0026-895X/98/020195-07$3.00/0

MOLECULAR PHARMACOLOGY, **53:**195–201 (1998).

ACCELERATED COMMUNICATION

DL-*threo*-β-Benzyloxyaspartate, A Potent Blocker of Excitatory Amino Acid Transporters

KEIKO SHIMAMOTO, BRUNO LEBRUN, YOSHIMI YASUDA-KAMATANI, MASAHIRO SAKAITANI, YASUSHI SHIGERI, NOBORU YUMOTO, and TERUMI NAKAJIMA

Suntory Institute for Bioorganic Research, Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618, Japan (K.S., B.L., Y.Y-K., M.S., T.N), and Osaka National Research Institute (Agency of Industrial Science and Technology, Ministry of International Trade and Industry), Midorigaoka, Ikeda, Osaka 563, Japan (Y.S., N.Y.)

Received October 6, 1997; Accepted November 4, 1997

This paper is available online at http://www.molpharm.org

ABSTRACT

DL-*threo*-β-Benzyloxyaspartate (DL-TBOA), a novel derivative of DL-*threo*-β-hydroxyaspartate, was synthesized and examined as an inhibitor of sodium-dependent glutamate/aspartate (excitatory amino acid) transporters. DL-TBOA inhibited the uptake of [^{14}C]glutamate in COS-1 cells expressing the human excitatory amino acid transporter-1 (EAAT1) (K_i = 42 μM) with almost the same potency as DL-*threo*-β-hydroxyaspartate (K_i = 58 μM). With regard to the human excitatory amino acid transporter-2 (EAAT2), the inhibitory effect of DL-TBOA (K_i = 5.7 μM) was much more potent than that of dihydrokainate (K_i = 79 μM), which is well known as a selective blocker of this subtype. Electrophysiologically, DL-TBOA induced no detectable inward currents in *Xenopus laevis* oocytes expressing human EAAT1 or EAAT2. However, it significantly reduced the glutamate-induced currents, indicating the prevention of transport. The dose-response curve of glutamate was shifted by adding DL-TBOA without a significant change in the maximum current. The K_b values for human EAAT1 and EAAT2 expressed in *X. laevis* oocytes were 9.0 μM and 116 nM, respectively. These results demonstrated that DL-TBOA is, so far, the most potent competitive blocker of glutamate transporters. DL-TBOA did not show any significant effects on either the ionotropic or metabotropic glutamate receptors. Moreover, DL-TBOA is chemically much more stable than its benzoyl analog, a previously reported blocker of excitatory amino acid transporters; therefore, DL-TBOA should be a useful tool for investigating the physiological roles of transporters.

Glutamate acts as an excitatory neurotransmitter in the mammalian central nervous system as well as a potent neurotoxin. The termination of neurotransmission is mediated by sodium-dependent high affinity glutamate/aspartate transporters. Glutamate transporters also play an important role in maintaining the extracellular glutamate concentration below neurotoxic levels and therefore contribute to the prevention of neuronal damage from excessive activation of glutamate receptors (Nicholls and Attwell, 1990; Rothstein *et al.*, 1996; Gegelashvili and Schousboe, 1997; Kanai, 1997; Tanaka *et al.*, 1997). Some serious neuronal diseases, such as epilepsy, amyotrophic lateral sclerosis, Alzheimer's diseases, and cellular damage from a stroke, may be linked to the failure of transporters. Five subtypes of EAATs (EAAT1–5) have been cloned from mammalian tissues (Kanai and Hediger, 1992; Pines *et al.*, 1992; Storck *et al.*, 1992; Tanaka, 1993; Shashidharan *et al.*, 1993, 1994; Arriza *et al.*, 1994, 1997; Kawakami *et al.*, 1994; Manfras *et al.*, 1994; Fairman *et al.*, 1995; Inoue *et al.*, 1995). These transporters couple the electrochemical gradient of three cotransported sodium ions and one countertransported potassium ion to that of glutamate (Zerangue and Kavanaugh, 1996). A proton also is cotransported. In addition, a substrate-dependent chloride conductance provides a potential mechanism for dampening cell excitability (Fairman *et al.*, 1995). The transporter subtypes notably differ in the magnitude of this chloride flux relative to the flux of glutamate (Fairman *et al.*, 1995; Wadiche *et al.*, 1995; Arriza *et al.*, 1997).

