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# *CONFORMATIONALLY RESTRICTED INHIBITORS OF THE HIGH AFFINITY L-GLUTAMATE TRANSPORTER*

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**Abstract:** A series of acidic amino acids has been prepared and evaluated in an effort to identify the structural features required for binding to and inhibiting the high affinity uptake system that clears L-glutamate from the synaptic cleft during excitatory amino acid-mediated neurotransmission in the mammalian CNS.

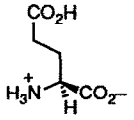
L-Glutamate is the major excitatory neurotransmitter in the mammalian CNS. This excitatory amino acid (EAA) or closely related ones, such as L-aspartate or L-homocysteate, mediate fast synaptic transmission at AMPA and KA receptors as well as participate in higher order processes coupled to $Ca^{++}$ (NMDA receptor) and phosphoinositide (ACPD receptor) signaling.[1] In addition to its role in normal neuronal communication, glutamate acts as a potent neurotoxin when its extracellular concentrations reach excessive levels. Glutamate-mediated neuronal injury, referred to as excitotoxicity, is believed to contribute to CNS pathology in a wide spectrum of disorders, including ischemia, hypoglycemia, epilepsy, Huntington's disease, amyotrophic lateral sclerosis (ALS), and Alzheimer's disease.[2] This dichotomy clearly suggests that levels of glutamate must be carefully maintained at concentrations sufficient to mediate excitatory transmission, yet not so high as to induce excitotoxic-mediated pathology. Specific high affinity transporters, which are integral membrane proteins present on presynaptic terminals and the astrocytes surrounding the synapse, catalyze the translocation of the glutamate and related acidic amino acids across the lipid bilayer of the plasma membrane and play a key role in maintaining this balance. Their ability to rapidly clear EAA agonists from the synaptic cleft is thought to be a critical step in terminating the excitatory signal, recycling the transmitter, and maintaining the extracellular concentration of glutamate below that which could induce excitotoxic injury.[3]

This crucial role has become evident as the potential pathological consequences of reduced function have been investigated. For example, the apparent inverse relationship between excitotoxic injury and transport capacity is consistent with the observation that much larger amounts of L-glutamate are required to produce lesions *in vivo* than of EAA agonists (e.g., kainate) that are not efficiently transported.[4] Similarly, Roberts and colleagues have reported that the neurotoxic action of β-threo-OH-aspartate (β-THA), a competitive inhibitor of glutamate uptake, results from its exacerbation of the excitotoxic action of glutamate.[5] *In vitro* studies with cortical cultures have demonstrated that reductions in transport capacity, produced by either decreasing astrocyte densities or attenuating transport directly, dramatically increase neuronal sensitivity to glutamate-mediated damage.[3b,6] Furthermore, data is now emerging that ties the pathological consequences of compromised transport to specific neurodegenerative diseases. Thus, Palmer et al.[7] have reported a decrease in glutamate transport sites in Alzheimer's disease on the basis of reduced substrate binding (i.e., $^3$H-D-aspartate), while Rothstein et al. have reported a marked reduction in the maximal velocity of glutamate uptake in synaptosomes prepared from the spinal cords of patients with amyotrophic lateral sclerosis (ALS).[8]

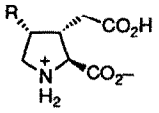
Considerable evidence suggests that EAA transport in the CNS is not mediated by a single system, but by a number of distinct transporters that have been difficult to fully differentiate owing to a lack of specific substrates and inhibitors. Pharmacological and kinetic studies have discerned variations as a function of tranporter location (neuronal vs. glial, forebrain vs. cerebellar, and synaptosomes vs. synaptic vesicles), as well as ion-dependence (sodium vs. chloride). Of the various systems, the high-affinity sodium-dependent glutamate transporter is the most thoroughly characterized in terms of its pharmacology, mechanism, and distribution.[3c,9] The significance of distinguishing and characterizing these systems goes well beyond interesting biochemical differences and is central to: (*i*) evaluating their roles in excitatory physiology and pathology, (*ii*) accurately identifying changes that accompany age or diseases, and (*iii*) assessing the consequences of reduced function. Toward this goal, we have attempted to prepare pharmacologically specific analogues of L-glutamate with which to probe transport biochemistry and function.

