-CM, PK15-NTD) (99, 100), or 4) using cells that produce a single transporter type (e.g., studying hENT1 in *S. cerevisiae*) (54, 55, 100). Generally, the use of radioisotopes is used to determine ligand flux for all the assays mentioned above, and for transporters that generate rapid bidirectional substrate flux the use of an inhibitor may need to be added prior to collection to prevent substrate efflux and subsequent loss of substrate from the luminal volume of proteoliposomes or the cytoplasm of cells.

Inhibition

The distinguishing characteristic of mammalian ENTs is that hENT1 is inhibited by nM concentrations of NBMPR, while hENT2 and 3 are less sensitive to NBMPR (hENT2 ≫ hENT3) (3, 65). Although NBMPR potently inhibits hENT1, it is not an effective combinatorial chemotherapeutic (101) agent due to significant off target effects in the cardiovascular system (102, 103). Dipyridamole and dilazep have also been shown to affect hENT1-3 (3, 65). In addition to these inhibitors, hENT1-2 have also been shown to be affected to a lesser degree by tyrosine- and serine/threonine-kinase inhibitors and benzodiazepines (104). Another potent inhibitor of ENT1 is CBD (cannabidiol) with a reported K_i of < 250 nM (105). It should be noted that this is not an exhaustive list of ENT inhibitors, but highlights the broad classifications of therapeutics that have been shown to interact with ENTs.

Interestingly, the transport of permeants by ENT1 is also inhibited in the presence of ethanol (106–108). Specifically, acute ethanol exposures has been show to inhibit ENT1-mediated transport in human lymphocytes (106), primary cultures of hepatocytes isolated from rat (109), human placental cells (110), human bronchial epithelial cells (108), HL-1 cardiomyocytes (111), S49 mouse lymphoma cells and a hybrid rodent neuronal cell line (NG108-15) (107, 112–114). Ethanol has also been observed to attenuate transport mediated by purified and reconstituted yeast ENT, FUN26 (Boswell-Casteel, unpublished data). Moreover, ENT1 sensitivity to ethanol has been shown to be regulated in a kinase-dependent manner by PKA and PKC (107, 111, 112), which have been previously shown to phosphorylate mouse ENT1 in the intercellular loop region between TMDs 6 and 7 (115). The means by which ethanol may modulate ENT function is unknown and the effect may be indirect of ENT-mediated binding. Furthermore, ENT1 contributes to the behavioral effects of ethanol (72), alcohol consumption and preference (72), and genetic polymorphisms of hENT1 are associated with alcoholism and an increased risk of alcohol withdrawal seizures (116). Additionally, the acute inhibition of ENT1 contributes to the regulation of glutamatergic neurotransmission by controlling adenosine flux (117). Studies have shown that adenosine inhibits neuronal activity by suppressing synchronous discharges associated with the Adora1 receptor (118–122). Specifically, adenosine acts via the Adora1 receptor presynaptically to inhibit glutamatergic synaptic transmission within the hippocampus (123, 124). Reduced adenosine signaling has also been implicated in decreased sensitivity to the intoxicating effect of ethanol and increased ethanol consumption in mice (125). Inhibition of the Adroa1 receptor increases glutamate-evoked postsynaptic transmission in the nucleus

accumbens (126), and ENT1 null mice have reduced Adora1-mediated inhibition of glutamate excitatory postsynaptic currents within the nucleus accumbens (72). It has been shown that short term inhibition of ENT1 reduces seizure load (117), however we postulate that chronic inhibition of ENT1 may contribute to seizure activity associated with substance withdrawal (e.g., alcohol, benzodiazepines) due to the reestablishment of adenosine flux eliciting changes in glutamate signaling. Given that chronic ethanol exposure/consumption evokes an adaptive response where increases in extracellular adenosine levels are no longer observed due to downregulation of ENT1 gene expression (113).

