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Substrate inhibitors and blockers of excitatory amino acid transporters in the treatment of neurodegeneration: critical considerations

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Abstract

Excessive glutamate release (mediated by reversed uptake) or impaired reuptake contributes to the etiopathology of many neurodegenerative disorders. Thus great effort has been devoted to the discovery of agents that can interfere with high-affinity Na^+-dependent glutamate transport, with the aim of finding new therapeutics against neurodegenerative diseases. We developed two different 3D-pharmacophore models for substrate inhibitors and blockers, which led to the rational design of novel and potent glutamate and aspartate analogues that selectively interact with excitatory amino acid transporters (EAAT). Our results indicated that all analysed EAAT ligands share the same orientation of the acidic functions and the protonatable nitrogen, even though the distance between the carboxylic carbons varies from 3.7 to 4.9 Å. This distance does not discriminate between substrate inhibitors and blockers, but between glutamate and aspartate derivatives. In contrasts differences in the volume distribution of the rest of the molecule with respect to the axis connecting the two carboxylic groups are responsible for the difference in activity between transportable and nontransportable inhibitors. Thus our 3D receptor interaction model for EAAT substrates and nontransportable inhibitors could lead to the rational design of selective EAAT ligands as possible neuroprotective agents. However, some critical points, such as which glutamate transporter is present on glutamatergic nerve terminals and which glutamate transporter mediates reversed glutamate uptake, still remain to be elucidated.

Keywords: Glutamate transporter; Neurodegeneration; Glutamate uptake; Substrate; Inhibitor

1. Introduction

Glutamate is the major excitatory amino acid in the mammalian central nervous system (CNS), being implicated in several physiological processes. Termination of excitatory activity is mediated by high-affinity Na^+-dependent glutamate transporters, principally located in glial cells surrounding synapses and in post-synaptic neurons. To date, five different glutamate transporters have been cloned from the mammalian CNS, namely, the excitatory amino acid transporters 1–5 (EAAT1-5) in humans. The homologues in rodents are designated as glutamate–aspartate transporter GLAST (EAAT1), glutamate transporter GLT1 (EAAT2) and excitatory amino acid carrier 1, EAAC1 (EAAT3), while EAAT4 and EAAT5 maintain the same nomenclature. Each of the five types of transporter is encoded by a different gene; however, analysis of the amino acid sequence revealed 38% to 65% homology among the five different transporters and a very high sequence conservation (>90% identity) among species homologues (Gegelashvili and Schousboe, 1997; Arriza et al., 1997; Danbolt, 2001; Kanner and Borre, 2002).

The neurotoxic properties of glutamate are well known: they were first shown by Lucas and Newhouse (1957), who demonstrated the lethal effects of glutamate on cultured neurons. Over the last 50 years a direct correlation between neuroexcitatory and neurotoxic properties of glutamate has been established. Direct glutamate-mediated excitotoxicity is linked to an excessive and prolonged presence of the neurotransmitter in the synaptic cleft and therefore to an excessive activation of post-synaptic glutamate receptors. Overactivation of the ionotropic NMDA and AMPA/kainate receptors by exogenous glutamate is associated with an

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increased influx of Na^+ and Ca^{2+} ions. These events are followed by activation of different intracellular pathways that can lead to neuronal death, such as alteration of membrane potential, activation of degradative enzymes (proteases, endonucleases, phospholipases), production of reactive oxygen species and increased energy consumption (Sonnewald et al., 2002).

The potential involvement of glutamate-mediated toxicity in both acute (stroke, ischaemia, epilepsy, traumatic brain injury) and chronic (amyotrophic lateral sclerosis, Alzheimer's, Parkinson's and Huntington's diseases) neurodegenerative diseases has been demonstrated in several studies. Excessive pre-synaptic release or impaired reuptake of glutamate is the principal mechanism believed to be involved in these processes. In particular conditions, such as in energy failure (ischaemia, stroke, hypoglycaemia), when the electrochemical gradient is dramatically reduced or disrupted, glutamate transporters can function in a reversed mode, thus releasing glutamate in the extracellular space, with consequent overactivation of post-synaptic receptors (Attwell et al., 1993).

