Comprehensive Profiling of Inhibitory Effects on Major CNS Transporters For Drug Safety Assessment

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Xuexiang Zhang, Dominique Bridon, Yong Huang
Optivia Biotechnology Inc., 115 Constitution Drive, Suite 7, Menlo Park, CA 94025, USA

OPTIVIA Biotechnology Inc.
www.optiviabio.com

ABSTRACT

Membrane transporters play crucial roles on brain physiology not only as the gatekeepers controlling CNS entry of nutrients and drugs across the blood-brain-barrier (BBB), more importantly, they directly modulate key biological processes such as neurotransmission, energy metabolism and antioxidant defense. Pharmacological modulations of transporters including SERT, DAT, NET and GAT1 have been very successful in treating CNS disorders such as depression and epilepsy; on the other hand, undesirable intervention of certain CNS transporters such as EAATs and xCT are believed to be associated with both acute and chronic CNS adverse effects such as excitotoxicity and Parkinsonism. Despite its critical implication in understanding and predicting CNS toxicity, information about inhibition of drugs against the major 30+ transporters expressed in the CNS (in contrast to on the BBB) is sparse. Against this backdrop, we have developed cell-based assays for more than 15 key CNS transporters and tested nearly 40 pharmaceuticals (CNS- and peripherally acting) and neuron toxins for their inhibitory effects on these transporters. It is not surprising that the prevalence of transporter inhibition by neuron toxins was found to be higher than marketed CNS-acting drugs; for example, L-Quisqualic acid, a naturally occurring excitotoxic agent, was found to be a potent inhibitor of xCT, a transporter with major role on glutathione (GSH) synthesis and homeostasis in the brain, suggesting that GSH depletion could be one mechanism of L-Quisqualate's toxicity to neurons. Surprisingly, adenine synthesis inhibitor L-alanosine was found to inhibit EAATs and xCT, raising the potential for CNS adverse effects by this experimental drug.

BACKGROUND

xCT (SLC7A11) and EAATs (SLC1A1-3) are currently subjects of high interest within the pharmaceutical industry from both a therapeutic and an imaging standpoint. L-Glutamate (L-Glu) is the primary excitatory neurotransmitter in the mammalian CNS. Through its activation of a wide variety of excitatory amino acid receptors, L-Glu-mediated signaling contributes to synaptic neurotransmission. Concentrations of L-Glu in the CNS are regulated by a family of excitatory amino acid transporters (EAATs) that rapidly concentrate it in glia cells and neurons, and thereby limit its extracellular accumulation. In addition to glutamate transporters, levels of extracellular glutamate are controlled by the cystine/glutamate antiporter xCT, which releases intracellular glutamate in exchange for cystine for the production of glutathione, the major cellular antioxidant. Minor alterations of extracellular glutamate levels by xCT and EAATs in the brain therefore have the potential to drastically alter glutamate neurotransmission. In addition, the glutamate signaling is also regulated by other transporters such as asc-1 (SLC7A10) and ASCT2 (SLC1A5).

The neurotoxin β-Methylamino-L-alanine (BMAA) a is considered to be one agent responsible for the high rate of ALS/Parkinson dementia observed in the island of Guam. Recent work has demonstrated that BMAA interacts with the X_c^- transport system and modifies glutamate homeostasis, leading to neurodegeneration. Similar observations have been made for Oxalyldiaminopropionic acid (ODAP), which is a structural analogue of the neurotransmitter glutamate and is the neurotoxin responsible for lathyrism.

Investigating the possible interactions of drugs/compounds with these transporters would have a large impact on CNS therapeutic development, through explaining causes of certain CNS side effects of drugs, revealing potential new therapeutic indications of existing drugs, and discovering potential therapeutic benefits of inhibiting key neurotransmitter transporters in treating various CNS diseases.

OBJECTIVES

Our objectives are: 1) To screen 30 marketed CNS acting drugs in different categories against EAAT2 - a major EAAT responsible for glutamate recycling 2) To investigate a small library of amino acid analog neurotoxins/compounds on the transporters related to glutamate signaling, including EAAT1-3, xCT, asc-1, and ASCT2.

MATERIALS AND METHODS

MDCK-II cells were maintained in DMEM and seeded in Millipore Millicell 96-well insert plate (PCF-0.4 µm). Cells were transfected using a novel *in situ* transfection technology, Opti-Expression™, which allows consistent and effective transfection of polarized cell monolayers. Cells were transfected with plasmids encoding either one of the human EAAT transporters (EAAT1, EAAT2, EAAT3), asc-1, ASCT2, or xCT together with 4F2HC. A plasmid encoding GFP was used as a mock control.

Uptake assays started 48 hours after transfection. Cells were pre-incubated with assay buffer for the appropriate amount of time. Transport was initiated by adding radio-labeled probe substrates or substrate / inhibitor (testing compound) mixture. Following the incubation, cells were washed with PBS and solublized with 50% acetonitrile to measure the intracellular accumulation of substrate. The amount of substrate was quantified by radiometric counting.