As has been the case for the EAA receptors, pharmacological characterization of the EAA transporters is closely tied to their prevalence and to the availability of pharmacologically selective ligands. Thus, previous work

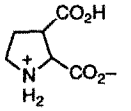
has focused primarily on the sodium-dependent transporter, defining its basic specificity by quantifying the ability of a wide range of EAA analogues to reduce the accumulation of radiolabeled substrates (e.g., $^3$H-L-glutamate and $^3$H-D-aspartate). The transport inhibitors generally share the common feature of being α-amino acids with a second acidic group separated from the α-COOH by 2–4 methylene groups.[3c,9a] Structure/activity studies indicate that the distal COOH group can be derivatized to a hydroxamate, as in L-aspartate-β-hydroxamate, or replaced by a sulfonate group, as in cysteic acid. Some modification of the carbon backbone is also tolerated: β-THA and dihydrokainate (DHK) are well known competitive inhibitors. Interestingly, while the transporter shows a strong preference for binding L-glutamate over the D-enantiomer, both L- and D-aspartate are excellent substrates.



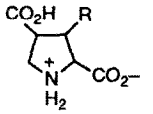
L-Glutamate



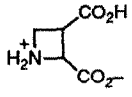
Kainic acid (R = 2-propenyl)
Dihydrokainate (R = *i*-propyl)
Trans-2,3-homo-PDC (R = H)



2,3-PDC



2,4-PDC (R = H)
3-Methoxyethyl (R = $CH_3OCH_2CH_3$)
3-Acetic acid (R = $CH_2CO_2H$)



2,3-ADC

Despite the usefulness of these compounds in the initial characterization of the uptake systems, their lack of selectivity is a serious drawback. For each there are several energetically accessible conformations, which allows binding at sites other than the transporter. As a result, these compounds cannot inhibit glutamate uptake without also activating one (or more) of the EAA receptors. Similarly, such compounds would be expected to be of little value in distinguishing the subtle differences that may exist among the EAA transport systems. Improved selectivity would thus constitute an important advance in this area, and one way to achieve this objective would be the use of analogs with more rigid structures resembling the conformer(s) of glutamate required for binding to the transporter(s) but not to the excitatory receptors.[10] Recently this approach has led to the identification of several interesting transport inhibitors, including L-α-(carboxycyclopropyl)glycine derivatives (L-CCG-III, Figure 6)[11] and *cis*-1-aminocyclobutane-1,3-dicarboxylate (CACB, Figure 6).[12] Several years ago we began a systematic study of azacyclic dicarboxylate conformer mimics and their interactions with the EAA receptors and transporters. In this report we describe structure/activity data for all twelve isomers of pyrrolidine 2,3-dicarboxylate (2,3-PDC), pyrrolidine 2,4-dicarboxylate (2,4-PDC), and azetidine 2,3-dicarboxylate (2,3-ADC), as well as several more highly substituted derivatives of 2,4-PDC, at the sodium-dependent transporter in synaptosomes prepared from rat forebrain.

### *Design and Synthesis of Potential Transport Inhibitors.*

The initial targets were simple 3- and 4-membered ring dicarboxylates (ADCs and PDCs). The analogous 6-membered ring compounds have been investigated at the EAA receptors,[10] but resultant structure/activity data are somewhat equivocal because two chair conformations are accessible for each isomer. In order to reduce this type of ambiguity, we prepared the PDCs (which can exist in several envelope conformers, but these differ from one another less than the corresponding piperidine chairs do), and the ADCs (in which the ring may only pucker slightly). Preliminary molecular modeling studies provided support for the notion that relative positions of the charged heteroatoms in some of these dicarboxylates match up reasonably well with some of the nine staggered glutamate conformers[13] that they are intended to mimic. While the 2,3 isomers could be considered to be aspartate analogs, some of them do overlay with folded glutamate conformations. The 2,4-PDCs, on the other hand, contain embedded glutamate-like conformations that are more extended.