Functional Characterization of Non-mammalian ENT Proteins

Given the high pharmacological value of ENTs, multiple studies of non-mammalian ENTs have been conducted. A number of studies have been performed on ENTs from parasitic protozoa and plants, [reviewed in (7, 127, 128)]. Table 1 contains a representative list of transportable substrates for non-mammalian ENTs. ENTs are invaluable in the lifecycle of parasitic protozoa, because they lack the ability to synthesize purines *de novo* and are therefore reliant on salvage pathways mediated my plasma membrane nucleoside and nucleobase transporters to provide substrate-specific permeation routes (7). This reliance make ENTs of parasitic protozoa prime therapeutic targets for the delivery of subversive substrates (129), but development of inhibitors directed at the ENTs of parasitic protozoa have been limited due to the multiplicity of expressed purine nucleoside and nucleobase transporters (7). Parasitic protozoa transporters LdNT1.1, LdNT1.2, and LdNT2 (*Leishmania donovani*) display 20 to 100-fold increase in substrate affinity compared to mammalian ENTs, they are electrogenic proton symporters, and are less effected by the mammalian inhibitors NMBPR, dipyridamole, and dilazep (7). The transporters from *Trypanosoma brucei* also exhibit a higher affinity for substrates than mammalian ENTs (7), while transporters from *Plasmodium falciparum* have affinities more comparable to mammalian ENTs. Another frequently studied family of ENT proteins comes from *Arabidopsis* [reviewed in (128)]. Transporters from *A. thaliana* display broad substrate selectivity and affinity (3 to 100 μM), function as substrate-proton symporters (with exception to AtENT7), and are insensitive to the inhibitors NMBPR, dilazep, and dipryidamol (128). In addition to these transporters, FUN26 (Function Unknown Now 26) from *Saccharomyces cerevisiae* was recently identified as a broadly selective, high-affinity, nucleoside and nucleobase transporter (59). FUN26 is not stimulated by a pH differential nor is it sensitive to NBMPR (59, 130). Like hENT3, FUN26 has also demonstrated limited ability to transport nucleotides, albeit with lower affinity than nucleosides or nucleobases (Boswell-Casteel, unpublished data). Importantly, FUN26 and AtENT7 are the first ENTs to be functionally characterized in purified form (59, 60), and this marks a major advancement in efforts to obtain a molecular structure of an ENT protein.

Mutagenesis Studies of ENTs

Site specific mutagenesis has been utilized to identify critical residues conferring function and structure of ENTs in the absence of an atomic resolution structure. The architecture of ENTs consists of 11 TMDs, a cytoplasmic N-terminus and an extracellular C-terminus that was confirmed by glycosylation scanning mutagenesis and through the use of antipeptide

antibodies as topological probes (131, 132). ENT1 was found to be N-glycosylated at N48 (133), ENT2 also contains glycosylation sites at residues N48 and N57 (134). ENT3 is expected to be glycosylated, but this is yet to be proved (65). Multiple studies have identified residues that affect substrate transport or inhibitor binding (8, 25, 36, 67, 82, 135–153), mitochondria targeting (144), and targeting to the plasma membrane (59, 142, 149, 150). A list of mutated residues with their extrapolated function is available in Table 2 for human ENTs and Table 3 for the non-mammalian ENTs. A recent study focusing on deletion mutants (deletion of intra- and extra-cellular loops as well as TMDs 9–11 and TMD11) demonstrated that the C-terminal TMDs were essential for proper trafficking and protein folding, while the loop regions appeared to be dispensable (154). Studies of ENT chimeras have also revealed important functional information surrounding the putative translocation pore and inhibitor binding sites in TMDs 3–6 (137, 140). Studies in *S. cerevisiae* have shown that SNPs conserved in ENT3 and FUN26 have functional overlap (59) (Boswell-Casteel, unpublished data). Collectively, these studies suggest that highly conserved residues throughout the ENT family will have overlapping functional duties, and be critical in unraveling ENT function. However, additional mutagenesis data is needed to fully understand the breadth of ENT-mediated substrate transport.