In patients with amyotrophic lateral sclerosis, synaptosomal preparations from affected CNS regions show decreased glutamate uptake, reflecting the selective loss of the glial transporter EAAT2 (Rothstein et al., 1992). In Alzheimer's disease, β-amyloid peptide accumulation has been linked to oxidative impairment of GLT1 (Maragakis and Rothstein, 2001), although this result is controversial (Danbolt, 2001). A role for glutamate transporters has been hypothesised also in epilepsy, where a reduction in the number of glial transporters GLT1 and GLAST, and of the neuronal transporter EAAC1 is found in patients and in some animal models (Roettger and Amara, 1999; Maragakis and Rothstein, 2001).

Thus great effort has been devoted in the last decade to the discovery of agents that can interfere with high-affinity Na^+-dependent glutamate transport, with the aim of finding new therapeutics against neurodegenerative diseases. An exhaustive review of the field has recently been published (Campiani et al., 2003). Here, we summarize the experimental approach that we have followed for the rational design of new EAAT transportable or nontransportable inhibitors (Campiani et al., 2001) and raise some critical considerations that still represent important open questions.

2. Design of glutamate and aspartate analogues as selective substrates and nontransportable inhibitors of excitatory amino acid transporters

To identify the structural and conformational features responsible for pharmacological activity, we performed a molecular modelling study with known EAAT substrate inhibitors (L-*threo*-3-hydroxyaspartic acid (L-*threo*-3-OHAsp), L-*threo*-4-hydroxyglutamic acid (L-*threo*-4-OHGlu), L-*trans*-pyrrolidine-2,4-dicarboxylic acid (L-*trans*-2,4-PDC), *cis*-1-aminocyclobutane-1,3-dicarboxylic acid (*cis*-ACBD), (2*S*,3*R*,4*R*)-2-(2-carboxycyclopropyl)glycine (L-CCGIII), 2,4-methanopyrrolidine 2,4-dicarboxylate (2,4MPDC)) and nontransportable inhibitors (blockers) (L-*anti-endo*-3,4-methanopyrrolidine dicarboxylate (L-*anti-endo*-3,4-MPDC), L-*trans*-pyrrolidine-2,3-dicarboxylic acid (L-*trans*-2,3-PDC), L-*threo*-β-benzyloxyaspartic acid (L-*threo*-3-BnOAsp), kainic acid, dihydrokainic acid, DL-*threo*-β-benzoyloxyaspartic acid (L-*threo*-3-BzAsp)) (Fig. 1A,B). Accordingly, we developed two different 3D-pharmacophore models for substrate inhibitors and blockers (Fig. 2), which led to the rational design of novel and potent glutamate and aspartate analogues that selectively interact with EAAT (Fig. 1C). Our results indicated that all analysed EAAT ligands share the same orientation of the acidic functions and the protonatable nitrogen, even though the distance between the carboxylic carbons may vary from 3.7 to 4.9 Å. This distance does not discriminate between substrate inhibitors and blockers, but between glutamate and aspartate derivatives. In contrast, differences in the volume distribution of the rest of the molecule with respect to the axis connecting the two carboxylic groups are responsible for the difference in activity between transportable and nontransportable inhibitors. From Fig. 2, it can be concluded that a constrained folded glutamate-like conformation, e.g., L-*trans*-2,3-PDC and L-*anti-endo*-3,4-MPDC (orange and green in Fig. 2B, respectively), forces the volume excess to occupy the edge opposite to the one where carboxylic groups are located (blockers), while a constrained unfolded glutamate-like conformation, e.g., L-*trans*-2,4-PDC and 2,4-MPDC (white and green in Fig. 2A, respectively), allows a quite uniform volume distribution on both sides of the backbone (substrate inhibitors).