The 2,4-PDCs were prepared from hydroxyproline as described previously.[14] A laborious route that we had developed to obtain the 2,3-PDCs from aspartic acid[14b] was dramatically improved by making use of a Rapoport β-alkylation[15] of N-benzyl-N-phenylfluorenyl-dimethyl aspartate with allyl iodide followed by ozonolysis, intramolecular reductive amination, and deprotection. Both D- and L-aspartate are readily available, and the alkylation can be controlled to give either diastereomer of the allylated aspartate and hence of the 2,3-PDCs.[16] The ADCs were prepared similarly, substituting bromochloromethane for allyl iodide and intramolecular $S_N2$ displacement (induced by an *in situ* Finklestein reaction of the resultant β-chloromethyl aspartate derivative) for the reductive amination. The enantiomeric purity of all fourteen dicarboxylates was > 95% based on proton NMR with chiral shift reagents. Finally, recognizing that the 4-isopropenyl group of kainic acid represents a latent carboxyl group, several 3-substituted 2,4-PDCs appeared to be easily prepared from this commercially available, albeit moderately expensive, natural product. The triacid L-trans-2,4-PDC-3-acetic acid was prepared by minor modification of the literature procedure.[17] Modification of the carboxymethyl side chain for the synthesis of methoxyethyl-2,4-PDC requires selective manipulation of one of the two carboxyl groups of kainate, which is easily accomplished by protection as the N-phenylfluorenyl dimethyl ester, allowing the selective

reduction of the γ-carboxyl group of the diester with DIBAH followed by alkylation of the resultant primary alcohol with methyl iodide. Ozonolysis of the isopropenyl group to the methyl ketone, further oxidation to the acid ($Br_2/HO^-$), and deprotection gives the methoxyethyl-2,4-PDC.

***Determination of Transport Inhibition.***

Each of the compounds was tested for its ability to attenuate the sodium-dependent uptake of $^3$H-D-aspartate into synaptosomes prepared from rat forebrain as previously described.[14a] The various analogues were added simultaneously with the substrate (5 μM) at the concentrations indicated in the Table. Transport was allowed to proceed for 2 min at 25°C, after which the assay was terminated by the rapid filtration and rinsing of the synaptosomes with ice cold buffer. Radioactivity present in the retained synaptosomes was quantified by liquid scintillation counting. The data (n = 3-6 sets of duplicate assays) are reported as % of control $^3$H-D-aspartate transport (i.e., absence of inhibitors and corrected for leakage and background) which was typically 0.99 ± 0.15 nmol/min/mg protein (n = 12 sets of duplicate assays). As reported in the Table, the two most potent inhibitors are L-glutamate, which is the primary endogenous substrate, and the rigid glutamate mimic L-trans-2,4-PDC. Substitution in the 3-position of L-trans-2,4-PDC, as in the kainate-derived L-trans-2,4-PCD-3-acetic acid (PCDA) and the 3-methoxyethyl PDCs greatly attenuates its potency as a transport inhibitor. Among the other series of compounds tested, L-trans-2,3-PDC, L-trans-2,3-ADC, D-trans-2,4-PDC, and L-cis-2,4-PDC were more comparable to the activity of dihydrokainate (DHK), a well-characterized weak competitive inhibitor. Interestingly, there is an inverse relationship between steric bulk at the 4-position and transport inhibition in the kainate-like derivatives: DHK is the most active, kainate itself intermediate, and the L-trans-2,3-homo-PDC (i.e., DHK lacking the isopropyl group) the least active.