ABBREVIATIONS: EAAT, excitatory amino acid transporter; EAAC, excitatory amino acid carrier; THA, *threo*-β-hydroxyaspartate; TBzOAsp, *threo*-β-benzoyloxyaspartate; TBOA, *threo*-β-benzyloxyaspartate; *t*-2,4-PDC, L-*trans*-pyrrolidine-2,4-dicarboxylic acid; L-CCG-III, (2*S*,1′*S*,2′*R*)-2-(2-carboxycyclopropyl)glycine; L-CCG-IV, (2*S*,1′*R*,2′*S*)-2-(2-carboxycyclopropyl)glycine; 2S4R4MG, (2*S*,4*R*)-4-methylglutamate; PCR, polymerase chain reaction; DHKA, dihydrokainate; KA, kainate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; mGluR; metabotropic glutamate receptor.

Inhibitors of glutamate transporters are essential for elucidation of the intrinsic properties and physiological roles of transporters. A number of pharmacological agents have been shown to inhibit glutamate transport (Ferkany and Coyle, 1986; Bridges *et al.*, 1991, 1993, 1994; Nakamura *et al.*, 1993; Robinson *et al.*, 1993; Arriza *et al.*, 1994; Yamashita *et al.*, 1995; Vandenberg *et al.*, 1997). Most of them indeed act as competitive substrates, inducing a transport current and a substrate-dependent chloride flux. Blocker-type inhibitors, which are not transportable, inhibit the transport of glutamate while letting transporters to be electrically silent. Moreover, they also suppress voltage-dependent pre-steady state charge movements, allowing kinetic information on the transporters to be obtained (Wadiche *et al.*, 1995). KA and DHKA block EAAT2 in the low-micromolar range, whereas ≈10 mM is required to block transport by EAAT1, EAAT3, and EAAT4 (Arriza *et al.*, 1994; Fairman *et al.*, 1995). Recently, 2S4R4MG was shown to be a very potent blocker for EAAT2 and a substrate for EAAT1 (Vandenberg *et al.*, 1997). However, these compounds also activate ionotropic glutamate receptors (Gu *et al.*, 1995). As such, they can be valuable pharmacological tools for the study of EAAT2 in heterologous expression systems but cannot be used to determine the physiological role of this transporter in complex preparations. THA and *t*-2,4-PDC were found to be blockers for EAAT5 (Arriza *et al.*, 1997) and substrates for EAAT1–4 (Arriza *et al.*, 1994; Fairman *et al.*, 1995). THA also was demonstrated to be a ligand for *N*-methyl-D-aspartate receptors (Jane *et al.*, 1994); therefore, pharmacological agents that are able to block EAATs without being transported and without affecting glutamate receptors are needed.

Our approach to the development of blockers of EAATs was the synthesis of derivatives of THA. We recently demonstrated that DL-TBzOAsp is a competitive blocker for bovine EAAT1 (glutamate/aspartate transporter type) (Lebrun *et al.*, 1997). However, because DL-TBzOAsp has an ester bond, it is unstable in aqueous solution (ester cleavage or acyl migration); therefore, we synthesized a more stable ether-type derivative, DL-TBOA. DL-TBOA showed potent inhibitory activity on the [^{14}C]glutamate uptake in COS-1 cells expressing human EAAT1 or EAAT2. Moreover, it proved to be highly selective for EAATs versus the glutamate receptors. Electrophysiological analysis demonstrated that DL-TBOA is a competitive blocker for human EAAT1 and EAAT2; it is indeed the most potent blocker for both EAAT1 and EAAT2 described to date.

Experimental Procedures

Materials. L-Glutamate was obtained from Nacalai Tesque (Kyoto, Japan). THA was from Sigma Chemical (St. Louis, MO). *t*-2,4-PDC, DHKA, and 2S4R4MG were from Tocris Cookson (Bristol, UK). L-[^{14}C]Glutamate, [^{3}H]CGS 19755 (*cis*-4-phosphono-methyl-2-piperidine carboxylic acid), [^{3}H]KA, and [^{3}H]AMPA were from DuPont-New England Nuclear (Botson, MA). L-CCG-III and L-CCG-IV were synthesized as described previously (Shimamoto *et al.*, 1991). DL-TBOA was synthesized in the same manner as DL-TBzOAsp, except for the use of benzyl bromide instead of acyl chloride (Lebrun *et al.*, 1997). The structure and purity (>95%) of the compound were confirmed with 400-MHz NMR. Stock solutions (100 mM) of the inhibitors, except for DL-TBzOAsp, were made in 0.1 M NaOH and stored at −20°. Stock solutions for DL-TBzOAsp were made in 50% dimethylsulfoxide without NaOH. DL-TBOA was stable for ≥1 week at room temperature; no noticeable decomposition was observed by NMR.