The new substrate inhibitors that we developed (1a–c, Fig. 1C) potently interact with EAAC1 and evoke currents similar to glutamate itself, but, unlike all the known substrates, show weak-to-negligible affinity for ionotropic glutamate receptors. The oxazoline-based EAAT blockers (1d,e, Fig. 1C) represent the first examples of glutamate transporter ligands characterized by the lack of a free protonatable amine function. These compounds are completely inactive at ionotropic glutamate receptors but inhibit glutamate transport and the leak-anion conductance in the absence of transported substrates, a behaviour typical for competitive glutamate transporter inhibitors. Our study generated two 3D receptor interaction models for EAAT substrates and nontransportable inhibitors that could facilitate the rational design of selective EAAT ligands as possible neuroprotective agents.

Although it is accepted that a defect in glutamate uptake may play an important role in neurodegenerative disorders, it is not clear how this process can be modulated. For the sake of clarity, in the present review we limit this discussion to the case of reversed transport occurring during brain

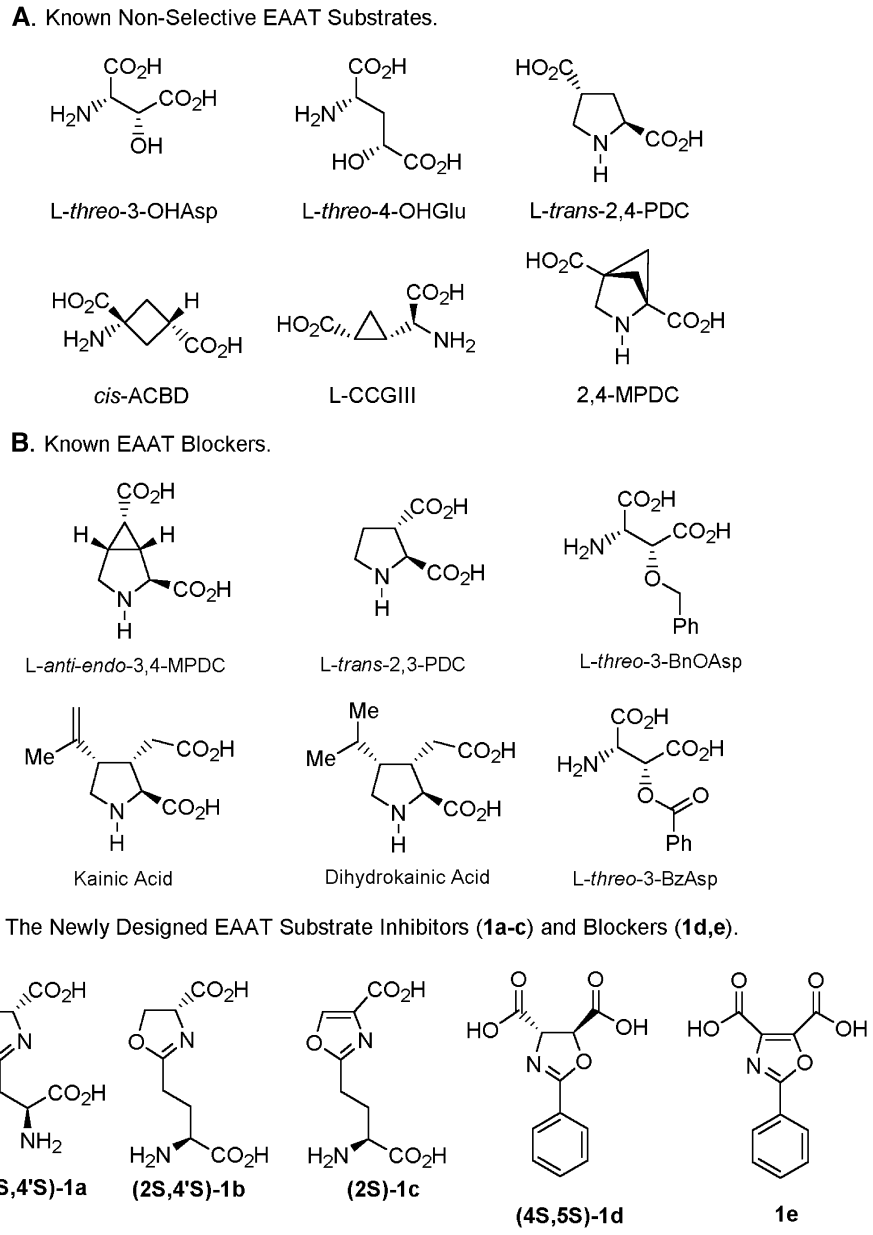

Fig. 1. Chemical structures of the considered and newly designed EAAT substrate inhibitors and blockers. (A) Known nonselective EAAT substrates: L-*threo*-3-hydroxyaspartic acid (L-*threo*-3-OHAsp); L-*threo*-4-hydroxyglutamic acid (L-*threo*-4-OHGlu); L-*trans*-pyrrolidine-2,4-dicarboxylic acid (L-*trans*-2,4-PDC); *cis*-1-aminocyclobutane-1,3-dicarboxylic acid (*cis*-ACBD); (2*S*,3*S*,4*R*)-2-(2-carboxycyclopropyl)glycine (L-CCGIII); 2,4-methanopyrrolidine 