Table 1. Probing substrates and inhibitors used in the transport assay

	EAAT1	EAAT2	EAAT3	xCT	asc-1	ASCT2
Probe substrate	Glutamic acid	Glutamic acid	Glutamic acid	Glutamic acid	Glycine	Glutamine
Standard inhibitor	Cysteic acid (1 mM)	Cysteic acid (1 mM)	Way213613 (0.3 mM)	Sulfasalazine (0.1 mM)		L-Alanine (1 mM)

RESULTS

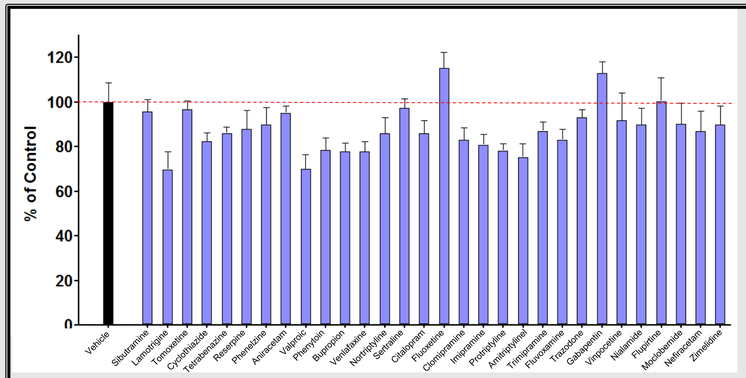

Fig 1. Lack of suppressing effect of 30 marketed CNS drugs on EAAT2 activity. None of the 30 CNS drugs screened at 50 µM were found to dramatically inhibit the EAAT2-mediated uptake of glutamic acid. P>0.05 vs. control (ANOVA). All data are expressed as mean±SEM.

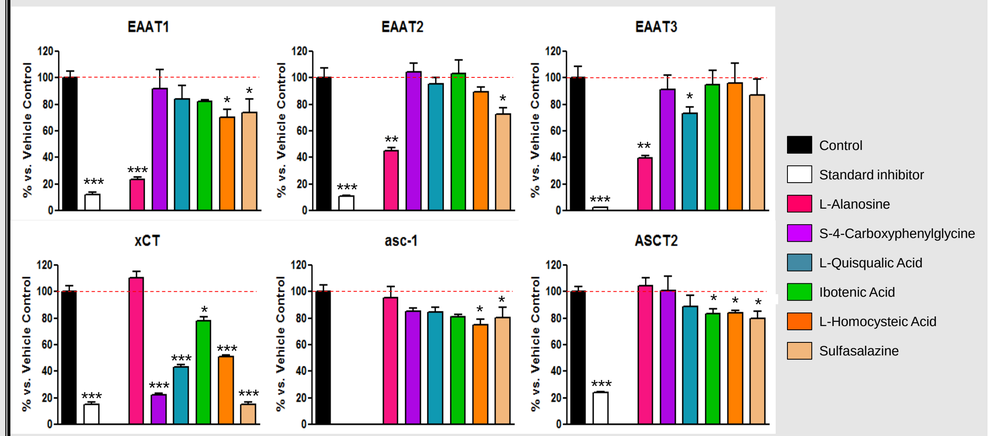

Fig 2. Inhibition of uptake transport of probing substrates by amino acid analog neurotoxins and compounds. Among the six amino acid analogs, L-Alanosine (100 µM) was identified as a potent inhibitor of all three EAATs, while it failed to affect xCT, asc-1 or ASCT2. L-Quisqualic acid induced robust inhibition of xCT transport, but had no effect on asc-1 or ASCT2. All compounds were tested at 100 µM except for the standard inhibitors. *P<0.05, **P<0.01, ***P<0.001 vs. vehicle control.

RESULTS (cont'd)

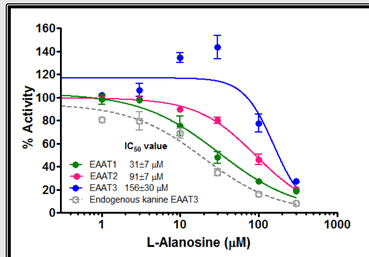

Fig 3. IC_{50} values of L-Alanosine on EAATs. L-Alanosine inhibited the human EAAT1, 2, 3 transporters, with a possible allosteric modulation on EAAT3. MDCK-II cells have high background of endogenous kanine EAAT3, which was inhibited by L-Alanosine as well. (IC_{50}=20±4 µM)

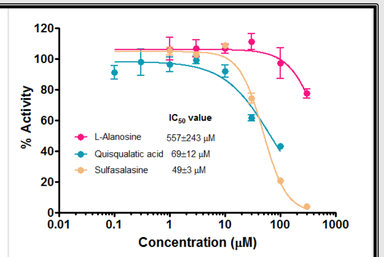

Fig 4. Concentration-dependent inhibition of xCT transport of glutamic acid. L-Quisqualic acid and Sulfasalazine dose-dependently reduced glutamic acid uptake mediated by xCT. L-Alanosine may also inhibit xCT activity at concentrations greater than 100 µM.

CONCLUSIONS

None of the 30 marketed CNS drugs were observed to dramatically inhibit the activity of EAAT2---a major EAAT responsible for glutamate recycling.

In a screening study involving six amino acid analog neurotoxins / compounds, L-Alanosine was found to significantly inhibit all three EAATs. It also attenuated the activity of xCT at higher concentrations. Although L-alanosine is being developed as an anti-tumor drug acting peripherally, BBB leakage is present in late stage cancer patients, raising the concerns of potential CNS adverse effects.

The remaining five compounds at 100 µM significantly suppressed xCT uptake. Quisqualic acid demonstrated a robust reduction of xCT activity with an IC_{50} of 69 µM. This inhibition may result in disruption of GSH production and play a role in its well-known neurotoxicity.

asc-1 and ASCT2 were either not affected or only moderately suppressed by the six amino acid analogs.

ACKNOWLEDGEMENTS

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