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Structure modeling of the neutral amino acid transporters from the SLC6 family

Thesis submitted for the degree of Doctor of Philosophy in medical sciences

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Kraków 2025

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Abstract

Proteins of the SLC6 family primarily mediate the transport of small polar soluble molecules across the cell membrane utilizing an electrochemical gradient as a driving force. This family is divided into four subfamilies, grouped by sequence similarity and substrate specificity, namely the transporters for monoamines, glycine, γ-aminobutyric acid, and neutral amino acids. They play vital roles in neurotransmission, nutrition, cellular and whole-body homeostasis. Dysregulation of their function and expression is linked to a variety of diseases and disorders. Brain-expressed SLC6 transporters, due to their critical role in nerve signaling, are key targets for medications used to treat psychiatric conditions. The neutral amino acid transporter subfamily consists of six members, consecutively SLC6A15–SLC620. They are widely distributed throughout the body, covering many systems such as the central nervous, renal, or intestinal system, supplying cells into molecules used in biochemical, signaling, and building pathways afterward. Brain-expressed neutral amino acid transporters of SLC6 transporters constitute significant medium for the provision of neurotransmitter precursors. In the kidney and intestine, they enable the reabsorption and absorption of amino acids and thus participate in nutrition. Altered function and expression of neutral amino acid transporters are associated with a variety of diseases and disorders, including Hartnup disease, major depression, iminoglycinuria, and mental retardation.

This thesis begins by introducing the role of membrane transporters within the SLC6 family, focusing on their significance in solute transport. Key aspects of their overall structure and transport mechanisms are discussed to provide a foundational understanding. The subsequent subsection of the introduction reviews the expression patterns, catalytic properties, and physiological functions of the neutral amino acid transporter subfamily within the SLC6 family. Additionally, the thesis compiles information on specific mutants of these transporters, highlighting their involvement in various disorders and genetic diseases. Finally, the introduction concludes with an exploration of the therapeutic relevance of selected neutral amino acid transporters, setting the stage for the research presented in this work.

The primary aim of this thesis is structural characterization of the neutral amino acid transporters from the SLC6 family, namely SLC6A15 (B^0AT2), SLC6A17 (NTT4), and SLC6A20 (SIT1). B^0AT2, NTT4 and SIT1 are transporters widely expressed in central nervous system embracing glutaminergic and GABAergic system. Their main role is to ensure the supply of neutral amino acids, which are precursors for the biosynthesis of glutamate and GABA neurotransmitters, among others. In this regard, they are implicated in the maintenance of neural homeostasis. The significance of the above transporters is underlined by the fact that their dysfunction has been reported to be related to psychiatric disorders. Notably, inhibiting these transporters has been proposed as a potential therapeutic strategy for conditions such as depression, anxiety, and schizophrenia. Understanding the molecular basis of the transport mechanism, substrate selectivity, and inhibition of neutral amino acid transporters facilitates the search for and development of drugs that target these proteins. A deeper understanding of these aspects not only sheds light on their physiological roles but also provides a platform for the design of chemicals against targeted transporters. Compounds that modulate their activity serve as invaluable tools for exploring their biological functions and therapeutic potential, offering insight into their relevance in health and disease. This knowledge is particularly meaningful given the involvement of transporters in critical neurological processes and their association with psychiatric disorders, as highlighted earlier.

The experimental part of this thesis focuses on homology modeling the abovementioned transporters in different conformational states. Models of B^0AT2, NTT4 were used to characterize elements of their structures crucial for transport of substrate. Detailed analysis of B^0AT2 and NTT4 structures, combined with substrate docking studies, shed a light on the molecular determinants of their substrate selectivity. Both transporters share critical residues in key functional regions, such as the vestibule, extracellular and intracellular gates, main substrate binding site, and ion pockets, which likely underlie their similar substrate profiles. Moreover, the hydrophobic nature of their main substrate binding site further explains their preference for amino acids with hydrophobic side chains over polar ones. Studies on the molecular basis of B^0AT2 inhibition by loratadine, employing a variety of computational methods such as molecular docking, molecular dynamics simulations, and MM-GBSA free

energy calculations, revealed that this inhibitor might be accommodated in both the outward-open and inward-open states of the transporter. Furthermore, models of B^0AT2 in different conformational states were used for virtual screening to identify new inhibitory agents. The applied structure-based approach led to the discovery of tiagabine and an L-proline analog as B^0AT2 inhibitors. Subsequently, the developed computational protocol was utilized to screen for SIT1 inhibitors, resulting in the identification of tiagabine as an inhibitor of this transporter as well. In addition, studies on NTT4 mutations that cause mental retardation by means of molecular dynamics simulations revealed that they disrupt the structural stability and function of the transporter, providing a molecular basis for their pathogenic effects.

The findings presented here contribute to the understanding of the structural properties of neutral amino acid transporters, which will guide further experimental studies, including the design of new inhibitors with desirable properties.

Streszczenie

Białka z rodziny SLC6 umożliwiają transport małych, polarnych, substancji rozpuszczalnych przez błonę komórkową, wykorzystując gradient elektrochemiczny jako siłę napędową. Z uwagi na podobieństwo sekwencji i specyficzność substratową, rodzina ta dzieli się na cztery podrodziny, mianowicie transportery dla monoamin, glicyny, kwasu γ-aminomasłowego (GABA) oraz aminokwasów obojętnych. Pełnią one kluczowe role w przekaźnictwie nerwowym, odżywianiu oraz homeostazie komórkowej i całego organizmu. Zaburzenia ich funkcji katalitycznej i ekspresji są powiązane z różnymi chorobami oraz zaburzeniami. Transportery SLC6 ulegające ekspresji w ośrodkowym układzie nerwowym, ze względu na ich kluczową rolę w sygnalizacji nerwowej, są ważnymi celami dla terapeutyków stosowanych w leczeniu chorób psychiatrycznych. Podrodzina transporterów aminokwasów obojętnych składa się z sześciu członków, kolejno SLC6A15–SLC6A20. Są one szeroko rozpowszechnione w organizmie, obejmując wiele układów, takich jak ośrodkowy układ nerwowy, nerki czy jelita, dostarczając komórkom aminokwasy wykorzystywane w szlakach biochemicznych, sygnalizacyjnych i budulcowych. Transportery aminokwasów obojętnych z rodziny SLC6, których ekspresja obejmuję ośrodkowy układ nerwowy stanowią ważne medium dla dostarczania prekursorów neuroprzekaźników. W nerkach i jelitach umożliwiają resorpcję i wchłanianie aminokwasów, uczestnicząc w ten sposób w procesach odżywiania. Zaburzona funkcja i ekspresja transporterów aminokwasów obojętnych są powiązane z rozmaitymi chorobami i zaburzeniami, w tym m.in. z chorobą Hartnupa, depresją, iminoglicynurią czy też opóźnieniem w rozwoju umysłowym.

Niniejsza rozprawa doktorska rozpoczyna się od wprowadzenia roli transporterów błonowych z rodziny SLC6, ze szczególnym uwzględnieniem ich znaczenia w transporcie substancji rozpuszczalnych. Omówiono kluczowe aspekty ich ogólnej struktury i mechanizmu transportu, aby zapewnić podstawowe zrozumienie tematu. Kolejna część wprowadzenia przedstawia wzorce ekspresji, właściwości katalityczne oraz funkcje fizjologiczne poszczególnych członków podrodziny transporterów aminokwasów obojętnych w obrębie rodziny SLC6. Dodatkowo, praca ta zawiera informacje na temat mutacji tych transporterów, podkreślając ich udział w różnych chorobach genetycznych i zaburzeniach. Wprowadzenie

kończy się dyskusją nad znaczeniem terapeutycznym wybranych transporterów aminokwasów obojętnych, stanowiąc podstawę dla badań przedstawionych w tej pracy.