2,4-dicarboxylate (2,4-MPDC). (B) Known EAAT blockers: L-*anti-endo*-3,4-methanopyrrolidine dicarboxylate (L-*anti-endo*-3,4-MPDC); L-*trans*-pyrrolidine-2,3-dicarboxylic acid (L-*trans*-2,3-PDC); L-*threo*-β-benzyloxyaspartic acid (L-*threo*-3-BnOAsp); DL-*threo*-β-benzoyloxyaspartic acid (L-*threo*-3-BzAsp). (C) Newly designed EAAT substrate inhibitors (1a–c) and blockers (1d,e): (2*S*,4′ *S*)-2-amino-3-(4′-hydroxycarbonyloxazolin-2-yl)propionic acid ((2*S*,4′ *S*)-1a); (2*S*,4′ *S*)-2-amino-4-(4′-hydroxycarbonyloxazolin-2-yl)butyric acid ((2*S*,4′ *S*)-1b); (2*S*)-2-amino-4-(4′-hydroxycarbonyloxazol-2-yl)butyric acid ((2*S*)-1c); (4*S*,5*S*)-2-phenyloxazoline-4,5-dicarboxylic acid ((4*S*,5*S*)-1d); 2-phenyl-oxazole-4,5-dicarboxylic acid (1e).

ischaemia, but the basic concepts can be easily extrapolated to other pathologies.

The source of the reversed transport of glutamate is probably pre-synaptic glutamatergic neurons, where glutamate is stored in concentrations higher than in glial cells or other neurons (Danbolt, 2001). Substrates lacking affinity for post-synaptic receptors, which could be transported by the same EAATs utilised by the endogenous neurotransmitter, could, in principle, be useful when reversed transport occurs. But in such a case, the neurons involved in reversed transport would have to be filled with the chemical substrate before stroke or immediately before reperfusion, which at present seems unlikely. Thus nontransportable inhibitors could be more useful in this situation. However, also in this case, at least two important questions remain to be answered in the rational discovery of such neuroprotective agents,