cDNA cloning. The cDNA clones coding for the human EAAT1 or EAAT2 were obtained by PCR performed on human brain cDNA (Clontech, Palo Alto, CA) using Ex *Taq* polymerase (Takara Shuzo, Shiga, Japan). To amplify the full-length EAAT-coding regions, sets of primers were designed according to the published nucleotide sequences (Shashidharan *et al.*, 1993, 1994; Arriza *et al.*, 1994; Kawakami *et al.*, 1994; Manfras *et al.*, 1994) (set for EAAT1, 5′-AGCTGGAGCTCCACCCCTTACAAAATCAGAAAAGTTGTGTTTTC-3′ and 5′-AATTGGGTACCTGGTGCTCAAGAAAGTGTTTCTTTATGTTAGTC-3′; set for EAAT2, 5′-AGCTGGAGCTCACCCCGGCGTCCGCTTTCTCCCTCGCCCACATC-3′ and 5′-AATTGGGTACCATAGGATACGCTGGGGAGTTTATTCAAGAATTTG-3′). Thirty cycles of amplification were carried out using a thermal cycle program (EAAT1, 1 min at 94°, 1 min at 55°, and 1 min at 72°; EAAT2, 1 min at 94°, 1 min at 45°, and 1 min at 72°). The PCR products (1.8 kbp for EAAT1 and 1.9 kbp for EAAT2) were cloned into pCR II according to the manufacturer's instruction for the Original TA Cloning Kit (InVitrogen, San Diego, CA). The obtained clones were subsequently analyzed by DNA sequencing. The clones containing EAATs were subcloned into pBluescript II SK^{-} after digestion with *Sac*I and *Kpn*I for expression in *Xenopus laevis* oocytes. The plasmids were linearized with *Bss*HII, and cRNA was transcribed from each of the cDNA constructs with T3 RNA polymerase and capped using the MEGAscript kits and Cap Analog (Ambion, Austin, TX). The eukaryotic expression vector pKDEMSS (Kitano *et al.*, 1995) derived from pdKCR-dhfr was kindly donated by Dr. K. Kitano (Suntory Institute for Biomedical Research, Mishima-gun, Osaka, Japan). EAAT1 cloned in pCR II was digested with *Spe*I and *Xho*I and subcloned into the *Spe*I/*Sal*I sites of pKDEMSS. EAAT2 cloned in pCR II was subcloned into pKDEMSS after digestion with *Eco*RI.

Transfection. COS-1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37° under an atmosphere of 5% CO_2. Cells were transfected by electroporation (1×10^7 cells, 200 V, 975 μF) with 10 μg of the eukaryotic expression vector pKDEMSS containing each type of transporter cDNA. COS-1 cells transfected by the vector alone were used to control the level of the endogenous uptake of [^{14}C]glutamate. Transfected cells were seeded onto 96-well plates and cultured for 2 days before the uptake assay.

Measurements of [^{14}C]glutamate uptake in transfected COS-1 cells. The subconfluent cells were washed two times with 300 μl of modified phosphate-buffered saline that contained 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM D-glucose, pH 7.4 (Yamashita *et al.*, 1995) and preincubated in 300 μl of the same buffer at 37° for 12 min. After aspiration of the buffer, cells were incubated with 1 μM L-[^{14}C]glutamate in 100 μl of modified phosphate-buffered saline in the absence or presence of test compounds at various concentrations at 37° for 12 min. To terminate the uptake, cells were washed three times with ice-cold buffer and solubilized with 100 μl of 1 M NaOH. Radioactivity was measured by scintillation counting in 3 ml of ULTIMA-GOLD (Packard, Meriden, CT). Nonspecific incorporation was determined in sodium-free solution (140 mM choline chloride, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, and 10 mM D-glucose, pH 7.4) (Nakamura *et al.*, 1993). Specific uptake of [^{14}C]glutamate is given relative to the control. The inhibition constants (K_i values) were determined by Dixon and/or Lineweaver-Burk plots obtained for increasing concentrations of [^{14}C]glutamate of 0.5–5 μM. All values displayed are mean ± standard error of at least three determinations.