Głównym celem niniejszej rozprawy doktorskiej jest charakterystyka strukturalna transporterów aminokwasów obojętnych z rodziny SLC6, a mianowicie SLC6A15 (B^0AT2), SLC6A17 (NTT4), oraz SLC6A20 (SIT1). Ekspresja B^0AT2, NTT4 i SIT1 w ośrodkowym układzie nerwowym, obejmuje układ glutaminergiczny i GABAergiczny. Rolą tych transporterów jest zapewnienie właściwej podaży aminokwasów obojętnych, które są prekursorami dla biosyntezy neuroprzekaźników, takich jak glutaminian czy kwas γ-aminomasłowy. W tym kontekście są one zaangażowane w utrzymanie homeostazy nerwowej. Znaczenie tych transporterów dodatkowo podkreśla fakt, że ich dysfunkcja została powiązana z zaburzeniami psychiatrycznymi. Warto zauważyć, że inhibicja tych transporterów została zaproponowana jako potencjalna strategia terapeutyczna w leczeniu schorzeń takich jak depresja, lęk czy schizofrenia. Zrozumienie podstaw molekularnych mechanizmu transportu, selektywności substratowej oraz hamowania i aktywacji transporterów aminokwasów obojętnych umożliwia racjonalne poszukiwanie i projektowanie związków chemicznych o aktywności ukierunkowanej na te transportery. Ponadto głębsze zrozumienie tych aspektów stanowi cenne narzędzie do badania ich funkcji fizjologicznych i potencjału terapeutycznego, co za tym idzie umożliwia poznanie ich znaczenia w zdrowiu i chorobie. Ta wiedza jest szczególne ważna, ze względu na zaangażowanie transporterów w kluczowe procesy neurologiczne oraz ich powiązanie z zaburzeniami psychiatrycznymi.

Część eksperymentalna tej pracy koncentruje się na modelowaniu homologicznym wspomnianych transporterów w różnych stanach konformacyjnych. Modele B^0AT2, NTT4 zostały wykorzystane do scharakteryzowania elementów ich struktur kluczowych dla transportu substratu. Szczegółowa analiza struktur B^0AT2 i NTT4, połączona z badaniami dokowania substratów, rzuca światło na podstawy molekularne ich selektywności substratowej. Oba transportery mają wspólne kluczowe reszty, w ważnych dla transportu substratu, regionach funkcjonalnych, takich jak przedsionek, bramki zewnątrz i wewnątrzkomórkowe, główne miejsce wiązania substratu oraz kieszenie dla jonów. Wspomniana identyczność prawdopodobnie leży u podstaw podobnych profili substratowych

obu powyższych transporterów. Ponadto, hydrofobowy charakter ich głównego miejsca wiązania substratu dodatkowo wyjaśnia ich silniejszą preferencję wobec aminokwasów z hydrofobowymi łańcuchami bocznymi w stosunku do aminokwasów o polarnych łańcuchach bocznych. Badania nad podstawami molekularnymi hamowania B^0AT2 przez loratadynę, z wykorzystaniem różnych metod obliczeniowych, takich jak dokowanie molekularne, symulacje dynamiki molekularnej oraz obliczenia energii swobodnej wiązania MM-GBSA, ujawniły, że ten inhibitor może wiązać się z transporterem w jego stanie konformacyjnym otwartym na zewnątrz, jak i otwartym do wewnątrz. Ponadto, modele B^0AT2 w różnych stanach konformacyjnych zostały wykorzystane do screeningu wirtualnego w celu identyfikacji nowych inhibitorów. Zastosowane podejście oparte o strukturę celu doprowadziło do odkrycia tiagabiny i analogu L-proliny jako inhibitorów B^0AT2. Opracowana w ten sposób metoda w została później wykorzystana do poszukiwania inhibitorów SIT1, co zaowocowało identyfikacją tiagabiny jako inhibitora tego transportera. Dodatkowo, badania nad mutacjami NTT4 powodującymi upośledzenie umysłowe przy użyciu metod symulacji dynamiki molekularnej ujawniły, że zaburzają one stabilność strukturalną i funkcję katalityczną transportera. W ten sposób, na poziome molekularnym, wyjaśnione zostały efekty patogenne wspomnianych mutacji.

Przedstawione w niniejszej rozprawie doktorskiej wyniki przyczyniają się do lepszego zrozumienia właściwości strukturalnych transporterów aminokwasów obojętnych, co stanowi punkt wyjściowy dla dalszych badań eksperymentalnych.

Abbreviations

ADHD	Attention deficit hyperactivity disorder
AKI	Acute kidney injury
B^0AT1	SLC6A19, neutral amino acid transporter
B^0AT2	SLC6A15, neutral amino acid transporter
B^0AT3	SLC6A18, neutral amino acid transporter
BCAAs	Branched chain amino acids
ChEMBL	Database of bioactive molecules with drug-like properties
cryo-EM	Cryogenic electron microscopy
DAT	SLC6A3, Dopamine transporter
dG_{bind}	Gibbs free energy change
EL	Extracellular loop
GABA	Gamma-aminobutyric acid
GaMD	Gaussian accelerated molecular dynamics
GAT1	GABA transporter type 1
GATs	GABA transporters (GAT1, GAT2, GAT3 and GAT4)
HEK293	Human embryonic kidney 293 cells
IL	Intracellular loop
IUPHAR	International Union of Basic and Clinical Pharmacology
LeuT	Leucine transporter
LOR	Loratadine
LPA	L-proline analog
MD	Molecular dynamics
MM-GBSA	Molecular mechanics with generalised Born and surface area solvation
MSA	Multiple sequence alignment
NAATS	Neutral amino acid transporter subfamily
NTT4	SLC6A17, neutral amino acid transporter
NTT5	SLC6A16, neutral amino acid transporter
PDB	Protein Data Bank
PKU	Phenylketonuria
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PTEN	Gene encoding phosphatase and tensin homolog, a tumor suppressor gene
S1 site	Main substrate binding site, orthosteric site
S2 site	Secondary substrate site, vestibule
SIT1	SLC6A20, neutral amino acid transporter
SLC6	Solute carrier 6 family
TGI	Tiagabine
TM	Transmembrane

Publication records

This thesis is based on a series of four peer-reviewed publications, including a review (I) and three original papers (II-IV).

I. **Kukułowicz J**, Pietrzak-Lichwa K, Klimończyk K, Idlin N, Bajda M. The SLC6A15-SLC6A20 Neutral Amino Acid Transporter Subfamily: Functions, Diseases, and Their Therapeutic Relevance. *Pharmacol Rev*. **2023** 76(1):142-193. IF = 19.3

II. **Kukułowicz J**, Siwek A, Wolak M, Bröer A, Yadav A, Bröer S, Bajda M. Insight into the Structure of the Neutral Amino Acid Transporter B^0AT2 Enabled the Discovery of Tiagabine as an Inhibitor. *ACS Chem Neurosci*. **2024.** IF = 4.2

III. **Kukułowicz J**, Bajda M. *In silico* structural studies on the vesicular neutral amino acid transporter NTT4 (SLC6A17). *Comput Struct Biotechnol J*. **2024**. 10;23:3342-3347. IF = 4.5

IV. Bröer A*, Hu Z*, **Kukułowicz J***, Yadav A, Zhang T, Dai L, Bajda M, Yan R, Bröer S. Cryo-EM structure of ACE2-SIT1 in complex with tiagabine. *J Biol Chem*. **2024** 300(9):107687. IF = 4.0

* These authors contributed equally to the work.

The obtained results were presented at the international conferences:

Oral communication: **Kukułowicz J**, Bajda M, Structural studies on SLC6A15 neutral amino acid transporter, *7th International Electronic Conference on Medicinal Chemistry*, 1–30.11.2021, Basel, Switzerland.

Poster: **Kukułowicz J**, Bröer A, Yadav A, Bröer A, Bajda M, Structural studies of the neutral amino acid transporter B^0AT2 revealed tiagabine as a potent inhibitor, *32nd Annual GP2A Medicinal Chemistry Conference,* 28-30.8.2024, Coimbra, Portugal.

Oral communication: **Kukułowicz J**, Bröer A, Hu Z, Yadav A, Zhang T, Dai L, Yan R, Bröer S, Bajda M, Structural studies of neutral amino acid transporters of the SLC6 family coupled with discovery of new inhibitors, *42nd Annual Scientific Meeting of Australasian Neuroscience Community*, 2-4.12.2024, Perth, Australia.

Research included in this thesis was supported by the **Strategic Program Excellence Initiative at Jagiellonian University** (U1C/W42/NO/28.19) and **Jagiellonian University Medical College grant** (N42/DBS/000375). The SPRm analysis was carried out with the use of research infrastructure co-financed by the **Smart Growth Operational Programme** POIR 4.2 project no. POIR.04.02.00-00-D023/20. The collaboration with the Australian National University was facilitated by the **Polish National Agency for Academic Exchange** (NAWA) (Mobility under **STER Programme**).

1. Introduction

1.1. Transporters of SLC6 family

Solute carriers (SLC) are proteins that facilitate the transport of small, polar molecules across cell membranes, supporting various physiological functions such as neural signaling, energy metabolism, and biochemical pathways. This superfamily, comprising over 400 transporters categorized into 67 families, is pivotal in maintaining cellular and systemic homeostasis [1–4]. Transporters of SLC6 family are primarily responsible for the transport of monoamines and amino acids with the use of an electrochemical gradient as the driving force. According to the Union of Basic and Clinical Pharmacology (IUPHAR) nomenclature, this family is divided into four subfamilies, related by sequence similarity and substrate specificity, with a total of 20 members [5]. They are transporters for monoamine, glycine, γ-aminobutyric acid (GABA), and neutral amino acid (Figure 1). Well-studied SLC6 transporters are those for neurotransmitters such as monoamines (serotonin, norepinephrine, dopamine) and GABA. Inhibitors of these transporters are prescribed to treat depression, anxiety, attention deficit hyperactivity disorder (ADHD), and epilepsy [6].