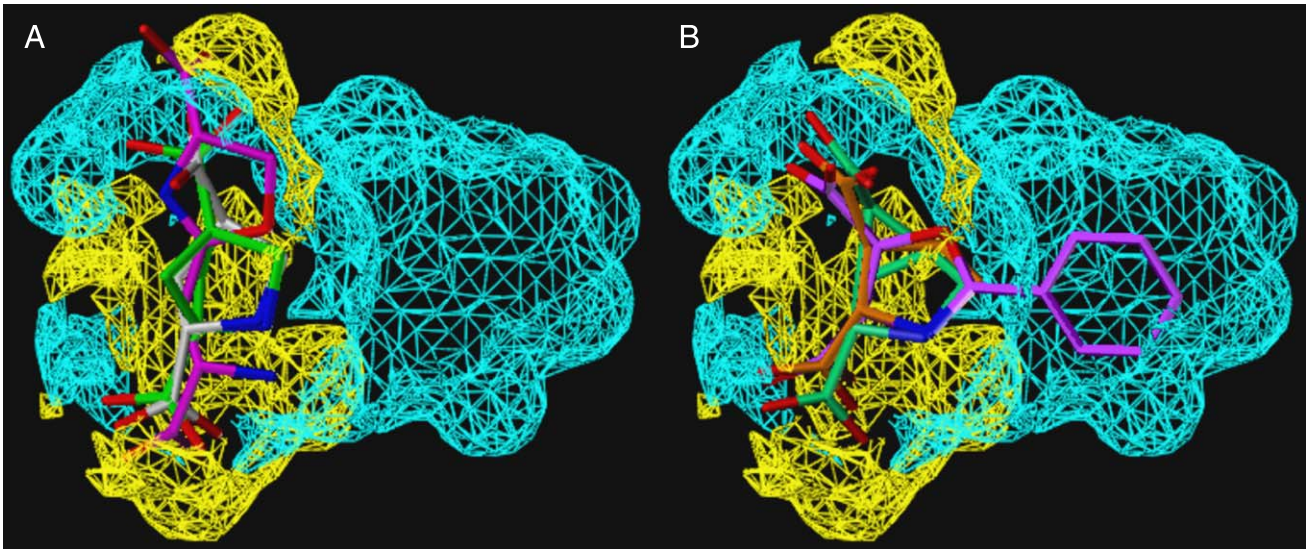

Fig. 2. Molecular volume of EAAT blockers (L-*anti-endo*-3,4-MPDC, L-*trans*-2,3-PDC, L-*threo*-BnOAsp, kainic acid, dhydrokainic acid, L-*threo*-3-BzAsp) with respect to substrate inhibitors (cyan framework), and for substrate inhibitors (L-*threo*-3-OHAsp, L-*threo*-4-OHGlu, L-*trans*-2,4-PDC, *cis*-ACBD, L-CCGIII, 2,4-MPDC) with respect to blockers (yellow framework). (A) Selective substrate inhibitor 1a (magenta) superimposed on L-*trans*-2,4-PDC (white) and 2,4-MPDC (green). (B) Selective blocker 1d (violet) superimposed on L-*trans*-2,3-PDC (orange) and L-*anti-endo*-3,4-MPDC (green). Superimpositions were generated by fitting the protonatable nitrogen (blue) and the two carboxylic carbons (oxygens in red). Hydrogens are omitted for clarity. Adapted from Campiani et al. (2001).

since, as summarised below, our knowledge of glutamate transporters is far from comprehensive.

3. Open questions

3.1. Which glutamate transporter is present on glutamatergic nerve terminals?

Both neurons and glia are capable of high-affinity Na^+-dependent glutamate transport, and the expression of the five transporters shows cellular and tissue specificity. EAAT1/GLAST and EAAT2/GLT1 are localised primarily in astrocytes, in membrane domains that immediately oppose the synaptic cleft or neuropil. EAAT3/EAAC1, EAAT4 and EAAT5 are expressed in neuronal membranes, in a perisynaptic distribution (Danbolt, 2001; Maragakis and Rothstein, 2001).

Experimental evidences suggest the existence of a pre-synaptic transporter located on glutamatergic nerve terminals. For instance, interruption of glutamatergic fibres caused a marked decrease in glutamate uptake in synaptosomes prepared from target regions (Fonnum et al., 1981; Taxt and Storm-Mathisen, 1984). Moreover, neurons are selectively retro-labelled after microinjection of [^{3}H]D-aspartate. Also, strong evidence for this hypothesis arises from immunocytochemical studies of D-aspartate reactivity that accumulates selectively in nerve endings (Gundersen et al., 1993, 1996).