Expression of EAAT in oocytes. Fifty nanograms of RNA coding for EAAT1 or EAAT2 was injected into stage V–VI defolliculated oocytes.

Electrophysiology. Two-electrode voltage-clamp recordings were made 2–4 days after injection with the use of the same apparatus and methods as described previously (Lebrun *et al.*, 1997).

Receptor binding assay. Rat brain synaptic membranes were prepared (Enna and Snyder, 1977) and modified (Murphy *et al.*, 1988) as described previously and stored at −80° until use. On the day of the assay, the membrane suspension was incubated in a buffer containing 0.04% Triton X-100 at 37° for 30 min (Murphy *et al.*, 1987, 1988). Triton X-100 was removed by centrifugation, and the pellet was washed twice with an assay buffer. Binding assays were performed according to published methods (London and Coyle, 1979; Murphy *et al.*, 1987, 1988; Kawai *et al.*, 1992). Incubation conditions are described in the legend for Table 3.

mGluR assay. CHO cells stably expressing mGluR1, mGluR2, or mGluR4 were kindly donated by Prof. S. Nakanishi (Kyoto University, Kyoto, Japan). Agonist and antagonist activities on mGluR1 were determined by intracellular Ca^{2+} concentration measurements on Fura-2/acetoxymethyl ester-loaded cells in the absence or presence of glutamate (10 μM), respectively, as described previously (Kawabata *et al.*, 1996). Agonist activities on mGluR2 or mGluR4 were evaluated by measuring the inhibition of forskolin-induced cAMP formation as described previously (Hayashi *et al.*, 1992). Antagonist activities were evaluated by measuring the rescue from the glutamate (10 μM)-induced inhibition of forskolin-induced cAMP formation.

Results

We recently obtained the first competitive blockers for bovine EAAT1 (glutamate/aspartate transporter) by introducing some bulky acyl substituents on the β-hydroxyl group of DL-THA (Lebrun *et al.*, 1997). However, these derivatives are unstable in aqueous solution, particularly at basic pH, due to the presence of an easily cleavable ester bond and a possible intramolecular acyl migration from oxygen to nitrogen. Furthermore, they might be susceptible to esterases in biological preparations. To obtain a more stable derivative, we synthesized the ether-type analog DL-TBOA (Fig. 1). This new compound proved to be stable even in a basic solution (disodium salt, pH 11.5) at room temperature. Because DL-TBOA was insensitive to the pH of a solution, it could be treated as a water-soluble sodium salt.

Cloning of human EAAT1 and EAAT2. To compare the inhibitory action of DL-TBOA with those of DL-TBzOAsp, our most potent blocker for EAAT1, and other known inhibitors on human EAAT1 and EAAT2, we first cloned these transporters by PCR from a human brain cDNA library. The sequence of our EAAT1 cDNA clone was identical to those of GenBank nos. D26443 (Kawakami et al., 1994), L19158 (Shashidharan and Plaitakis, 1993), and U03504 (Arriza *et al.*, 1994). On the contrary, there were some nucleotide differences between our EAAT2 cDNA clone and those of GenBank nos. U03505 (Arriza *et al.*, 1994), U01824 (Shashidharan *et al.*, 1994), and Z32517 (Manfras *et al.*, 1994). The nucleotide sequence of our clone has been submitted to the GenBank/EMBL/DDBJ Data Bank with accession no. D85884. The predicted human EAAT2 gene product reported here has 99% sequence identity with that of U03505, 98% with that of U01824, and 92% with that of Z32517. Three amino acids (His19, Val211, and Phe347) in the amino acid sequence predicted from our clone were replaced in a very similar sequence predicted from UO3505, whereas these amino acids were conserved in the sequences predicted by the other clones; therefore, the differences do not seem to be errors introduced by the *Taq* polymerase. Because the characteristics of EAAT2 described below were very similar to those reported previously, we postulate that the substitutions have little effect on the transporter activity.

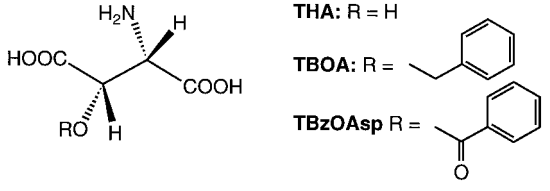

Fig. 1. Structures of DL-THA and its derivatives. Structures are indicated as L-isomers.