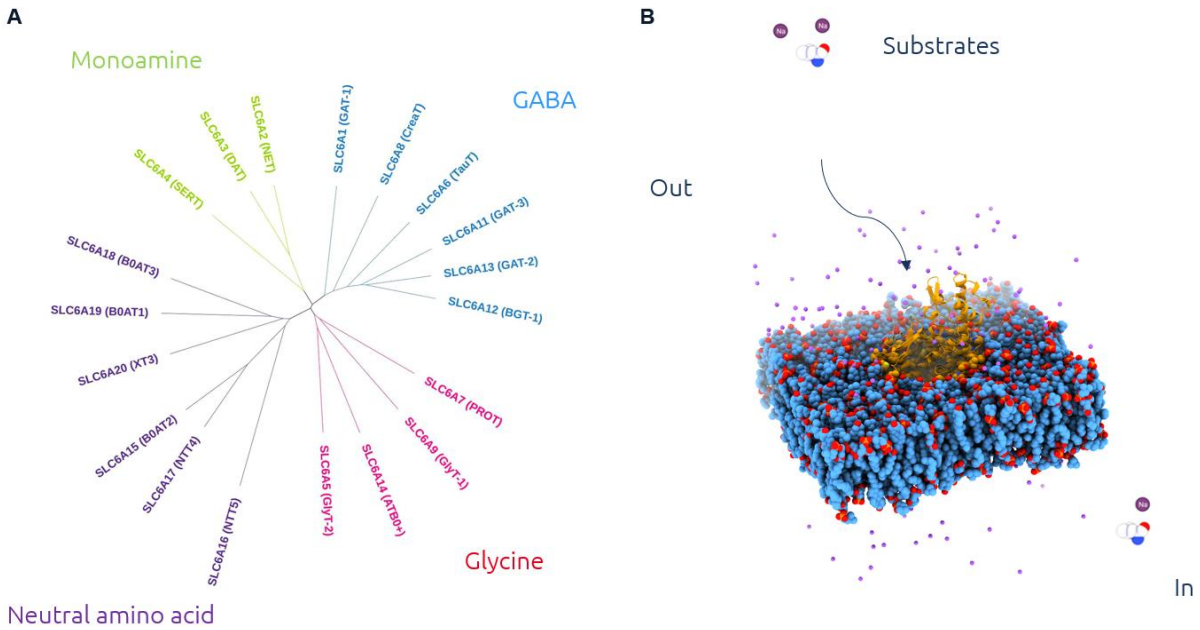

Figure 1 (A) The phylogenetic tree of the SLC6 family. Members and subfamilies are shown. (B) Visualization of the neutral amino transporter in the cell membrane.

1.2. Structure and substrate transport mechanism of SLC6 transporters

The SLC6 transporters share so-called LeuT fold, which is characteristic of the bacterial homolog of SLC6 - the *Aquifex aeolicus* leucine transporter [7]. This fold consists of 12 helical transmembrane domains (TM) connected by extracellular and intracellular loops (EL, IL), with both the N- and C- termini located intracellularly (Figure 2A). Such carriers are characterized by two repeats of five transmembrane segments (TM1–5 and TM6–10), related along a pseudo-two-fold symmetry axis parallel to the cell membrane. TM1, TM3, TM6, and TM8 form an inner ring that contains the main substrate binding site (S1) in the center of membrane. Above the S1 site, a vestibule called also secondary binding site (S2) is located. It is separated from the S1 site by residues forming an extracellular gate (Figure 2B).

The transport mechanism of SLC6 members is explained by an alternating-access model. This model is based on a rigid scaffold of TM3-5 and TM8-10, along with a mobile 'bundle' formed by TM1, TM6, and TM2, TM7 [8]. Such an arrangement allows the transporter for coordinated uptake of substrates from the extracellular side and their release into the cytosol. A key feature of this mechanism is the unfolded region in the middle of TM1 and TM6, which divides them into TM1a, TM1b, TM6a and TM6b. This region, also known as the hinge, is critical for substrate binding and facilitates conformational changes during the transport cycle. Transport turnover in SLC6 transporters begins with an outward-open conformation, allowing co-transported ions to enter the transporter and nest in their respective pockets adjacent to the S1 site. This is followed by the entry of the substrate, its passage through the vestibule, and binding at the S1 site. Subsequently, the extracellular gate closes, and the transporter becomes occluded. To complete the transport cycle, a transition to an inward-open state occurs, enabling the release of the substrate into the intracellular environment. This process is characterized by disruption of the interaction between residues of the intracellular gate and remarkable conformational changes in TM1a and TM6b as well as shifts in TM7 and TM2 (Figure 2C).

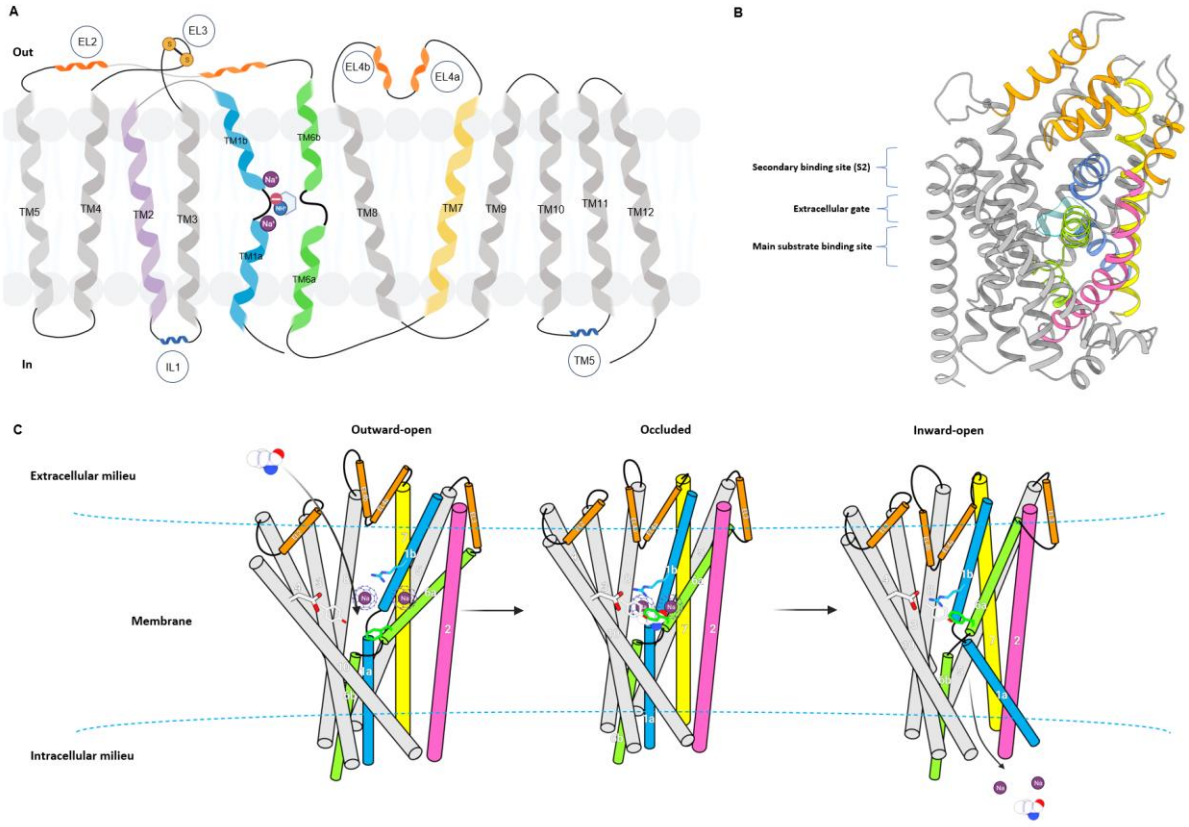

Figure 2 (A) Topology of SLC6 transporters. Transmembrane domains colored in gray constitute a rigid scaffold, whereas the others undergo conformational shifts during substrate transport. (B) Bacterial transporter for leucine in an occluded conformation (PDB code: 2a65). (C) Transition of SLC6 transporter between the distinct conformational states.