More detailed kinetic studies with L-trans-2,4-PDC demonstrated it to be a competitive inhibitor of both L-glutamate[14a] and D-aspartate transport. In the latter case, the inhibitor exhibited a $K_i$ of 1.3 ± 0.4 μM (mean ± SD, n=5) compared to a $K_m$ of 3.0 ± 0.7 μM (mean ± SD, n=12) for D-aspartate. Related studies have also demonstrated that L-trans-2,4-PDC was ineffective at inhibiting the binding of radioligands to the well-characterized EAA receptors (e.g., NMDA, KA, and AMPA).[14a] The combination of this selectivity and the fact that its glutamate-like conformation is well-defined, makes L-trans-2,4-PDC an ideal compound for molecular modeling studies of the binding site pharmacophore, as well as an excellent starting point for the generation of the next series of inhibitors.

***Molecular Modeling***

The fact that there are just two highly active inhibitors, L-glutamate and L-trans-2,4-PDC, among this group of nearly twenty structurally related diacids suggested that a structural comparison of the two might reveal a unique conformation preferred for optimal binding at the transporter. Thus, minimized conformations of the nine staggered conformers[13,18] of L-glutamate and several envelope forms of L-trans-2,4-PDC were compared,[19] yielding a best fit pair that defines the preferred conformations for the bound forms of both molecules, with a carboxyl-carboxyl distance of 4.58 ± 0.08 Å and a distal carboxyl-amino distance of 2.45 ± 0.02 Å. The PDC conformer is one in which the distal carboxyl group occupies an axial-like position at the flap of the pyrrolidine envelope, and the glutamate conformer corresponds to a relatively abundant solution conformation.[13] The glutamate conformer, which we believe is the one required for binding at the transport site, was then compared with the members of each series of dicarboxylates: the remaining 2,4-PDCs (Figure 1, which also includes the original glutamate/L-trans-2,4-PDC pair); the 2,3-PDCs (Figure 2); the 2,3-ADCs (Figure 3); and kainate and related derivatives (Figure 4). In all Figures, the glutamate conformer is red, with van der Waals radii shown only for the heteroatoms (60% for oxygen and 40% for nitrogen). The other structures, identified in each figure caption, are shown in colors according to decreasing activity: yellow (most active), white (less active), blue and purple (inactive). All hydrogens have been omitted for clarity.

There are several notable features of these comparisons, not the least of which is that the heteroatoms of the 2,3-PDCs and ADCs often overlap quite well with those of the 2,4-PDC isomers despite their relatively rigid structures and differing numbers of atoms in the backbones connecting the carboxyl groups. However, a derivative with heteroatoms occupying the same region of space as those in the active glutamate (or L-trans-2,4-PDC) conformer is not necessarily itself active. For example, the compared atoms of enantiomeric conformations overlap perfectly, but differ in two important ways: (i) in the overlaid structures, and presumably also in the binding site, their rings or chains protrude in opposite directions, and (ii) their carboxyl groups are oriented quite differently, i.e., they would converge on the complementary group in the binding site from different directions. The clear preference for the binding of L-enantiomers (Table) suggests that one or both of these factors are important in achieving optimal binding to the transporter. In particular, the orientation of the distal carboxyl group in L-trans-2,4-PDC matches more favorably with that of glutamate than any of the other 2,4-isomers (Figure 1).

