Neuroscience Letters 451 (2009) 212–216

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Discovery and characterization of potent small molecule inhibitors of the high affinity proline transporter☆

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ARTICLE INFO

Article history:
Received 22 September 2008
Received in revised form 5 January 2009
Accepted 6 January 2009

Keywords:
Proline transporter
Dopamine transporter
Glycine transporter
Proline transporter inhibitor

ABSTRACT

The mammalian proline transporter (PROT) is a high affinity Na^+/Cl^--dependent transporter expressed in specific regions of the brain. It is homologous to other neurotransmitter transporters such as glycine, norepinephrine, serotonin, and dopamine transporters. PROT is enriched in glutamatergic synaptic terminals and may play an important role in the regulation of excitatory neurotransmission. No non-peptide small molecule inhibitors have been described for this transporter. To study its physiological role in the central nervous system and evaluate its potential as a therapeutic target, we established cell lines that stably express recombinant hPROT and characterized its kinetic properties for proline uptake. We then screened for inhibitors and identified a series of compounds that inhibit hPROT-mediated proline uptake. A known compound, benztropine, was found to inhibit hPROT with an IC_{50} of 0.75 μM. A series of novel compounds were also found, one of which, LP-403812, showed an IC_{50} of approximately 0.1 μM on both recombinant human and mouse PROT without significant inhibition of glycine and dopamine transporters at concentrations up to 10 μM. This compound also inhibited proline transporter activity of mouse brain synaptosomes with the same potency. These inhibitors provide important tools for the understanding of PROT functions in the brain and may lead to the development of therapeutic agents for certain neurological disorders.

Certain amino acids or their metabolites have been shown to be able to modulate excitatory synaptic transmission in the central nervous system. Glycine or D-serine, for example, is required for activation of NMDA-type glutamate receptor [7,8]. Proline, an amino acid found abundantly in the brain, may also modulate activity of NMDA receptors. Physiological levels of proline (3 μM in CSF) have been shown to regulate glutamatergic transmission in rat hippocampal neurons [1,2]. Proline, albeit at mM concentrations, was also found to activate the NMDA receptor directly [10]. Therefore, proline, like D-serine and glycine, may modulate glutamatergic transmission in certain areas of the brain though the precise mechanism is unknown.

Rapid signaling of neurotransmitters in the synaptic space requires precise control of the magnitude and duration of activity. One of the hallmarks of neurotransmitters is the existence of specific mechanisms to terminate signaling through removal of the ligands. Several important ligands, such as dopamine, serotonin, and glycine are removed from synaptic spaces through rapid re-uptake by transporters [15]. Interestingly, a high-affinity transporter for L-proline was molecularly identified and designated PROT, an important piece of evidence for L-proline as a neurotransmitter [4,14]. Sequence analysis indicates that PROT belongs to the family of neurotransmitter transporters encompassing those for dopamine, serotonin, norepinephrine, and glycine with an overall sequence identity of 40–50% [14,16]. Furthermore, PROT was found to be localized specifically to a subset of excitatory nerve terminals with partial apposition to AMPA-type and NMDA-type glutamate receptors clusters, suggesting a presynaptic regulatory role of PROT at certain glutamatergic synapses [3,11].

The family of transporters containing PROT is integral membrane proteins with 12 transmembrane domains. Transporter activity is dependent on both Na^+ and Cl^-, and is powered by the transmembrane electrochemical ion gradient [13]. Recombinant expression of PROT showed that the transporter is highly specific for

Abbreviations: PROT, proline transporter; hPROT, human proline transporter; mPROT, mouse proline transporter; DAT, dopamine transporter; GLYT, glycine transporter; GGFL, des tyrosyl leu-enkephalin.

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doi:10.1016/j.neulet.2009.01.018

L-proline with an apparent K_m of approximately 6 μM [14]. Previously, three opioid peptide-related peptides were found to inhibit L-proline uptake by rat brain synaptosomes [12]. These peptides were confirmed to inhibit proline uptake using recombinant PROT [5,6].

However, these peptides are unstable *in vivo*, and have limited utility in studying the function of PROT. Given the potential role of L-proline in modulating excitatory neurotransmission, we initiated efforts in identifying and optimizing small molecule inhibitors of PROT that may lead to the discovery of therapeutics. Hereby, we describe the discovery and characterization of such high affinity PROT inhibitors and discuss their potential use in understanding the roles of PROT in the brain and in treating certain neurological disorders.

^{3}H-proline (55 Ci/mmol) and ^{3}H-glycine (30 Ci/mmol) were from American Radiolabeled Chemicals Inc. ^{3}H-dopamine (50 Ci/mmol) was purchased from GE Healthcare. Affinity purified rabbit anti-rat PROT (peptide) antibody was from Alpha Diagnostic International. GGFL, benztropine and other chemicals were from Sigma. The compound library used for screening is part of Lexicon Pharmaceutical's small molecule compound collection.

The full-length coding sequences of human and mouse PROT were obtained by standard PCR techniques using the human and mouse brain Marathon-Ready cDNA (Clontech) as template and cloned into pCR4–TOPO. The coding sequence was then subcloned into pcDNA 3.1 and transfected into COS1 cells. Drug-resistant clones were isolated with G418 (0.54 mg/ml), and clones stably expressing PROT were identified by proline-specific uptake. Clones stably expressing the glycine transporter 1 and dopamine transporter (GLYT1 and DAT, respectively) were created essentially by the same procedure.