1.3. Expression, catalytic and physiological function of neutral amino acid transporters

A subfamily of neutral amino acid transporters (NAATS) of SLC6 family includes the SLC6A15-SLC6A20 transporters [9]. SLC6A15 transporter (B^0AT2) and SLC6A17 transporter (NTT4) are widely expressed in the brain, including regions such as the striatum, cortex, hippocampus and amygdala. Regarding functional division, expression of these transporters occurs in neurons of glutamatergic and GABAergic systems. In terms of cellular localization, B^0AT2 is expressed in the soma and axons of neurons, whereas NTT4 is expressed in synaptic vesicles, albeit its membrane anchoring has also been proposed [10–13]. SLC6A18 transporter (B^0AT3), SLC6A19 transporter (B^0AT1) and SLC6A20 transporter (SIT1) are localized mainly in apical membranes of epithelial cells in the kidney proximal tubules and intestines [14–23]. Notably, glial tissue also expresses SIT1. [22,24,25]. Functional expression of B^0AT3, B^0AT1, and

SIT1 in the kidneys and intestines depends on angiotensin-converting enzyme 2 (ACE2) as an essential auxiliary protein that facilitates membrane trafficking and anchoring [26–30]. Transcript of gene encoding SLC6A16 transporter (NTT5) appears in both peripheral tissues and brain. In peripheral tissues, it is highly expressed in the testis (Table 1) [31].

Table 1 Body distribution of the neutral amino acid transporters of SLC6 family.

Transporter	**Main organ(s), structures, and systems**	**Remaining organs, structures**	**Subcellular localization**
SLC6A15 (B^0AT2)	Brain: neurons of striatum, cortex, hippocampus, amygdala, hypothalamus, pons, spinal cord, epithelial cells of choroid plexus, glial cells ependymal cells (bordering ventricles) Neurons of GABAergic, glutamatergic, serotoninergic, noradrenergic, and cholinergic system	Muscle, eye, placenta	Cell membrane: soma and axon
SLC6A16 (NTT5)	Testis, pancreas, prostate, uterus, liver, lungs Brain: spinal cord, corpus callosum, caudate nucleus, hippocampus, amygdala	Blood, bone	Vesicles
SLC6A17 (NTT4)	Brain: neurons of hindbrain, cerebellum, hippocampus, brain stem, thalamus, hypothalamus, cerebral cortex, spinal cord, amygdala, pons olfactory bulb, striatum, substantia nigra, pontine nuclei, epithelial cells of choroid plexus Neurons of GABAergic, and glutamatergic system	Skeletal muscle, intestine, liver, eye	Vesicles: presynaptic and post-synaptic terminal Putative localization in cell membrane
SLC6A18 (B^0AT3)	Kidney: proximal tubules Seg2, and Seg3	Intestine, liver, spleen	Cell membrane: luminal exposition for kidney, intestine

Transporter	Main organ(s), structures, and systems	Remaining organs, structures	Subcellular localization
SLC6A19 (B^0AT1)	Intestine: villi enterocytes of jejunum and ileum Kidney: proximal tubules Seg1, Seg2, and Seg3	Pancreas, skin, prostate, stomach	Cell membrane: luminal exposition, kidney, intestine
SLC6A20 (SIT1)	Kidney: proximal tubules Seg1, Seg2, Seg3	Brain: Glial cells Intestine: villi of enterocytes Lungs: alveolar cells type II Thymus, spleen	Cell membrane: luminal exposition for kidney, intestine

In vitro examination of activity of B^0AT2 and NTT4 displayed very close profile of transported substrates between these transporters. Although they transport a broad spectrum of neutral amino acids, they are high affinity transporters for branched-chain amino acids (BCAAs) such as leucine, isoleucine, and valine, as well as proline and methionine [10,13,32,33]. Notably, recently reported *in vivo* study showed that the primary role of NTT4 is to provide glutamine to synaptic vesicles [34]. The high affinity transporters B^0AT3 and SIT1 were shown to prefer glycine. In addition, SIT1 also preferentially accepts proline. [22,24]. It is worth noting that the function of B^0AT3 appears to be insignificant in humans, as the mutation resulting in a missense variant of the transporter does not negatively affect body homeostasis [35]. Low affinity B^0AT1 exhibits high promiscuity against a broad range of neutral amino acids excluding imino acids [18,36]. In the case of all the above transporters, transport is sodium dependent. In the case of B^0AT3 and SIT1, transport is also chloride dependent. Table 2 summarizes substrate profile of neutral amino acid transporters, their substrate specificity as well as stoichiometry of transport.

Table 2 Substrates of neutral amino acid transporters of SLC6 family, their Michaelis constant values and stoichiometry of transport.

Transporter	**Substrates**	**K_m values (mM)**	**Ratio of transport of substrate and co-substrate(s)**
SLC6A15 (B^0AT2)	Methionine Proline Valine Leucine Isoleucine Alanine Phenylalanine Glutamine Asparagine	0.04 / 0.1 0.2 / 0.38 0.16 0.81 / 0.16 0.058 / 0.08 0.67 1.05 5.3 n/a	$1Aa:1Na^+$
SLC6A16 (NTT5)	n/a	n/a	n/a
SLC6A17 (NTT4)	Methionine Proline Valine Leucine Isoleucine Glutamine Glycine	n/a 0.86 / 0.36 n/a n/a n/a 0.52 0.17	$1Aa:1Na^+$
SLC6A18 (B^0AT3)	Glycine Alanine Methionine Valine Leucine Isoleucine Serine	0.53 n/a n/a n/a 0.21 n/a n/a	$1Aa:2Na^+:1Cl^-$
SLC6A19 (B^0AT1)	Methionine Valine Leucine Isoleucine Phenylalanine Alanine Glutamine Asparagine Cysteine Glycine Threonine Proline Tyrosine Tryptophan Histidine	~1.0 ~1.0 ~1.0 ~1.0 ~4.0 ~4.0 ~4.0 ~4.0 ~4.0 ~11.0 ~11.0 ~11.0 ~11.0 ~11.0 ~11.0	$1Aa:1Na^+$
SLC6A20 (SIT1)	Proline Glycine	Brain transporter isoform: 0.157 Kidney isoform: 0.049 Brain isoform: 0.02 Kidney isoform: 0.31	$1Aa:2Na^+:1Cl^-$

B^0AT2 and NTT4 are involved in the transport of precursors for glutamate biosynthesis [10,32]. The expression of functional NTT4 is critical for the development of the central nervous system in the prenatal stage [37]. SIT1 is linked to glutaminergic regulation in the brain [24,25]. SLC6A18-SLC6A20 transporters play a role in amino acid absorption in the intestine and reabsorption in the kidneys, ensuring the supply of amino acids to the organism [21,38]. The substrates of NTT5 as well as its physiological role are unknown. Table 3 summarizes the physiological role of the NAATS according to their expression in the body.

Table 3 Physiological functions of the SLC6A15-SLC6A20 transporters according to body expression [9].

Transporter	**Organ, tissue, system**	**Physiological functions**
SLC6A15 (B^0AT2)	Brain: neurons Epithelium of the choroid plexus Brain neurons Glutaminergic system Brain neurons	Provision of neutral amino acids toward neurons Element of the blood-brain barrier; selective uptake of amino acids toward neurons Glutaminergic homeostasis Provision of precursor amino acids for glutamate biosynthesis Food intake control
SLC6A16 (NTT5)	n/a	n/a
SLC6A17 (NTT4)	Brain neurons: synaptic terminals of neurons Epithelium of choroid plexus Brain neurons	Packing vesicles into glutamine Element of the blood-brain barrier, selective uptake of amino acids towards neurons Neuritogenesis, synaptogenesis in prenatal development Food intake control
SLC6A18 (B^0AT3)	Kidney	Reabsorption of amino acids from proximal tubules
SLC6A19 (B^0AT1)	Intestine, kidney	Absorption and reabsorption of amino acids in proximal tubules of kidney and enterocytes of intestine
SLC6A20 (SIT1)	Intestine Brain	Reabsorption and absorption of amino acids in the proximal tubules of the kidney and enterocytes of the intestine Provision of neutral amino acids to glial cells and to a lesser extent to neurons, glutaminergic homeostasis

1.4. Mutations of neutral amino acid transporters related to a variety of disorders and causing genetic diseases

Polymorphisms of *slc6a15* gene were associated with major depressive disorder and anxiety [39,40]. Mutations of NTT4 have been reported in humans with mental retardation [37]. Mutations in the SLC6A18-SLC6A20 transporters contribute to hyperglycinuria and

iminoglycinuria. Hyperglycinuria is characterized by excessive glycine excretion in the urine, linked to the development of osteoporosis and kidney stones. Iminoglycinuria involves defective renal reabsorption of glycine, proline, and hydroxyproline and can manifest as hypertension, glycosuria, nephrolithiasis, mental retardation, gyrate atrophy, deafness, and blindness [38]. In addition, mutations of B^0AT1 are associated with Hartnup disease, while mutations of SIT1 have been linked with type 2 diabetes mellitus, retinal and age-related macular degeneration [19,21,41,42]. Hartnup disease results from impaired intake of neutral amino acids, leading to symptoms such as pellagra-like skin rashes, cerebellar ataxia, mood disturbances, diarrhea, photosensitivity, and fatigue, often exacerbated by niacin deficiency [19,21].