This also appears to be true for all of the other L-trans isomers (Figures 1-3), although poorer overlap is achieved for the L-trans- 2,3-PDCs and -ADCs. Overlap may also be affected by substituent effects within a given enantiomeric series. The aforementioned inverse relationship between potency of transport inhibition and steric bulk of the 4-substituent in DHK, kainate, and L-trans-2-3-homo-PDC may be due to differences in populations of the two pyrrolidine envelopes shown in Figure 6. For the most potent of the three, DHK, the conformer on the left is preferred and also aligns most closely with the preferred conformer of L-glutamate (Figure 4). As "R" decreases in size, the conformer on the right becomes more favorable, resulting in poorer overlap (see Figure 4, in which the L-trans-2-3-homo-PDC is in this conformation) and reduced potency (Table). This relatively subtle role of the 4-substituent may also play a determining role in the specificity with which these compounds bind to the various EAA receptor subtypes.

**Table.** Transport system pharmacology

| Analogue | UPTAKE OF $^3$H-D-ASPARTATE (5 μM) % of CONTROL | |
|---|---|---|
| | 250 μM | 25 μM |
| L-Glutamate | --- | 40 ± 7 |
| Dihydrokainate | 25 ± 2 | 83 ± 7 |
| Kainate | 39 ± 4 | --- |
| L-trans-2,3-homo-PDC | 74 ± 6 | --- |
| ***2,3-Pyrrolidine dicarboxylates*** | | |
| L-trans-2,3-PDC | 38 ± 6 | 94 ± 6 |
| D-trans-2,3-PDC | 108 ± 8 | --- |
| L-cis-2,3-PDC | 81 ± 8 | --- |
| D-cis-2,3-PDC | 107 ± 7 | --- |
| ***2,3-Azetidine dicarboxylates*** | | |
| L-trans-2,3-ADC | 53 ± 5 | 96 ± 6 |
| D-trans-2,3-ADC | 98 ± 5 | --- |
| L-cis-2,3-ADC | 109 ± 10 | --- |
| D-cis-2,3-ADC | 100 ± 8 | --- |
| ***2,4-Pyrrolidine dicarboxylates*** | | |
| L-trans-2,4-PDC | 0 | 18 ± 5 |
| D-trans-2,4-PDC | 54 ± 4 | 88 ± 13 |
| L-cis-2,4-PDC | 36 ± 4 | 82 ± 3 |
| D-cis-2,4-PDC | 105 ± 10 | --- |
| L-trans-2,4-PDC, 3-acetic acid | 95 ± 6 | --- |
| L-trans-2,4-PDC, 3-methoxyethyl | 55 ± 7 | --- |
| L-trans-2,4-homo-PDC | 74 ± 6 | --- |

