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Discovery of the First Selective Inhibitor of Excitatory Amino Acid Transporter Subtype 1

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Abstract: The discovery of the first class of subtype-selective inhibitors of the human excitatory amino acid transporter subtype 1 (EAAT1) and its rat orthologue GLAST is reported. An opening structure−activity relationship of 25 analogues is presented that addresses the influence of substitutions at the 4- and 7-positions of the parental skeleton 2-amino-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile. The most potent analogue **1o** displays high nanomolar inhibitory activity at EAAT1 and a >400-fold selectivity over EAAT2 and EAAT3, making it a highly valuable pharmacological tool.

The excitatory amino acid transporters (EAATs) belong to the solute carrier family 1 (*SLC1*), and carry out the concentrative uptake of (*S*)-glutamate (Glu) [and aspartate (Asp)] from the glutamatergic synaptic cleft.[1−3] Five EAAT subtypes have been identified to date, in humans termed EAAT1−EAAT5. For historical reasons the nomenclature differs in rodents: the orthologues of human EAAT1, EAAT2 and EAAT3 subtypes are termed GLAST, GLT-1 and EAAC-1, respectively, whereas the nomenclature for EAAT4,5 is conserved across the species.

The EAATs display differential localization and overall distribution patterns in the adult rodent brain. Whereas GLAST (EAAT1) and GLT-1 (EAAT2) are astroglial transporters, EAAC-1 (EAAT3) and EAAT4 are predominantly found in neurons.[4] GLT-1 (EAAT2) is the predominantly expressed subtype, found throughout the brain and spinal cord, and has been shown to be responsible for >90% of the Glu uptake in the adult brain.[4−6] GLAST (EAAT1) is found at the highest levels in the cerebellum with lower levels in the cortex and spinal cord,[7] and EAAC-1 (EAAT3) is expressed in high densities at postsynaptic terminals in the whole brain, particularly in the hippocampus, cerebellum, and basal ganglia.[4] EAAT4 is highly enriched in the Purkinje cells of the cerebellum, but the transporter has also been detected in the rat fore- and midbrain albeit in dramatically lower levels.[4,5,8,9] Finally, EAAT5 is found exclusively in the retina.[4,5,10] In agreement with the distribution and densities of the CNS subtypes, EAAT2 knockout mice have displayed pronounced loss of hippocampal neurons, seizures, and 50% mortality at 6 weeks of age. In contrast, knockout of GLAST (EAAT1), EAAC-1 (EAAT3), or EAAT4 in mice have been reported not to cause significant abnormalities in anatomy or in CNS-related phenotypes.[4,6] However, such studies may not be fully conclusive when it comes to elucidation of the physiological importance of the specific transporter subtype, as the absence of physiologic and phenotypic effects in the knockout mice may also be ascribed to compensatory mechanisms. Hence, we believe that valuable information about the physiological functions and thus therapeutic potentials of a specific EAAT subtype may be addressed in a more subtle way by studying the actions of a selective ligand.[4]

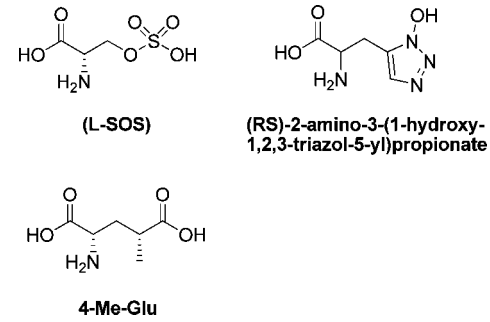

Figure 1. EAAT ligands L-serine-*O*-sulfate (L-SOS), (*RS*)-2-amino-3-(1-hydroxy-1,2,3-triazol-5-yl)propionate, and (4*R*)-4-methylglutamate (4-Me-Glu).