1.5. Inhibition of selected neutral amino acid transporters as strategy for the treatment of metabolic and psychiatric disorders

JNT-517 (Jnana Therapeutics), a small molecule chemical inhibitor of B^0AT1, is in phase III of clinical trials for a treatment of phenylketonuria (PKU) (https://clinicaltrials.gov study number: NCT06628128) (Figure 3). Research has demonstrated that genetic deletion or pharmacological inhibition of B^0AT1 significantly lowers phenylalanine levels in both plasma and the brain in PKU animal models. This effect is primarily attributable to decreased intestinal absorption and limited renal reabsorption of phenylalanine, resulting in its increased urinary excretion [43]. Another B^0AT1 inhibitor, MZE782 from Maze Therapeutics, is being studied for the treatment of acute kidney injury (AKI). It has been shown that knockout of B^0AT1 in mice attenuates tubular senescence and reduces the inflammatory and fibrotic response to nephrotoxic agents such as aristolochic acid, which is commonly used to model AKI [44]. It has also been suggested that inhibition of B^0AT1 may be relevant for the treatment of obesity and metabolic disorders such as type 2 diabetes mellitus, similar to gastric bypass surgery [43,45–47].

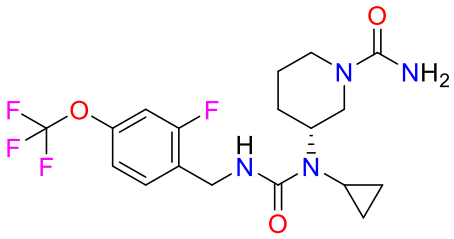

Figure 3 Structure of JNT-517, a B^0AT1 inhibitor in clinical trials for the treatment of PKU.

Recently reported studies suggest that the inhibition of B^0AT2, NTT4 and SIT1 might bring benefits in the treatment of some psychiatric disorders [24,48–50]. The inhibition of B^0AT2 was proposed to treat depressive disorders and anxiety. This is dictated by fact that behavioral studies in mice under basal conditions and subjected to persistent stressors showed that *slc6a15* gene knockout mice, compared to wild type individuals, revealed both reduced anxiety and a depressive phenotype under chronic stress conditions. In addition, mice overexpressing B^0AT2 exhibited anxiety disorders under basal conditions [51,52]. A recent report showed that suppression of NTT4 in adult mice results in increased levels of GABA and glutamate in synaptic vesicles [34]. This effect may be due to an altered balance in the glutamine-glutamate-GABA pathway as a result of a local buildup of glutamine [50]. Newly published studies have shown that mutation of *PTEN* gene encoding phosphatase and tensin homolog, a suppressor protein, leads to overexpression of SIT1 and consequent reduction in glycine levels and NMDA receptor function. In contrast, SIT1 knockout mice show both increased glycine levels and NMDA receptor signaling [24,25]. Consequently, the inhibition of NTT4 and SIT1 may be helpful for the enhancement of synaptic activity by neurotransmitters derived from glutamine, such as glutamate [53]. Such intervention may be relevant in the treatment of schizophrenia, which is associated with insufficiency of the glutaminergic system [54].

2. Aim of the thesis

Brain-expressed B^0AT2, NTT4, and SIT1 transporters remain unexplored in terms of their usefulness as biological targets for the treatment of psychiatric disorders. In this context, small molecule chemical compounds, such as inhibitors or activators, provide a valuable toolkit for validating their therapeutic relevance. However, the number of non-substrate active compounds against these transporters has been limited, with antihistaminergic loratadine and structurally related desloratadine analogs being the primary examples identified for B^0AT2.

Understanding the architecture of proteins and exploring their conformational space provide insight into their mechanism of action. From the point of view of medicinal chemistry, the variety of molecular target structures helps in the search for active compounds i.a. inhibitors, exploiting computational approaches. Determining the structure of proteins using experimental techniques like X-ray crystallography or cryogenic electron microscopy (cryo-EM) is challenging, expensive, and time-consuming. Therefore, using *in silico* methods to generate models of selected molecular targets can be a significant simplification in such cases.

In view of the above, the aim of the thesis was the structural characterization of selected neutral amino acid transporters, namely B^0AT2, NTT4, and SIT1. In addition, obtained structures were employed for a rational computer-aided search for inhibitors of the B^0AT2 and SIT1 as well as studies on molecular determinants of pathogenic mutations of NTT4 transporter.

Studies in this thesis included the generation of homology models for each of the aforementioned transporters in different conformational states, a detailed discussion on the structures of B^0AT2 NTT4 and an analysis of determinants of their substrate selectivity. In the case of NTT4, deep learning derived model supported structural studies. Furthermore, the binding mode of loratadine to B^0AT2 was examined employing molecular docking, molecular dynamics simulations and MM-GBSA binding free energies calculations, which ultimately facilitated the discovery of new scaffolds as inhibitors of this protein and evolutionary related neutral amino acid transporter, SIT1. This work also contains research on molecular determinants of pathogenic mutations of NTT4, aimed at the understanding of the transport

mechanism of NTT4. This aspect was investigated through molecular dynamics simulations using previously obtained models.

All computational investigations planned in this thesis were performed in the Department of Physicochemical Drug Analysis at the Faculty of Pharmacy of Jagiellonian University Medical College.

3. Methods

3.1. Homology modeling

Homology models of B^0AT2, NTT4 and SIT1 in different conformations were constructed using MODELLER 10.1 employing AutoModel class. A total of 50 models were produced for each conformation. Structures of SLC6 homologs obtained by X-ray crystallography and cryo-EM in an outward-open, occluded and inward-open were derived from the Protein Data Bank (PDB) and used as templates. These were transporters for dopamine (DAT) in an outward-open state (PDB code: 4xp9), and GABA (GAT1) in an inward-open state (PDB code: 7sk2). Notably, to build models of B^0AT2 and NTT4 in the occluded state, a chimeric template (LeuT-B^0AT1) was constructed using the structure of the LeuT in the occluded state (PDB: 2a65) as the core and the fragment of EL4 of the B^0AT1 transporter in the occluded state obtained by cryo-EM method (PDB: 6m17). To this end, B^0AT1 was superimposed with LeuT, then the helical fragment of the EL4 following TM7 was extracted from B^0AT1 (PDB code: 6m17) and transferred to the LeuT. In this way, in the LeuT-B^0AT1 template, original V-shaped conformation of EL4 facing the entrance cavity was retained. Models of B^0AT2 and NTT4 in the outward-open state were additionally obtained in SWISS-MODEL using the structure of DAT (PDB code: 4xp9) as a template (Publications II, III).

Prior to homology modeling, a multiple sequence alignment (MSA) of SLC6 transporters and aLeuT was performed in Clustal Omega. These pairwise alignments of target and template were then compared with the MSA results and findings from other published studies. Manual adjustments were made to the alignments of the transporters investigated and aLeuT as needed. The sequence of studied transporters for alignment was derived from the UniProt database. Furthermore, the sequence alignment input file did not include the N- and C- termini because no template sequence was allocated to these regions. Jalview and BioEdit programs were used to facilitate the alignments. They allowed for inspection of process and editing of sequences (Publications II, III).

The quality of the generated models was assessed based on the Discrete Optimized Protein Energy (DOPE) score, and the top 10% of models with the most favorable scores were selected

for further evaluation. These models were analyzed using Ramachandran plot, QMEAN and Verify3D (Publications II, III).

3.2. Deep learning derived model of NTT4

In the case of NTT4, an additional deep learning derived AlphaFold model was used, which is deposited in AlphaFoldDB (Publication III).

3.3. Visualizations of models

Visualization of the models was carried out in PyMOL, Maestro (Schrödinger Suite) and UCSF ChimeraX (Publications II, III).

3.4. Docking studies

Docking studies were performed in Glide (Schrödinger Suite version 2020-3). The ligands of interest were prepared using the LigPrep module with default settings. Ionization was predicted at a pH of 7.4 ± 0.2. The models were generated using the Protein Preparation Wizard with default parameters. Docking studies were performed with standard precision. Five poses for each ligand were saved, and post-docking minimization was performed. The OPLS3e force field was used for docking studies (Publications II, III).