Inhibition of radiolabeled glutamate uptake in transfected COS-1 cells. Transfections were performed according to the electroporation method as described in Experimental Procedures. Transfected cells accumulated 5–10-fold more [^{14}C]glutamate than vector-transfected cells. Uptake was reduced by >90% in Na^+-free solution. Michaelis constant (K_m) values for L-glutamate were 57 ± 6.0 μM for EAAT1 and 49 ± 10 μM for EAAT2.

Both DL-TBOA and DL-TBzOAsp markedly inhibited [^{14}C]glutamate (1 μM) uptake in transfected cells in a dose-dependent manner (Fig. 2). On EAAT1, DL-TBOA was more potent than DL-TBzOAsp, with almost the same level of ac-

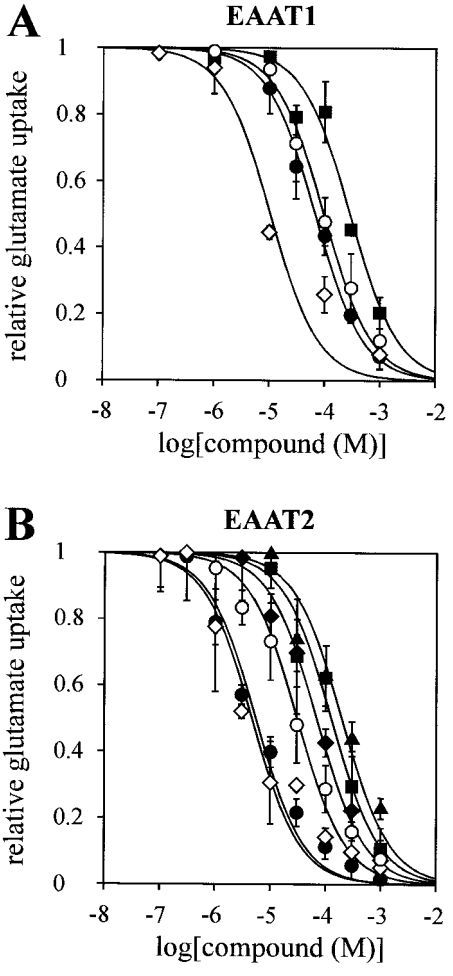

Fig. 2. DL-TBOA inhibits glutamate uptake in COS-1 cells expressing human EAAT1 (A) or EAAT2 (B) in a dose-dependent manner. Uptake of [^{14}C]glutamate (1 μM) was assessed in the presence of the indicated amount of inhibitors. Inhibitors were DL-TBOA (●), DL-TBzOAsp (■), DHKA (▲), 2S4R4MG (◆), DL-THA (○), and L-CCG-III (◇). Specific uptake of [^{14}C]glutamate were expressed as relative ratio to control. Values are presented as mean ± standard error of at least three determinations.

TABLE 1

Inhibition of [^{14}C]glutamate uptake in COS-1 cells expressing human EAAT1 or EAAT2

IC_{50} values are obtained from the dose-dependent inhibition curve (Fig. 2). Values are from at least three independent experiments.

	IC_{50}	
	EAAT1	EAAT2
	μM	
DL-TBOA	67 ± 7.5	5.5 ± 1.0
DL-TBzOAsp	286 ± 46	126 ± 29
DL-THA	96 ± 13	31 ± 5.8
DHKA	>1000	196 ± 56
2S4R4MG	105 ± 21	69 ± 9.7
t-2,4-PDC	65 ± 7.1	14 ± 6.8
L-CCG-III	11 ± 0.6	4.6 ± 1.5
L-CCG-IV	900 ± 87	673 ± 46