Over the past 4 decades, the interest in glutamatergic neurotransmission as a therapeutic target has prompted the synthesis of an astonishing number of ligands targeting ionotropic and metabotropic Glu receptors.[11−13] In contrast, medicinal chemistry efforts in the EAAT field have been far more limited.[14,15] The vast majority of EAAT ligands published to date are analogues of endogenous substrates Glu and Asp and display nonselective activities as substrates or inhibitors at the EAAT1, EAAT2, and EAAT3 subtypes.[14,15] However, the EAAT2 subtype appears to be more susceptible to inhibition by such amino acid analogues compared with EAAT1 and EAAT3, given the number of ligands found to display preference or selectivity for this subtype.[14,15] In contrast, no truly EAAT1-selective ligands have been reported. Nevertheless, the interest in such compounds is reflected in the notable attention paid to the few compounds that display a bare minimal of preference for the subtype.[3,15] Best two examples are (*RS*)-2-amino-3-(1-hydroxy-1,2,3-triazol-5-yl)propionate and L-serine-*O*-sulfate (L-SOS) (Figure 1). The former displays very weak activity at the EAAT1 subtype ($IC_{50} \approx 100\ \mu M$) and only 3- and >10-fold lower activities at EAAT2 and EAAT3, respectively.[16] It has not been established whether it is a substrate or an inhibitor of the transporters. L-SOS displays weak substrate activity at EAAT1 and EAAT3 ($IC_{50} \approx 100\ \mu M$) and only a 10-fold lower activity at EAAT2.[17] Although the Glu analogue (4*R*)-4-methylglutamate (4-Me-Glu) is a completely nonselective EAAT ligand, it is actually the most EAAT1-discriminating ligand described to date, being a substrate at EAAT1 and an inhibitor of EAAT2,3.[18,19] However, since the compound will inhibit Glu uptake through EAAT1−3 with similar potencies and furthermore is a very potent iGluR5 agonist, it is not well-suited as a pharmacological tool for studies of EAATs in native tissues.[13]

In this paper, we present the discovery of the first subtype-selective inhibitor of EAAT1 and the opening of the structure−activity-relation (SAR) which investigates the influence of the substitutions at the 4- and 7-positions of the parental skeleton 2-amino-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile for the subtype-selective EAAT1-activity.

A small commercially available compound library consisting of 3040 compounds (obtained from Chembridge Corporation)

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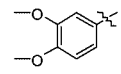
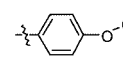
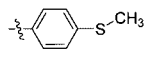
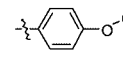
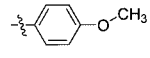
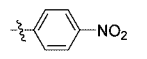
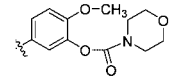
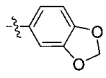
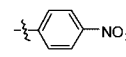
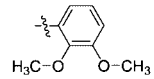
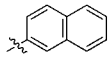
Table 1. Inhibition of [^{3}H]-D-Asp Uptake in HEK293 Cells Stably Expressing Human EAAT1, EAAT2, and EAAT3[a]

	R^1	R^2	EAAT1	EAAT2	EAAT3		R^1	R^2	EAAT1	EAAT2	EAAT3
1a			4.7 [5.32 ± 0.03]	>100 [<4.0]	>100 [<4.0]	**1n**			11 [4.97 ± 0.05]	>300 [<3.5]	>300 [<3.5]
1b			2.7 [5.56 ± 0.05]	>100 [<4.0]	>100 [<4.0]	**1o**			0.66 [6.18 ± 0.08]	>300 [<3.5]	>300 [<3.5]
1c			2.3 [5.64 ± 0.05]	>100 [<4.0]	>100 [<4.0]	**1p**			7.3 [5.13 ± 0.03]	>300 [<3.5]	>300 [<3.5]
1d			3.6 [5.44 ± 0.04]	>100 [<4.0]	>100 [<4.0]	**1q**			~100 [~4.0]	>300 [<3.5]	>300 [<3.5]
1e			4.0 [5.40 ± 0.05]	>300 [<3.5]	>300 [<3.5]	**1r**			9.9 [5.00 ± 0.04]	>300 [<3.5]	>300 [<3.5]
1f			~ 50 [~4.3]	>100 [<4.0]	>100 [<4.0]	**1s**			~ 30 [~4.5]	>300 [<3.5]	>300 [<3.5]
1g			14 [4.85 ± 0.04]	>100 [<4.0]	>100 [<4.0]	**1t**			>100 [<4.0]	>100 [<4.0]	>100 [<4.0]
1h			4.7 [5.33 ± 0.04]	>100 [<4.0]	>100 [<4.0]	**1u**			~ 30 [~4.5]	>100 [<4.0]	>100 [<4.0]
1i			7.4 [5.13 ± 0.05]	>100 [<4.0]	>100 [<4.0]	**1v**			9.2 [5.04 ± 0.05]	>300 [<3.5]	>300 [<3.5]
1j			3.6 [5.44 ± 0.04]	>100 [<4.0]	>100 [<4.0]	**1w**			>300 [<3.5]	>300 [<3.5]	>300 [<3.5]
1k			2.1 [5.67 ± 0.04]	>100 [<4.0]	>100 [<4.0]	**1x**			>300 [<3.5]	>300 [<3.5]	>300 [<3.5]
1l			4.3 [5.37 ± 0.03]	>100 [<4.0]	>100 [<4.0]	**1y**			>300 [<3.5]	>300 [<3.5]	>300 [<3.5]
1m			3.2 [5.49 ± 0.04]	>300 [<3.5]	>300 [<3.5]						