3.5. Molecular dynamics simulations

All molecular dynamics (MD) simulations followed a consistent protocol across the studies. Complexes subjected to MD simulations were positioned within the membrane using the OPM server. For MD simulations in DESMOND 6.3 simulated systems were constructed using the System Builder module within the Schrödinger Suite (2020-3), employing TIP3P water and POPC membrane models. Protein-ligand complexes were placed within an orthorhombic box measuring 15 Å × 15 Å × 15 Å, and the system was neutralized using Na^+ and Cl^- ions to achieve a final concentration of 0.15 M. System minimization and pre-equilibration were performed following the standard protocol implemented in DESMOND 6.3. Final production simulations were conducted in the NPT ensemble at 300 K and 1.013 bar, using a 2 fs timestep and a

recording interval of 100 ps, with the random seed and default settings for other parameters. The OPLS3e force field was employed (Publication II, III). Gaussian Accelerated MD (GaMD) simulations were conducted using the NAMD program. Input files were prepared using the CHARMM-GUI server, applying its enhanced sampler option. Complexes were embedded in a system sized at 125 Å × 125 Å (X and Y dimensions) with a POPC membrane, solvated using the TIP3P water model, and neutralized with Na^+ and Cl^- ions to achieve a final 0.15 M NaCl concentration. System equilibration was followed using the six-step protocol proposed by CHARMM-GUI, with additional short MD runs and equilibration to collect potential statistics for GaMD simulations. These productions were carried out in NAMD 2.13 using the CHARMM36m force field applying NPT ensemble at 303.15 K. The timestep was 2 fs, the total simulation duration was 20 ns, and data were recorded at intervals of 10 ps (Publication II).

3.6. MM-GBSA binding free energy calculations

MM-GBSA binding free energies calculations were carried out in Prime MM-GBSA v3.0 Schrödinger Suite (2020-3) with applied of the VSGB solvation model and the OPLS3e force field. Protein flexibility, water, and additional ions were skipped in the computations (Publication II).

4. Results and discussion

4.1. Structure modeling of neutral amino acid transporters of SLC6 family

4.1.1. Structure of B^0AT2 and NTT4 transporters and molecular determinants of their substrate selectivity

General structures of B^0AT2 and NTT4 resemble cryo-EM structures of NAATS members such as B^0AT1 and SIT1 [55–57]. They consist of 12 helical transmembrane domains (TM), connected by extracellular (EL) and intracellular (IL) loops displaying the conserved LeuT-like fold. Moreover, modeled structures showed characteristic for NAATS transporters significantly elongated EL3, EL4 and EL6. Notably, cryo-EM structures within this subfamily revealed a unique conformation of EL4 with two distinct segments: a large helix following TM7 and a V-shaped fragment oriented toward the substrate transport pathway. Importantly, it was shown that in both B^0AT1 and SIT1, the helical fragment following TM7 forms polar interactions with ACE2, stabilizing the heteromeric complex (Publications II, III).

Interestingly, comparison of the pairwise sequence alignments of B^0AT2 and NTT4 and insight into their models revealed that subsites important for substrate transport are strictly conserved. This means that the vestibule, extracellular gate, main substrate binding site, ion pockets, cytosolic vestibule, and intracellular gate of these proteins share the same residues. Given the above, this might explain the similar profile of transported substrates between B^0AT2 and NTT4 (Publications II, III).

The vestibule of SLC6 transporters has been shown to initially recognize substrate. This pocket can be divided into hydrophobic and ionic regions. Furthermore, the hydrophobic pocket allows interactions with the side chains of the substrates, while the charged residues of the ionic site interact with the amine and carboxyl groups of the transported amino acid. Based on models of B^0AT2 in different conformations and their comparison with other SLC6 structures, as well as analysis of multiple sequence alignments of SLC6 homologs, the hydrophobic pocket of this transporter is composed of Val521, Trp164, Ile161, Tyr157, Trp85, and Phe443, while the ionic site contains Arg86 and Asp525. Although the ionic site residues are conserved across the SLC6 family as part of the extracellular gate, the hydrophobic pocket

composition varies between transporter types, potentially affecting substrate selectivity (Publication II).

Examination of ion binding pockets adjacent to the S1 site of the substrate free B^0AT2 model in the occluded state revealed that one sodium ion (Na^+1) is surrounded by TM1, TM6, and TM7, while another sodium ion (Na^+2) interacts with TM1 and TM8. Both the $Na^+(1)$ and $Na^+(2)$ pockets exhibited the tetrahedral geometry of coordinated ions (Publication II).

The main binding site in the B^0AT2 models in the occluded state closely mirrors that of the template structures and consists of residues from TM1, TM3, TM6 and TM8. The S1 pocket, which is thought to play a critical role in substrate recognition, is predominantly surrounded by hydrophobic amino acids. Access to the S1 site from the extracellular environment is controlled by an extracellular gate formed by Tyr158 (TM3) and Phe308 (TM6a), which act as a lid over the substrate in the occluded conformation. Phe77 from TM1a occupies the pocket's bottom. Examination of TM1b and TM8 revealed that the main chains of Leu81, Gly82, and Asn83 (TM1b), along with the side chains of Ser474 and Thr478 (TM8) form a polar subsite within S1. TM3 from the S1 site contains a hydrophobic Val154 near the extracellular gate, while residues from TM6 facing the main substrate binding pocket are two phenylalanines (Phe308 and Phe314) and Ala309 (Publication II).

The overall hydrophobic nature of S1 site of B^0AT2 favors the binding of amino acid substrates with hydrophobic side chains rather than those with polar unionized or charged ones. This is reflected by docking studies of biogenic amino acids, i.e. proline, to the orthosteric pocket of this transporter in the occluded state where this amino acid was consistently accommodated (Figure 4) (Publication II).

The above results of studies on determinants of transport mechanism and selectivity of B^0AT2 are also applicable to NTT4. This is justified by the identity of regions implying these determinants (Publication III).

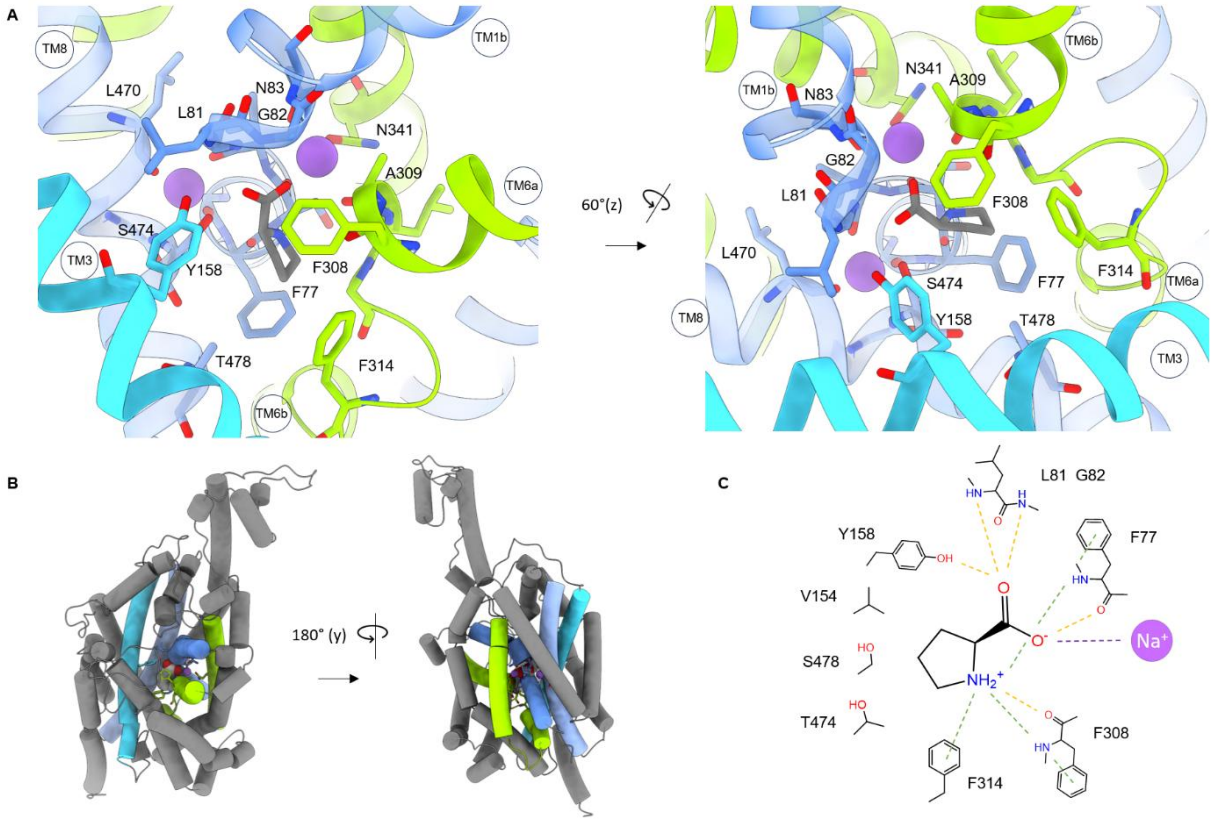

Figure 4 (A) and (B) Proline at main substrate binding site of B^0AT2. (C) Interaction diagram of proline with residues of the orthosteric site of B^0AT2. The carboxylic acid fragment of the substrate interacts with key residues of S1 site, including the main chain of Phe77, Leu82, Gly82. In addition, this moiety forms a hydrogen bond with the side chain of Tyr158 and an ionic interaction with sodium of the Na^+1 pocket. Secondary amine of proline forms hydrogen bond with Phe308 along with cation-π interactions with Phe77, Phe308 and Phe314. Substrate aliphatic moiety surrounded by hydrophobic S1 subsite.