Structural Modeling and Mechanism of ENT Transport

ENTs are members of the Major Facilitator Superfamily (MFS), (155) due to observations of structural commonality between other members of the MFS (e.g., LacY, GlpT), putative structural models (homology and *ab initio*) have been constructed (148, 150, 156, 157). In the absence of an atomic resolution structure, computational models provide a platform for future studies aimed at probing structure-function relationships. Based on a canonical MFS fold (158), the computational ENT models predict an inner bundle of transmembrane domains (TMDs) 1, 2, 4, 5, 7, 8, 10, and 11 surrounding a central hydrophobic cavity, while TMDs 3, 6, and 9 are peripheral to the central pore and face the surrounding membrane (148, 150, 156, 157). The use of these models in combination with mutagenesis or cysteine-crosslinking data has shown that TMDs 5, 7, and 8 are important to transporter function (148, 157). Aromatic residues at the distal ends of TMDs 1, 2, 7, and 6 are expected to form the extracellular gate (or cytoplasmic gate for intracellular ENTs) in the inward-open configuration. Additionally, the intracellular gate (luminal gate) is expected to contain residues from TMDs 4, 5, 10, and 11 (150). Finally, TMDs 1, 8, 10, and 11 are expected to participate in the permeation pathway (148). Collectively, these studies point to an alternating access mechanism, which is common among MFS transporters (155, 158). In this model, substrate binds to a central cavity in the open configuration, followed by a series of intermediate, occluded, states (159) leading to conformational switching which, ultimately, leads to the release of bound substrate on the opposite side of the membrane bilayer (158). This model predicts that each substrate will be able to reciprocally inhibit the uptake of other permeants. However, there is evidence that some parasitic ENTs exhibit nonreciprocal inhibition, which implies that the simple model of competitive inhibition by structurally similar substrates binding to overlapping sites may not always hold true for ENTs (160, 161). Additionally, biphasic uptake was observed for the fluorescent probe FuPmR by ENT1, but is believed to be the result initial uptake combined with intracellular metabolism

of FuPmR (162). Another transport mechanism was recently suggested for members of the MFS and it is termed the clamp-and-switch model (163). In this model transitions between the inward-open, outward-open, and multiple occluded states involves rigid-body rotation between the N- and C- domains, but also includes changes in the individual TMDs – particularly the A helices (TMDs 1, 4, 7, and 10) that bend to form the occluded states (clamp) (163). Once the occluded state (clamp) has been formed, the N- and C- domains rotate to expose a binding pocket to the opposite face of the membrane (switching) (163). While structural modeling can serve as a platform for directing future structure-function studies, until multiple atomic resolution structures of various conformations have been ascertained the alternating access or clamp-and-switch model of transport will remain ambiguous.

Physiological Roles of ENT Function

The ability of ENTs to transport nucleosides and nucleobases contributes to maintaining cellular nucleoside homeostasis. Nucleoside recycling is essential for nucleotide, DNA, and RNA synthesis, intracellular signaling pathways (e.g., cAMP, cGMP) and phospholipid synthesis (e.g., CDP) (1, 2). ENTs also have essential roles in delivering nucleoside analogs to intracellular targets for the treatment of numerous hematological and solid tumors, and viral infections (e.g., HIV, hepatitis C) (164, 165). Specifically, hENT1 expression has been associated with increased patient survival for pancreatic cancer patients receiving gemcitabine treatment (Figure 2) (41–49). Nucleoside analogs are routinely used in conjunction with platinum-based chemotherapeutics, such as cisplatin, and have been shown to have an enhanced effect when compared to individual treatments for a wide range of cancers (e.g., pancreatic, breast, non-small-cell lung cancers) (166–169). ENT inhibitors are also used to treat epilepsy and various cardiac conditions that require use of antiplatelet agents, calcium channel blockers, and vasodilators (31, 32, 81, 104).