[a] The IC_{50} for analogues **1a**−**y** are given in μM with pIC_{50} ± SEM in brackets (n = 3−7).

was screened for inhibitory activity at EAAT1-, EAAT2-, and EAAT3-expressing HEK293 cell lines in a [^{3}H]-D-Asp uptake assay. Interestingly, we found **1a** to inhibit the [^{3}H]-D-Asp uptake through EAAT1 selectively, showing no inhibitory activity at EAAT2 or EAAT3. A subsequent verification of this preliminary screening result gave IC_{50} values for **1a** of 4.7 μM, >100 μM, and >100 μM at EAAT1, -2, and -3, respectively (Table 1). In comparison, the standard EAAT inhibitor DL-*threo*-β-benzyloxyaspartate (TBOA) inhibited [^{3}H]-D-Asp transport through all three EAAT subtypes with IC_{50} values in the low micromolar range (data not shown).[20,21]

The discovery of **1a** prompted us to commence the building of a SAR for this class of EAAT1 inhibitors by exploring the 4- and/or 7-position. The parental skeleton 2-amino-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile comprising substituents in the 4- and/or 7-positions (**1a**−**y**) is readily available as a mixture of the four stereoisomers from the multicomponent reaction (MCR) of 1,3-cyclohexandinone (which delivers the R^1 substituent), an aldehyde (which allows the incorporation of the R^2 substituent), and malononitrile (Scheme 1). The MCR has been described under a large variety of reaction conditions such as in EtOH/amine base,[22] H_2O/catalytic HTMAB,[23] H_2O/DAHP,[24] and solvent-free/NaBr under microware conditions.[25]

In all, 25 compounds were synthesized by us or obtained from commercial suppliers and their pharmacological activities determined at the EAAT1-, EAAT2-, and EAAT3-expressing HEK293 cell lines in the [^{3}H]-D-Asp uptake assay. The IC_{50} values of the analogues are summarized in Table 1, and concentration−inhibition curves for selected analogues are shown in Figure 2. The inhibitory activities displayed by the analogues at EAAT1 were found to be dependent on the nature of the R^1 and R^2 substituents (Table 1). None of the analogues **1a**−**y** showed any significant inhibitory activity at the EAAT2 and EAAT3 subtypes at high-micromolar concentrations (all IC_{50} values >100 μM, Table 1).

Building the SAR, analogues **1a**−**t** which bear one aromatic substituent in both the 4- and 7-positions show differential inhibitory potencies at EAAT1 with **1o** being the most potent inhibitor (IC_{50} = 0.66 μM). In detail, inhibitory activity is maintained while the R^1 substituent spans a phenyl (non-, mono-, or disubstituted), a naphthyl, or a five-membered hetereoaromatic moiety (furan and thiophene). For the R^2 substituent a mono or disubstituted phenyl ring is allowed; however, larger *o*-substituents may result in decreased activity (*o*-fluoro in **1a** vs the *o*-methoxy group in **1f**). The volume size of the pocket occluding the R^2-substituent is probed successfully with a naphthyl group (**1i**,**j**), a biphenyl group (**1k**), and an additional trisubstituted phenyl ring attached via a short linker (**1l**). But limitations to the bulk of the R^2-substituent are also apparent given that **1q** shows a marked decrease in inhibitory activity ($IC_{50} \approx 100$ μM at EAAT1). As for analogues in which R^2 is a heteroaromatic group, the marked decrease in inhibitory potency displayed by **1s**,**t** points to disfavored