Synaptosomes were prepared as described previously [6]. Briefly, mouse forebrain was dissected and homogenized in 7 ml ice-cold homogenization buffer consisting of 0.32 M sucrose, 1 mM $NaHCO_3$, and protease inhibitor cocktail (Roche). The brain homogenates were centrifuged at 1,000 × *g* for 10 min to remove nuclei. Supernatant was collected and re-centrifuged at 20,000 × *g* for 20 min to pellet crude synaptosomes. The synaptosomes were resuspended in ice-cold assay buffer: 122 mM NaCl, 3.1 mM KCl, 25 mM HEPES, 0.4 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 10 mM dextrose at pH 7.4. Resuspended synaptosomes were centrifuged again at 20,000 × *g* for 20 min, and the pelleted synaptosomes were resuspended in the same buffer. Protein concentration was measured by the DC protein assay kit (BioRad).

Transporter assay: Proline uptake assay was performed essentially as described previously [6,14], with minor modifications. Cells stably expressing PROT were seeded at 15,000 cells per well in a 384 well poly D-lysine coated plate (or 30,000 cells per well in a 96 well plate) and grown overnight in DMEM plus 10% FBS and 0.54 mg/ml G418. The cells were then washed twice with Krebs-Ringer's-HEPES-Tris (KRHT) buffer, pH 7.4, containing 120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 10 mM D-glucose, 10 mM HEPES and 5 mM Tris. The cells were then incubated with 50 μl of KRHT buffer containing 45 nM ^{3}H-proline for different periods of time at room temperature. Proline uptake was terminated by removing the radiolabeled proline and washing the cells rapidly for three times with 100 μl of ice-cold KRHT buffer. Scintillation fluid (50 μl per well) was added and the amount of radioactivity present was determined using a Packard TopCount scintillation counter. Nonspecific uptake was determined by measuring ^{3}H-proline uptake in the presence of 2 mM unlabeled proline. High throughput screening for PROT inhibitors was performed under the same conditions in 384 well plates in the presence of 2 μM compound, except that the uptake time was 20 min. All data were processed by a Graphpad Prism 4 for Windows software using nonlinear sigmoidal dose response model.

Glycine uptake assay was performed in the same way as proline uptake assay using COS1 cells expressing GLYT1 and 166 nM ^{3}H-glycine instead of ^{3}H-proline. The incubation time was 10 min at room temperature. Nonspecific uptake was determined by measuring ^{3}H-glycine uptake in the presence of 2 mM unlabeled glycine. Dopamine uptake assays were performed in a similar way with slight modifications of the uptake buffer as described previously [9]: Krebs-Ringer's-HEPES-Tris (KRHT) buffer, pH 7.4, containing 125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM D-glucose, 25 mM HEPES, 1 mM sodium ascorbate, and 1.2 mM KH_2PO_4. The uptake was carried out for 10 min at room temperature in the presence of 1 μM ^{3}H-dopamine. Nonspecific uptake was determined by measuring ^{3}H-dopamine uptake in the presence of 250 μM benztropine.

Proline transport assay was performed in a 100 μl reaction mixture consisting of 10 μg synaptosomes, 1 μCi of ^{3}H-proline (0.24 μM) in the same assay buffer as the recombinant PROT for 0–20 min at room temperature. The reaction was terminated by rapid filtration through GF/B filter plate (Millipore), followed by three rapid washes of 200 μl ice-cold assay buffer. Scintillation fluid (50 μl per well) was added to each well and incubated for 2 h. ^{3}H-proline transport was determined by radioactivity counting. Nonspecific activity was measured in the presence of 0.3 mM GGFL in the reaction.

The human PROT coding sequence was cloned into the vector pcDNA 3.1 and transfected into COS1 cells and HEK293 cells. COS1 cells were chosen for most of studies because the cells adhere to assay plates much better than HEK293 cells and permit automated washing. Drug-resistant colonies were isolated and screened for robust, high affinity proline uptake. One of those clones (E12) was found to have strong proline uptake activity and further characterized in detail. Western blot analysis showed expression of hPROT with an apparent molecular weight (MW) of approximately 85 kD (Fig. 1A). The predicted MW of hPROT is about 70.8 kD. Shafqat et al. [14] reported an apparent MW of 68 kD for hPROT in human CNS, and Renick et al. [11] reported of a MW of 69 kD for rat PROT expressed in HEK293 cells. Since this family of transporters is known to be glycosylated, the difference in MW is most likely due to different levels of glycosylation depending on how the transporter is expressed.

The kinetics of proline uptake by hPROT in the E12 clone was then determined. Transport of proline was linear up to 60 min at room temperature (data not shown), much longer than the 20 min time found at 37 °C [6]. The K_m for proline uptake is about 13.2 μM, between the value of 6.2 μM reported by Shafqat et al. [14] and 20.1 μM by Galli et al. [6]. The proline transport activity of PROT was absolutely dependent on Na^+, as expected, as shown in Fig. 1B. The Hill coefficient of Na^+ is 2, suggesting the binding of 2 Na^+ ions is required to transport each proline molecule, consistent with the previous report [6] and the recent crystal structure of the bacterial leucine transporter from Aquifex aeolicus [17]. The K_m of 65 mM is slightly higher than previous reported 40.7 mM [6]; this could be due to different cell background or assay conditions. In addition, we also tested the activity of the transporter in the presence and absence of Cl^-, and found it was, as expected, totally inactive in the absence of Cl^- (data not shown).

Several opioid peptides, including the peptide des-tyrosyl leu-enkephalin (GGFL) were found to be inhibitors of PROT [12]. The inhibitory activity of GGFL was confirmed under our assay conditions, with an IC_{50} of 1.5 μM, similar to the value of 3.5 μM reported previously [6]. Assays were optimized under room temperature to allow high throughput screening at room temperature for better temperature control and data consistency. At room temperature, hPROT showed similar K_m for proline and affinity to GGFL, the only known inhibitor as reported previously, suggested that it is feasible to identify inhibitors by assaying hPROT at room temper-