By extension, ENTs contribute to the regulation of a plethora of metabolic functions and cell signaling cascades by controlling intra- and extracellular adenosine concentrations. Dihydropyridine-type calcium channel blockers (e.g., nimodipine, nitrendipine, nifedipine, nicardipine) are a class of vasodilators that also inhibit the cellular uptake of adenosine by targeting hENT1 and hENT2 (2). Nimodipine inhibits hNET1 at nM concentrations and nifedipine, nitrendipine, and nimodipine inhibit hENT2 at μM concentrations (2). Acting through distinct GPCRs, adenosine signaling mediates a variety of physiological responses such as vasodilation, coronary blood flow, myocardial oxygen supply-demand balance, inflammation, neurotransmission, hypoxia, trauma, and ischemia (32, 81, 94, 170–174). In fact, adenosine analogs have been exploited in clinical settings for their antiarrhythmic and cardioprotective effects. Inhibitors of ENTs confer a protective advantage in ischemia, trauma, hypoxia, and certain types of seizure disorders by blocking adenosine uptake (32, 81, 94, 117, 172, 173, 175). Also, as described above, hENT3 is associated with a number of autosomal recessive disorders. Given the far-reaching effects of ENTs on human physiology, it is imperative that a continued focused effort be maintained, especially in the area of drug delivery, cardiology, neurology, and functional/structural characterization.

Perspective

Major advances have been made in the ability to heterologously express, purify, and functionally characterize ENTs. The use of computational structural models has served as an insightful guide for designing mutagenesis strategies and aiding in understanding substrate/inhibitor interactions. However, despite progress unraveling the individual roles of ENTs, understanding the molecular architecture of the ENT family, the transport mechanism, and the significance of the ENT family for whole organism physiology remains at its infancy. Accumulating a greater understanding of ENT structure and function will require the use of transgenic animal models, studies using purified protein, and ultimately the acquisition of multiple atomic resolution structures. Headway in these areas will be essential to developing novel therapeutics and exploiting the remedial potential of ENTs.

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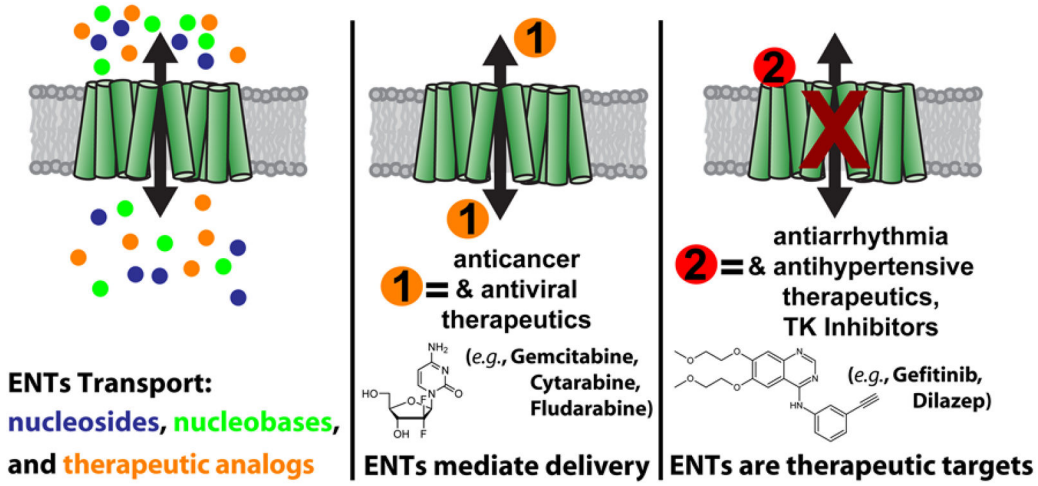

FIGURE 1. ENTs are diverse transporters and serve as major pharmaceutical targets

ENTs regulate the flux of pyrimidine and purine nucleosides, nucleobases, and therapeutic analogs. ENTs also modulate therapeutic efficacy by mediating the transport of medications across cellular membranes to their ultimate site of action (orange “1”) or serve as the direct target for drug binding (red “2”) resulting in transporter inhibition.