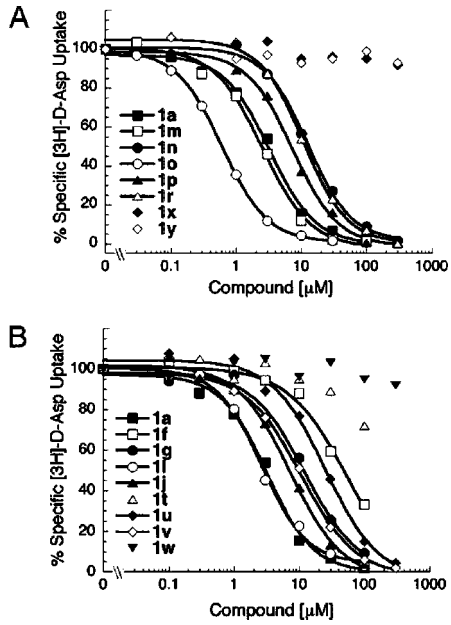


Figure 2. Concentration−inhibition curves for selected analogues at the EAAT1-HEK293 cell line in the [^{3}H]-D-Asp uptake assay using 30 nM [^{3}H]-D-Asp as tracer concentration. (A) Influence of inhibitory activity of the chemical nature of R^1 by comparing analogues **1a**,**m**−**p**,**r**,**x**,**y** which bear comparable R^2 substituents ((un)substituted phenyl groups). (B) Influence of inhibitory activity of the chemical nature of R^2 by comparing analogues with conserved R^1 substituents (phenyl). The figure depicts representative experiments. For experimental details, see Supporting Information.

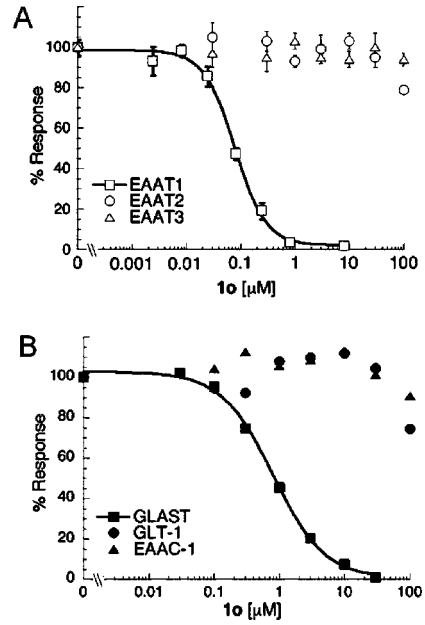
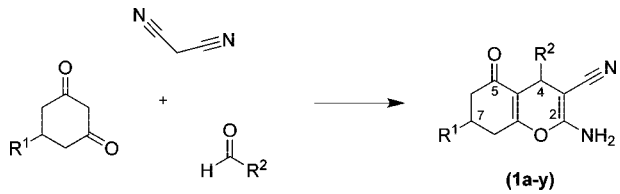


Figure 3. Pharmacological characterization of **1o** at human and rat EAATs in the FLIPR Membrane Potential Blue assay: (A) concentration−inhibition curves for **1o** at the EAAT1-, EAAT2-, and EAAT3-HEK293 cell lines using 50 μM Glu as substrate concentration; (B) concentration−inhibition curves for **1o** at the rat EAAT subtypes GLAST (EAAT1), GLT-1 (EAAT2), and EAAC-1 (EAAT3) transiently expressed in tsA-201 cells using 50 μM Glu as substrate concentration. The figure depicts representative experiments. For further details, see Supporting Information.

Scheme 1. Multicomponent Reaction (MCR) Methodology Applied for the Synthesis of **1a**−**y**[a]



[a] For details, see *Supporting Information*.

electrostatic interactions with the transporter protein. The suitable physical chemical nature of the R^2 substituent is investigated further in the three analogues **1u**−**w**: a benzyl group, **1u**, results in a 10-fold decrease in potency, whereas a methyl group, **1v**, is fully acceptable. In contrast, no substituent (**1w**; R^2 = H) results in the complete loss of activity. This finding is quite intriguing, and its origin will be addressed in future studies. Analogously, a distinct change in pharmacology is also observed when R^1 is a methyl group, **1x**, or a hydrogen, **1y** (Table 1). Again, all inhibitory activity is lost, which points to the loss of an essential π−cation or π−π stacking interaction.

The EAAT1-selective inhibitory activity displayed by this class of compounds in the [^{3}H]-D-Asp uptake assay was verified for the most prominent compound in the series, **1o**, in a different functional assay. In a previous study, we have found the pharmacological properties displayed by a series of standard EAAT substrates and nonsubstrate inhibitors at the EAAT1-, EAAT2-, and EAAT3-HEK293 cell lines in a fluorescence-based high throughput assay, the FLIPR Membrane Potential Blue (FMP Blue) assay, to be in good agreement with those observed for the compounds in other assays.[20] In the FMP Blue assay, Glu displayed K_m values of 16 μM (pK_m ± SEM, 4.79 ± 0.04, n = 4), 27 μM (pK_m ± SEM, 4.57 ± 0.05, n = 3), and 21 μM (pK_m ± SEM, 4.68 ± 0.04, n = 4) at EAAT1, EAAT2, and EAAT3, respectively. Application of compound **1o** at concentrations up to 100 μM onto the three cell lines did not give rise to fluorescent responses in this assay, which demonstrates that **1o** is not a substrate at the EAATs (data not shown). However, compound **1o** was found to be a potent inhibitor of EAAT1, inhibiting the fluorescent response induced by 50 μM Glu in EAAT1-HEK293 cells in a concentration-dependent manner with an IC_{50} of 120 nM (pIC_{50} ± SEM, 6.91 ± 0.03, n = 4), whereas the compound did not display any significant inhibition of the Glu-induced responses in EAAT2- or EAAT3-cells at concentrations up to 100 μM (Figure 3A). In contrast to the EAAT1-selectivity displayed by **1o** and in agreement with the findings of a previous study,[20] the competitive EAAT inhibitor TBOA displayed low-micromolar K_i values at all the three subtypes (data not shown).