4.2. Molecular modeling of B^0AT2 inhibition by loratadine

SLC6 transporters in complex with inhibitors adopt outward-open, occluded and inward-open conformations. Therefore, models in all these conformational states were used to study the inhibition of B^0AT2 by loratadine (LOR) (Publication II).

Preliminary docking studies of loratadine with B^0AT2 in all three primary conformations revealed that LOR was unable to dock to the transporter in its occluded state. This outcome can be attributed to the size of LOR exceeding the dimensions of the S1 site and the steric hindrance caused by the closed extracellular gate, which prevents the compound from being accommodated within this binding pocket (Publication II).

LOR docked to the model of B^0AT2 in an outward-open state occupied both the S1 and S2 sites. The tricyclic moiety was positioned near the extracellular gate with its chloride-substituted benzene ring surrounded by Asp525 (TM10), Tyr158, Ile161 (TM3), and Trp85 (TM1b). The pyridine ring was located near Arg86 (TM1b), Phe308 (TM6), and Thr530 (TM10), and the alkyl fragment connecting the aromatic benzene to the pyridine ring was located near Gln437 from EL4. The substituted piperidine ring of LOR extended toward the S1 site, where its carbonyl oxygen interacted with Gly82 (TM1b), while the alkyl was enveloped by hydrophobic residues of the S1 pocket (Figure 5A). LOR docked to B^0AT2 in an inward-open state occupied the S1 site, positioning the ethyl group extending from the urethane fragment close to the hydrophobic side chains of phenylalanine residues Phe308 and Phe314 (TM6). The carbonyl group of LOR was directed toward Gly82 (TM1b), while its tricyclic system was opposite to Phe77 (TM1a), allowing π-π stacking interactions of the LOR aromatic fragments with this residue (Figure 5B) (Publication II).

The stability of the above complexes has been studied using MD simulations in DESMOND and GaMD simulations in NAMD. As a result, LOR complexed with B^0AT2 in the inward-open state was shown to be more stable than LOR complexed with this transporter in the outward-open conformation. Interestingly, MM-GBSA binding free energy (dG_{bind} (kcal/mol)) calculations for the initial complex of LOR with B^0AT2 as well as of relaxed complexes on MD simulations showed that inhibition of B^0AT2 by LOR is energetically more favorable when the transporter conforms to the inward-open state, suggesting its non-competitive mode of inhibition (Publication II).

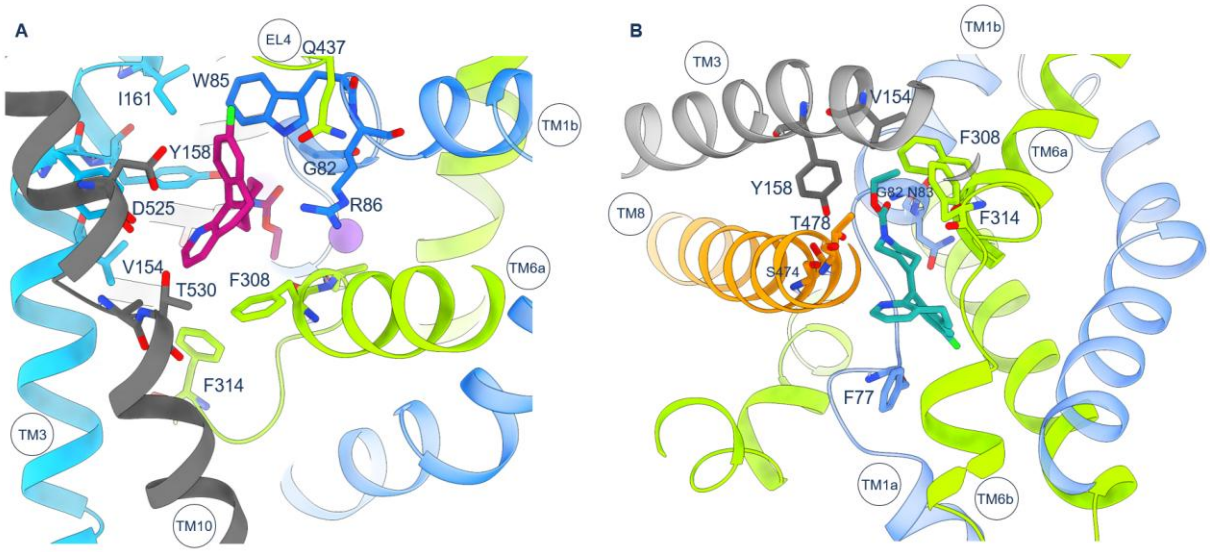

Figure 5 (A) Interaction of LOR with B^0AT2 in the outward-open conformation. (B) Binding of LOR in the inward-open state of B^0AT2.

4.3. Virtual screening in the search for B^0AT2 and SIT1 inhibitors

Loratadine is a histamine H_1 receptor antagonist and a weak inhibitor of transporters for GABA (GATs) of the SLC6 family [58]. Considering the above, it was assumed that other GABA transporter inhibitors may inhibit B^0AT2. Accordingly, GATs inhibitors were derived from ChEMBL (database of bioactive molecules with drug-like properties) and docked to the models utilized to examination of the binding modes of LOR with B^0AT2. As a result, derivatives of nipecotic acid-based GAT inhibitors docked well to B^0AT2 in an outward-open and inward-open state. In particular, the potent GAT1 inhibitor tiagabine (TGI) showed remarkable binding characteristics with B^0AT2 similar to those of LOR in complex with this transporter in both outward-open and inward-open states (Figure 6). Furthermore, the dataset of compounds containing fragments of B^0AT2 substrates was docked to the models from studies on LOR binding mode. In this way, the L-proline analog (LPA) has been shown to be a potential candidate for a further inhibitor of B^0AT2 (Publication II).