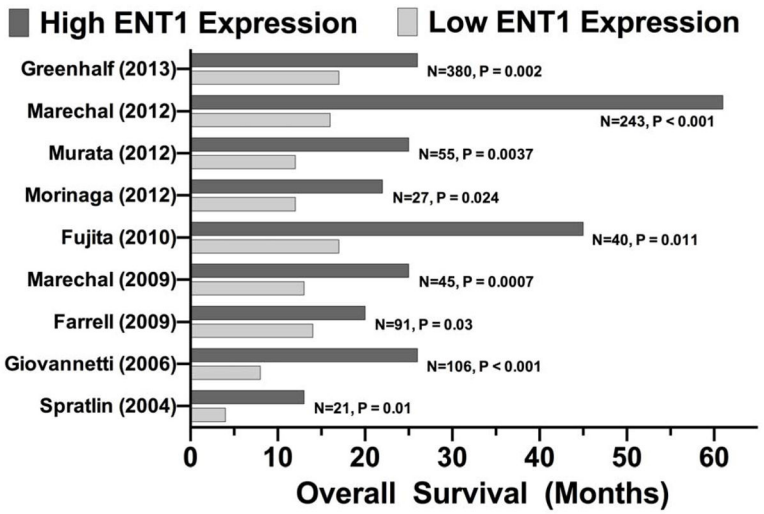

FIGURE 2. Human ENT1 expression levels correlate to increased patient survival

Multiple studies have shown that higher levels of hENT1 expression results in increased patient survival for pancreatic cancer patients receiving gemcitabine treatment. Data was compiled from multiple previously published works (41–49), red bars indicate high hENT1 expression levels, while blue bars represent low hENT1 expression levels.

Table 1

Permeant Selectivities of Nucleoside Transporters

Substrate[a]	Human (K_m (mM)[b])*	Yeast	Parasitic	Plant
Nucleosides and Nucleobases				
2-Deoxyuridine		ScENT1		
Adenine	hENT1 (0.12 – 3.2), hENT2 (1.1 – 1.8), hENT3	ScENT1	LmaNT3, TbNT8.1	
Adenosine	hENT1 (0.011 – 0.040), hENT2 (0.1 – 0.14), hENT3 (1.86), hENT4 (0.78 – 7.8)	ScENT1	LdNT1.1, TbNT2, TbNT5, TbNT6, TbNT7, TbNT9, TbNT10	AtENT1, AtENT3, AtENT6, AtENT7, StENT1, HvENT1, OsENT2
Cytidine	hENT1 (0.21 – 0.58), hENT2 (5.6), hENT3	ScENT1	LdNT1.1	AtENT1, AtENT3, AtENT4, AtENT6, AtENT7, StENT3, HvENT1
Cytosine	hENT2	ScENT1		
Guanine	hENT1, hENT2		LmaNT3, TbNT8.1	
Guanosine	hENT1 (0.048 – 0.14), hENT2, hENT3	ScENT1	LdNT2, TbNT2, TbNT5, TbNT6, TbNT7, TbNT9, TbNT10	AtENT3, AtENT4, AtENT6, AtENT7, StENT1,
Hypoxanthine	hENT1 (0.096 – 6.0), hENT2 (0.70 – 1.5)	ScENT1	LmaNT3, TbNT5, TbNT6, TbNT7, TbNT8.1, TbNT9	
Inosine	hENT1 (0.029 – 0.17), hENT2 (0.05 – 0.18), hENT3	ScENT1	LdNT2, TbNT2, TbNT5, TbNT6, TbNT7, TbNT9, TbNT10	
Thymidine	hENT1 (0.30), hENT2 (0.71), hENT3	ScENT1	LdNT1.1	AtENT3
Thymine	hENT1 (6.3), hENT2 (6.0), hENT3			
Uracil	hENT1, hENT2 (2.6)	ScENT1		
Uridine	hENT1 (0.043 – 0.40), hENT2 (0.2 – 0.73), hENT3 (2.0)	ScENT1	LdNT1.1	AtENT1, AtENT3, AtENT4, AtENT6, AtENT7, StENT1, StENT3, HvENT1, OsENT2
Xanthine			LmaNT3, TbNT8.1	
Nucleotides or Triphosphorylated Compounds				
ATP	hENT3			
UTP		ScENT1		
Therapeutic Analogs				
5-Fluorouracil	hENT1 (2.3), hENT2 (2.6)			
6-Mercaptopurine	hENT1 (1.2), hENT2 (1.1)			