The nature of this neuronal glutamate transporter is not well established, though neurons express GLT1mRNA (Danbolt, 2001) and several splice variants of GLT1 are known. The pre-synaptic transporter could be an isoform of GLT1 not recognised by classical antibodies made against the glial form. In line with this hypothesis, Chen et al. (2002) identified a variant form of GLT1 with a shorter C-terminal (GLT1b), a form that seems to be selectively expressed in glutamatergic nerve endings. This neuronal GLT1b has a pharmacological profile very similar to that of the glial GLT1a, with similar K_m for glutamate (28 ± 3 μM for glial GLT1a and 32 ± 3 μM for neuronal GLT1b), and very similar affinity for dihydrokainate, L-*trans*-2,4-PDC, kainate and L-serine-*O*-sulphate. Microscopic analysis with silver-immunogold labelling shows that this transporter is located mainly in pre-synaptic terminals and also in post-synaptic spines and dendrites, thus confirming the presence of glutamate uptake by pre-synaptic nerve endings.

The demonstration of the presence of a pre-synaptic glutamate transporter in glutamatergic nerve endings is, however, problematic, in part because synaptosomal preparations, used for biochemical assay of [^{3}H]glutamate uptake or heteroexchange, are likely to be contaminated by glial elements. It has been reported that glutamate uptake by synaptosomes prepared from rat brain and spinal cord is principally mediated by a GLT1-like component, based on the sensitivity to specific GLT1 inhibitors (Wang et al., 1998; Bridges et al., 1999) and on the finding that synaptosomes from mice knock-out for GLT1 show very low glutamate uptake (Tanaka et al., 1997). However, it is difficult to make a univocal interpretation of these data. We can conclude that either

a different transporter, with marked similarity to glial GLT1, is present in pre-synaptic terminals or that the synaptosome preparation is contaminated by glial elements. Interestingly, however, there is no GLAST-mediated uptake in synaptosomes (Lieb et al., 2000). Thus new approaches using subfractions enriched in neuronal (or glial) components (Nakamura et al., 1993; Daniels and Vicroy, 1998) will help clarify this matter (Suchak et al., 2003).

3.2. Which glutamate transporter mediates reversed uptake?

In brain ischaemic episodes, glutamate is released when the energy supply is compromised. Evidence shows that the first source of this glutamate is reversed uptake from the cytoplasm. The current hypothesis is that this reversed uptake is mediated, in principle, by neuronal transporters, since the bulk of this transmitter is stored in pre-synaptic neuronal vesicles (Danbolt, 2001). Moreover, astrocytes are less sensitive to hypoxic conditions (Danbolt, 2001) and are less affected by changes in membrane potential. This does not exclude the possibility of a combined release from neurons and astrocytes in ischaemia. There is evidence that in vivo reversed uptake is mediated by GLT1 (Ogata et al., 1992; Seki et al., 1999; Phillis et al., 2000); however, in contrast with these findings, Rossi et al. (2000) reported that dihydrokainate, a selective inhibitor of GLT1, had no effect in reducing glutamate release by reversed uptake.

For the pharmacological treatment of diseases in which glutamate toxicity is linked to the reversed function of transporters, it is of particular interest to design and develop compounds that are able to selectively block this reversed uptake. In these cases, an ideal compound should act as a nontransportable blocker of the EAATs and not as a substrate inhibitor, since the latter could increase reversed uptake by heteroexchange. Such a compound must interact selectively with glutamate transporters, showing low affinity for ionotropic receptors. An important issue is whether selectivity for the different EAATs is needed, and which transporter mediates this action. If reversed uptake occurs mainly from neurons, the neuronal GLT1b could represent an interesting target for compounds with neuroprotective activity.

The GLAST-mediated component has to be considered, because this transporter is highly expressed in CNS regions affected by neurological disorders and is likely to be involved in reversed uptake (since at least some component of this process is not dihydrokainate sensitive). A minor role seems to be played by the neuronal transporter EAAT3, which is expressed post-synaptically and with functions linked to signal transmission. Therefore it would not be suitable to block these transporters, since they are probably not involved in the pathogenesis of neurodegeneration.

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