The pharmacological profile of **1o** was also determined at the rat EAAT subtypes GLAST (EAAT1), GLT-1 (EAAT2), and EAAC-1 (EAAT3) transiently expressed in tsA-201 cells using the FMP Blue assay. In this assay, Glu displayed K_m values of 37 μM (pK_m ± SEM, 4.43 ± 0.04, n = 3) at GLAST (EAAT1), 45 μM (pK_m ± SEM, 4.35 ± 0.05, n = 3) at GLT-1 (EAAT2), and 35 μM (pK_m ± SEM, 4.45 ± 0.04, n = 3) at EAAC-1 (EAAT3). In concordance with its EAAT1 selectivity at the human transporters, **1o** was shown to be a selective inhibitor of GLAST (EAAT1), displaying an IC_{50} values of 970 nM (pIC_{50} ± SEM, 6.02 ± 0.05, n = 4) at this subtype. No significant inhibition of the Glu-induced responses in GLT-1 (EAAT2)- or EAAC-1 (EAAT3)-expressing cells was observed for **1o** at concentrations up to 100 μM (Figure 3B).

The compounds in this study are structurally distinct from the endogenous EAAT substrates Glu and Asp. Thus, it is not surprising that the active analogues in the **1a**−**y** series are not substrates but rather inhibitors of the EAAT1. Whether the compounds target the orthosteric site in EAAT1, i.e., the binding

site for Glu and Asp, or are negative allosteric (noncompetitive) modulators inhibiting the uptake through EAAT1 via binding to other regions in the transporter is currently under investigation.

It is important to stress that the 25 analogues only have been characterized pharmacologically at three of the five EAAT subtypes. Thus, we cannot exclude the possibility that the compounds in addition to their EAAT1 activity could target EAAT4 and/or EAAT5. However, the EAAT1−3 and EAAT4,5 subtypes make up two distinct subgroups within the EAAT family in terms of transporter function and ion conductance, despite the fact that a 50−60% sequence homology exists between all of the subtypes.[3,9,10] We are currently investigating whether **1o** and other compounds in the series target EAAT4, the fourth CNS EAAT subtype. Nevertheless, we find it very unlikely that a compound exhibiting >400-fold selectivity for one subtype within the EAAT1−3 subgroup would possess any significant activity at EAAT4,5.

In conclusion, we have presented the first class of compounds that show fully selective inhibition of the human EAAT1 subtype over EAAT2 and EAAT3. A SAR was built from the pharmacological investigation of 25 analogues, varying the substituents in the 4- and 7-positions of the parental skeleton: 2-amino-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile. From this study, we have shown that the presence of an aromatic ring in the 7-position (R^1-substituent) is crucial for the inhibitory activity at EAAT1. On the other hand, the 4-position (R^2-substituent) may accommodate small and larger groups, not being restricted to aromatics only, although a substituent in this position is mandatory. The most potent analogue in the series, **1o**, displayed high nanomolar inhibitory activity ($IC_{50} = 0.66\ \mu M$) at EAAT1, with more than 400-fold selectivity compared to EAAT2 and EAAT3. This selectivity profile was also observed at the rat orthologues, which in all makes **1o** a highly attractive pharmacological tool for future explorations of the physiological role of the EAAT1 subtype. Expansion of the SAR, studies addressing the bioavailability and pharmacokinetic properties of **1o**, and detailed investigations of its binding mode at EAAT1 are all ongoing projects in our laboratories.

Acknowledgment. We thank the Lundbeck Foundation, the Carlsberg Foundation, and the Danish Research Council for financial support. Drs. S. G. Amara and T. Rauen are thanked for their generous gifts of cDNAs for the human and rat EAATs, respectively. Shahrokh Padrah is thanked for technical assistance regarding synthetic work.

Supporting Information Available: Experimental procedures for the synthesis of analogues **1c**,**n**,**o**,**w** and for the pharmacological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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