Substrate[a]	Human (K_m (mM)[b])*	Yeast	Parasitic	Plant
Cladribine	hENT1 (0.023), hENT2, hENT3			
Clofarabine	hENT1 (0.11 – 0.15), hENT2 (0.33), hENT3			
Cytarabine	hENT1, hENT2			
Didanosine	hENT1 (2.3 – 3.0), hENT2, hENT3			
Fludarabine	hENT1 (0.11), hENT2, hENT3			
Gemcitabine	hENT1 (0.16), hENT2 (0.74), hENT3			
Ribavirin	hENT1 (0.16 – 3.5), hENT2 (0.33 – 3.8)			
Zalcitabine	hENT1, hENT2 (>7.5), hENT3			
Zebularine	hENT3			
Zidovudine	hENT2, hENT3			
5′-Deoxy-5-fluroyridine	hENT1			
Pentostatin	hENT1, hENT2			
Azacutidine	hENT1, hENT2, hENT3			
Decitabine	hENT1, hENT2			
Imaging Agent				
3′-Deoxy-3′-fluorothymidine	hENT1 (3.4), hENT2 (2.6)			

[a]Substrate selectivities compiled using the works of Young, *et al.*(3), Landdear, *et al.*(7), Girke, *et al.*(128), Molina-Arcas, *et al.*(90), Damaraju, *et al.* (22), Pastor-Anglada, *et al.*(176), Boswell-Casteel, *et al.* (59), and unpublished data from Boswell-Casteel, *et al.* Empty cells signify either untested substrates or substrates that were deemed untransportable.

[b]K_m values in different expression systems vary, however, relative affinities are generally consistent

[c]K_m values for human ENTs are obtained from the works of Young, *et al.* (3), Parkinson, *et al.*(104), Bone, *et al.* (177), Molina-Arcas, *et al.* (90), SenGupta, *et al.* (141), Ward, *et al.*, (99), Visser, *et al.* (135), Visser, *et al.* (94), and Yao, *et al.* (89) and are meant to demonstrate the differences in subtype preference. A range of published Km values are shown for substrates that have multiple reported values.

Table 2

Mutagenesis of Human ENTs

Residue	Location	Studied Mutation(s)	Functional Role	Reference(s)
hENT1				
G24	TMD1	A/R/E	Nucleoside recognition and uptake	(147)
W29	TMD1	C/G/T	inhibitor sensitivity, substrate selectivity, nucleoside transport efficacy	(36)
M33	TMD1		inhibitor sensitivity	(36, 136)
N48	loop between TMD 1 & 2	Q	glycosylation	(131)
P71	loop between TMD 1 & 2	L	mitochondrial targeting	(144)
E72	loop between TMD 1 & 2	G	mitochondrial targeting	(144)
N74	loop between TMD 1 & 2	P	mitochondrial targeting	(144)
C87	TMD2	S	inhibitor sensitivity	(178)
M89	TMD2	C/L/V/T/Q	inhibitor sensitivity, nucleoside transport efficacy	(143)
L92	TMD2	P/Q	inhibitor sensitivity, nucleoside transport efficacy	(143)
G154	TMD4	S	inhibitor sensitivity	(141)
S160	TMD4	C/N	inhibitor sensitivity	(143)
G179	TMD5	A/L/V/C/S	inhibitor sensitivity, nucleoside transport efficacy	(142)
G184	TMD5	A/L/V/C/S	Targeting protein to plasma membrane	(142)
C193	TMD5	S	inhibitor sensitivity	(178)
C213	TMD6	S	nucleoside transport efficacy	(178)
I216	TMD6	T	ethanol sensitivity, nucleoside transport efficacy	(68)
C222	TMD6	S	inhibitor sensitivity	(178)
F334	TMD8	Y	catalytic turnover	(145)
N338	TMD8	Q/C	protein folding	(145)
C387	loop between TMD 9 & 10	S	inhibitor sensitivity, protein folding	(179)
C414	loop between TMD 10 & 11	S	nucleobase transport	(89, 179)
C416	loop between TMD 10 & 11	S/A	inhibitor sensitivity, substrate selectivity, nucleoside transport efficacy	(179)
C439	TMD11	A	inhibitor sensitivity, nucleoside transport efficacy	(179)
L442	TMD11	I	substrate selectivity, nucleoside transport efficacy	(36, 135)
hENT2				
D5	N-terminus	Y	nucleoside transport efficacy	(67)
I33	TMD1	M/S/A/C	inhibitor sensitivity	(94, 135)
N48	loop between TMD 1 & 2	D	glycosylation	(134)
N57	loop between TMD 1 & 2	D	glycosylation	(134)
hENT3				
M116	TMD2	R	retention in endoplasmic reticulum, nucleoside transport selectivity, nucleoside transport efficacy	(8, 82)
R363	TMD8	Q		(180)