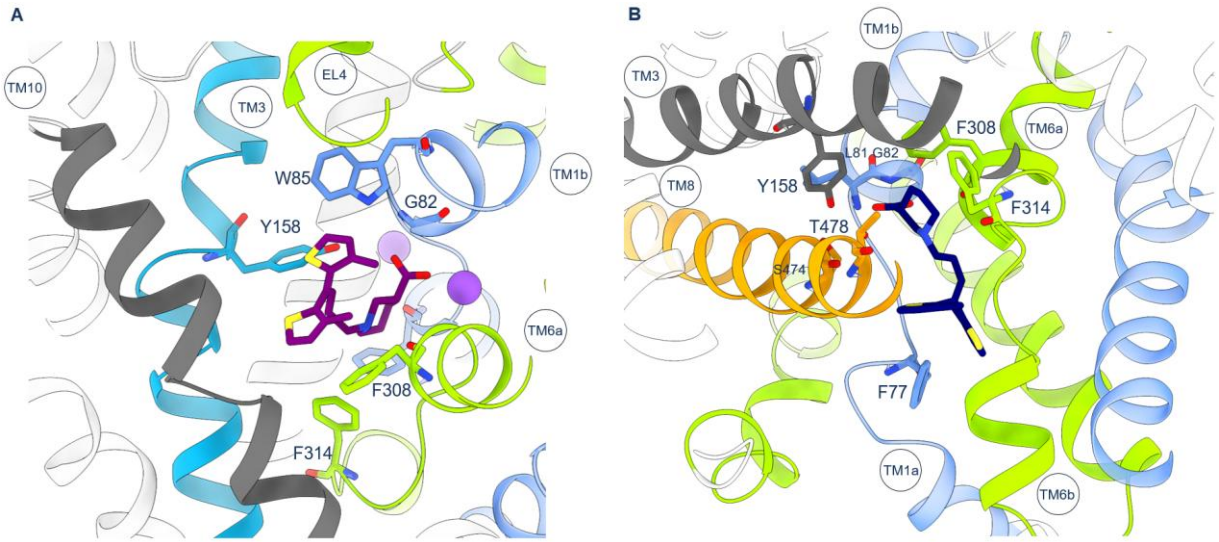

Figure 6 (A) TGI complexed by B^0AT2 in the outward-open conformation. Notable interactions include those of the carboxylic acid of TGI with sodium of the Na^+1 pocket and with Gly82. In addition, the charged amine moiety formed a hydrogen bond with Phe308, while the thiophene rings showed hydrophobic interactions with Trp85 and Phe308. (B) TGI complexed with B^0AT2 in the inward-open state. The nipecotic acid fragment occupied the S1 site, allowing interactions of the carboxylic acid of TGI with Leu81, Gly82, and Tyr158). Protonated nitrogen atom created cation-π interactions with phenylalanine 308 and 314. Thiophene rings approached Phe77. The stability of the above binding modes was confirmed by molecular dynamics simulations performed in DESMOND and GaMD in NAMD.

The activity of loratadine, tiagabine, and L-proline analog was evaluated in a preliminary *in vitro* assay measuring the uptake of 100 µM [^{14}C]leucine by B^0AT2 expressed in *Xenopus laevis* oocytes in the presence of the above candidate inhibitors at a screening concentration of 100 µM (Figure 6). As a result, TGI stood out, inhibiting leucine transport by 90%, while for LOR and LPA these values equaled 57% and 63% respectively (Publication II). In an extended biological evaluation uptake of [^{3}H]proline was measured at a concentration of 50 nM by HEK293 cells overexpressing B^0AT2 in the presence of loratadine or tiagabine or LPA at three concentrations: 1 mM, 100 µM and 10 µM. As a result, tiagabine inhibited radiolabeled substrate transport by 100% at 1 mM, 84% at 100 µM and 23% at 10 µM inhibitor. For loratadine the corresponding values were 100%, 100%, 85% and for LPA they were 100%, 70% and 25% (Publication II). For each of these compounds in the presence of 50 nM radiolabeled proline, a dose-response curve was plotted for concentrations of inhibitor ranging from 1 nM to 1 mM. The IC_{50} obtained was 22.2 µM (± 3.7) for tiagabine, 2.1 µM (± 0.5) for loratadine, and 42.2 (± 8.7) for LPA. Moreover, the results surface plasmon resonance microscopy (SPRm)

assay HEK293 cell lines showed K_d values of 9.27 µM and 3.48 µM for tiagabine and 53.9 µM and 12.3 µM for loratadine (Publication II).

The computational protocol developed for the screening of B^0AT2 inhibitors in Publication II was applied to the screening of SIT1 inhibitors published in Publication IV. In doing so, tiagabine was also discovered as an inhibitor of SIT1 with a determined IC_{50} for [^{14}C]leucine uptake by *Xenopus laevis* oocytes overexpressing SIT1 of 18 µM. Moreover, the inward-open structure of SIT1 in complex with TGI was resolved by cryo-EM method (PDB code: 8wm3), which is consistent with the results of molecular modeling predictions (Publication II).

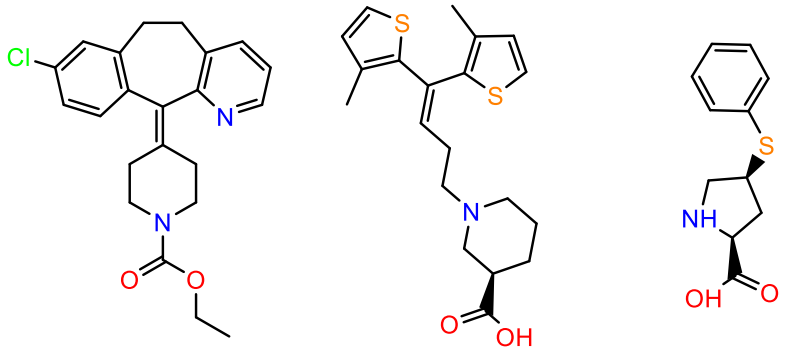

Figure 7 Structures of the B^0AT2 inhibitors: LOR (left), TGI (middle), and LPA (right).

TGI is an antiepileptic drug that until recently was thought to be a selective inhibitor of GAT1, however its precise pharmacology remains unclear [59,60]. On the other hand, seizures have been reported in clinical cases of tiagabine overdose [61]. Notably, TGI is currently in phase III clinical trials for the treatment of schizophrenia (ClinicalTrials.gov, ID: NCT00179465). Interestingly, TGI is also prescribed off-label for anxiety and depression [62]. In GAT1-depleted mice subjected to the elevated plus maze test, reduced anxiety behavior was observed following TGI administration, suggesting that its antidepressant and antianxiety effects may involve mechanisms beyond GAT1 inhibition [63]. In view of the above, newly discovered molecular targets of TGI will drive further studies on the mechanism of action of TGI, as it cannot be excluded that unclear effects of administration of this drug may result from inhibition of some NAATS. In addition, TGI and LPA can be used as starting points for the design of new B^0AT2 and SIT1 inhibitors.

4.4. Studies on pathogenic mutations of NTT4

Inherited congenital mutations Pro633Arg and Gly162Arg in NTT4 have been identified as pathogenic variants that impair transporter function, leading to intellectual disability. Experimental data have shown that the Pro633Arg mutation of NTT4 is associated with a failure of the transporter to localize to the membrane of synaptic vesicles. In contrast, the Gly162Arg mutant reaches the membrane but is incapable of transporting substrates [34,37]. To understand how the above mutations affect NTT4 expression in the cell membrane and the mechanism of transport, MD simulations were performed in DESMOND (Publication III).

Three runs of 500 ns MD simulations revealed that the Pro633Arg mutation of NTT4 model derived from AlphaFoldDB, located in the TM12, membrane-flanking region of the transporter, induces structural instability. The substitution of proline with a positively charged arginine rapidly disrupts the local membrane environment, leading to the displacement of the POPC membrane. The Gly162Arg mutation was found within TM3, adjacent to the EL4, a structural element critical for substrate transport. Three repetitions of 100 ns MD simulations using a model of the Gly162Arg NTT4 mutant in the inward-open state demonstrated that the introduction of arginine in place of Gly162 created polar interaction with Asn517 contained in TM10. Notably, Asn517, which normally interacts with Thr438 from EL4 to stabilize the inward-open conformation, lost its native interaction due to interference with Arg162. This disruption likely impairs the transporter's transition to the inward-open state during substrate turnover. Consequently, the above MD simulations showed that the Gly162Arg NTT4 mutant is unable to maintain the specific interaction that occurs in its inward-open state. This finding may explain why this mutant is non-functional despite successful membrane insertion (Publication III).

5. Summary

This thesis encompasses structural studies utilizing homology models of B^0AT2, NTT4, and SIT1 in different conformations. Insights into the structures of B^0AT2 and NTT4, combined with docking studies of their substrates, contribute to the understanding of the molecular determinants of their substrate selectivity. Notably, both transporters share key residues that line crucial for transport subsites, such as the vestibule, extracellular gate, main substrate binding site, ion pockets, cytosolic vestibule, and intracellular gate. These shared structural features may explain their similar substrate profiles. Furthermore, the hydrophobic nature of the main substrate binding site in these transporters was revealed, accounting for their preference for amino acids with hydrophobic side chains over those with polar ones.

Studies on the molecular basis of B^0AT2 inhibition by loratadine, employing a variety of computational methods such as molecular docking, molecular dynamics simulations, and MM-GBSA free energy calculations, revealed that this inhibitor might be accommodated in both the outward-open and inward-open states of the transporter. Furthermore, models of B^0AT2 in different conformational states were used for virtual screening to identify new inhibitory agents. The applied structure-based approach led to the discovery of tiagabine and an L-proline analog as B^0AT2 inhibitors. Subsequently, the developed computational protocol was utilized to screen for SIT1 inhibitors, resulting in the identification of tiagabine as an inhibitor of this transporter as well. The discovery of new B^0AT2 and SIT1 inhibitors opens new avenues for the development of therapeutic agents targeting these transporters.

MD simulation studies on pathogenic mutations of NTT4 associated with intellectual disability suggest that one variant causes misfolding of TM12 leading to impaired membrane anchoring, while another may be associated with insufficient EL4 movements, which are essential for the alternating-access transport mechanism.

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