**Figures 1 – 5:** Stereoviews of glutamate/rigid analog overlays (see text).

**Figure 1. 2,4-PDCs**
Red: L-glutamate
Yellow: L-trans
White: L-cis
Blue: D-trans
Purple: D-cis

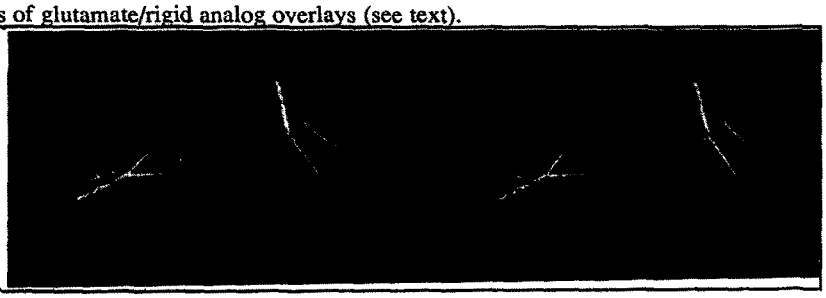


**Figure 2. 2,3-PDCs**
Red: L-glutamate
Yellow: L-trans
White: L-cis
Blue: D-cis
Purple: D-trans

**Figure 3. 2,3-ADCs**
Red: L-glutamate
Yellow: L-trans
White: D-trans
Blue: D-cis
Purple: L-cis

**Figure 4. DHK**
Red: L-glutamate
Yellow: DHK
White: kainate
Blue: L-t-2,3-homo-PDC

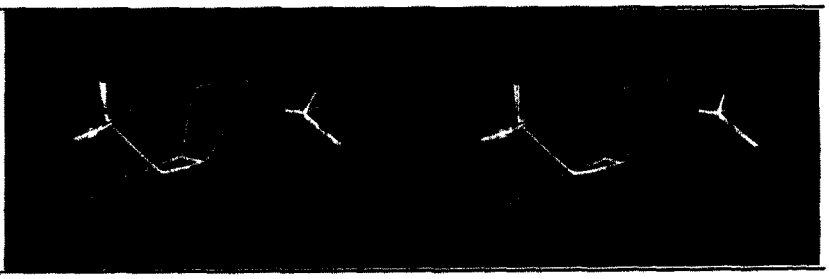
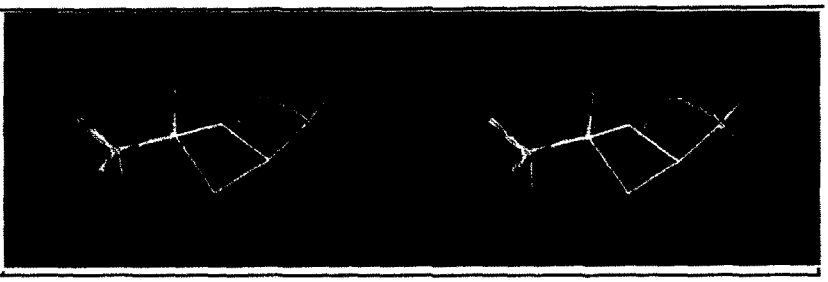
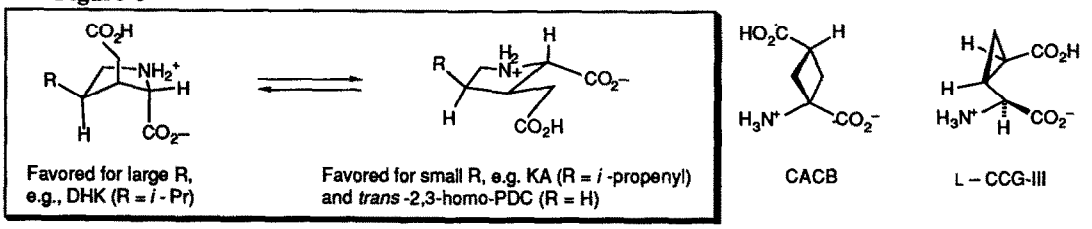


**Figure 5. Potent Transport Inhibitors**
Red: L-glutamate
Yellow: L-trans-PDC
White: CACB
Blue: L-CCG-III



These structure/activity studies show some consistent trends within this series of compounds that we believe provide new insight into binding interactions of EAAs at the high affinity, sodium-dependent synaptosomal glutamate transporter binding site. If our current model is correct, the other recently described conformationally restricted transport inhibitors CACB and L-CCG-III (Figure 6) should also be accommodated. As shown in Figure 5, the overlap of heteroatoms in these two diacids coincides with our predictions. Future studies will be directed toward further defining the relationships between conformational, steric, and orientational effects at this site, as well as at other EAA transporters and receptors.

**Figure 6**

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18. This analysis ignores carboxyl rotamers, which greatly increase the total number of conformers.
19. All molecular modeling was performed on a Silicon Graphics 4D Personal Iris workstation using Polygen's QUANTA software package (version 3.2.3). The Chemnote application was used to assign atom types and partial atomic charges and to construct structures. The crude structures were then energy minimized with the CHARMm force field using the Adopted-Basis Newton-Raphson algorithm with the distance-dependent dielectric term set at 80. The Molecular Similarity application was then employed to superimpose refined stuctures. The corresponding nitrogen, α-carbonyl carbon, and the distal carbonyl carbon of each molecule were overlaid using a Rigid-Body Fit to the target glutamate conformer.