Residue	Location	Studied Mutation(s)	Functional Role	Reference(s)
G427	TMD10	S/R/A/F/Y	nucleoside transport selectivity, nucleoside transport efficacy	(8)
G437	loop between TMD 10 & 11	R	catalytic turnover, nucleoside transport efficacy	(8, 82)
T449	loop between TMD 10 & 11	R	accumulation in late endosomes	(82)

Table 3

Mutagenesis of Non-Mammalian ENTs

Residue	Location	Studied Mutation(s)	Functional Role	Reference(s)
LdNT1.1				
M40	TMD1	D	nucleoside transport efficacy	(148)
F48	TMD1	A/W	nucleoside transport efficacy	(148)
W75	TMD2	A	nucleoside transport efficacy	(148)
E94	TMD2	Q/D	nucleoside transport efficacy	(148, 149)
E121	TMD2	Q/D	nucleoside transport efficacy	(149)
K153	TMD4	R/A	nucleoside transport efficacy, substrate selectivity	(149)
E157	TMD4	Q/D	substrate selectivity	(148)
Y161	TMD4	A	nucleoside transport efficacy	(150)
G163	TMD4	A	nucleoside transport efficacy	(150)
M175	TMD5	A	nucleoside transport efficacy, substrate selectivity	(150)
G183	TMD5	D/A/N	nucleoside transport efficacy	(151)
D215	TMD6	N/E	nucleoside transport efficacy	(149)
C337	TMD7	Y/F/S	nucleoside transport efficacy	(151)
F341	TMD7	A	substrate selectivity	(148)
R404	TMD9	A/K	targeting to plasma membrane	(149)
E429	TMD9	Q/D		(149)
V445	TMD10	A	nucleoside transport efficacy	(150)
G467	TMD11	A	nucleoside transport efficacy	(150)
I468	TMD11	A	targeting to plasma membrane	(150)
S469	TMD11	F	nucleoside transport efficacy	(148)
I470	TMD11	A	nucleoside transport efficacy	(150)
T478	TMD11	F	nucleoside transport efficacy	(148)
LdNT2				
R393		L/E/N/K	nucleoside transport efficacy, substrate selectivity	(152)
D389		N//E	targeting to plasma membrane	(152)
CeNT1				
I49	TMD1	M/A/L/T	nucleoside transport efficacy	(153)
I429	TMD11	L/T	inhibitor sensitivity	(153)
ScENT1				
G216	TMD5	A	loss of protein expression	(59)
F249	TMD6	I	nucleoside transport efficacy, substrate selectivity	(59)
L390	TMD8	A	nucleoside transport efficacy, substrate selectivity	(59)
G463	TMD10	A	nucleoside transport efficacy, substrate selectivity	(59)
AtENT3				
G281	7	R	fluorouridine sensitivity, pyrimidine transport	(181, 182)