tivity as that of DL-THA. Values of 50% inhibitory concentration (IC_{50}) were 67 μM for DL-TBOA, 286 μM for DL-TBzOAsp, and 96 μM for DL-THA. Some other compounds, well known inhibitors acting as competitive substrates, were examined in the same system (Table 1). Among them, L-CCG-III very significantly inhibited [^{14}C]glutamate uptake with an IC_{50} value of 11 μM, which is in accord with previous studies (Kawai *et al.*, 1992; Nakamura *et al.*, 1993; Yamashita *et al.*, 1995). L-CCG-IV, a stereoisomer of L-CCG-III, was reported to be nearly as potent as L-CCG-III on human glutamate transporter-1 expressed in COS-7 cells (Yamashita *et al.*, 1995). It showed only a weak affinity (IC_{50} = 900 μM) in this work, which is consistent with its poor inhibitory activity on the uptake by the glial plasmalemmal vesicle and synaptosomes (Kawai *et al.*, 1992; Nakamura *et al.*, 1993). On EAAT2, the inhibitory effect of DL-TBOA was much stronger than that of DL-TBzOAsp and DL-THA (IC_{50} = 5.5, 126, and 31 μM, respectively). The potency of DL-TBOA was comparable to that of L-CCG-III (IC_{50} = 4.6 μM). DHKA, known to be a selective blocker of EAAT2, inhibited the uptake on EAAT2 with an IC_{50} value of 196 μM, whereas it had no effect on EAAT1 at a concentration as high as 1 mM. Recently, 2S4R4MG was reported to be the most potent blocker for EAAT2 and a substrate for EAAT1 in an electrophysiological study using *X. laevis* oocytes expressing EAAT1 or EAAT2 (Vandenberg *et al.*, 1997). In this study, 2S4R4MG (IC_{50} = 69 μM) proved to be more potent than DHKA on EAAT2 but less potent than DL-TBOA.

The uptake inhibition mechanism of these compounds was examined by a kinetic analysis. Initial rates of glutamate uptake were measured for various concentrations of glutamate and the inhibitors (Fig. 3). Dixon plots gave straight lines, which intersected at a common point, indicating a competitive inhibition. The inhibition constants (K_i values) are summarized in Table 2.

Electrophysiological studies on *X. laevis* oocytes. To determine whether DL-TBOA is a substrate or a blocker of EAAT1 and EAAT2, we performed an electrophysiological analysis of its effect on *X. laevis* oocytes injected with cRNA encoding EAAT1 or EAAT2. DL-TBOA (100 μM) did not elicit a detectable current in voltage-clamped oocytes expressing EAAT1 or EAAT2. However, the inward current induced by glutamate (100 μM) was significantly reduced in the presence of DL-TBOA (100 μM) (Fig. 4).

A Schild analysis of the effects of increasing concentrations of DL-TBOA on glutamate dose responses demonstrated that

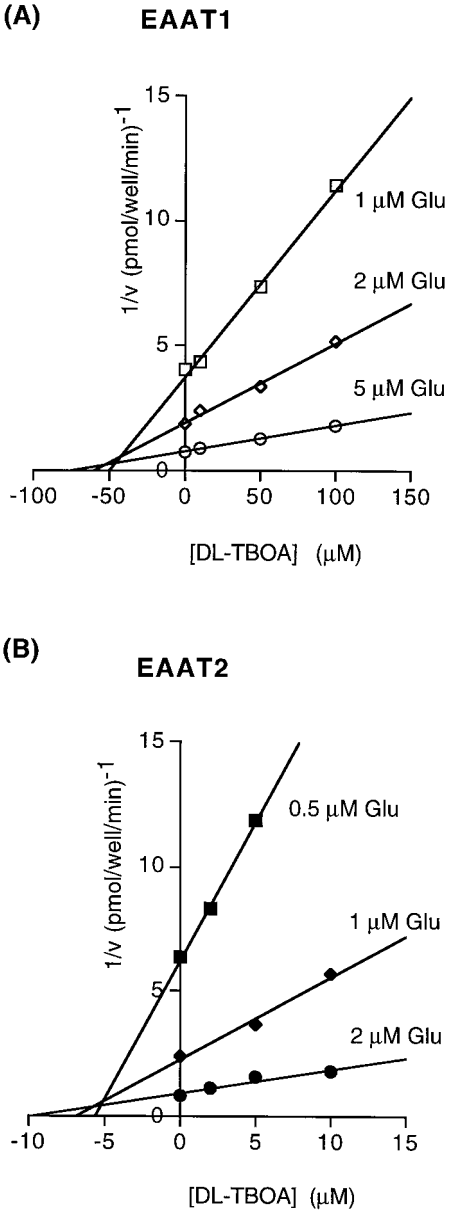

Fig. 3. DL-TBOA inhibits glutamate (*Glu*) uptake in a competitive manner. The initial rates of the uptake in COS-1 cells transfected with EAAT1 (A) or EAAT2 (B) were measured in the presence of (A) 1 (□), 2 (◇), and 5 μM (○) or (B) 0.5 (■), 1 (◆), and 2 μM (●) L-[^{14}C]glutamate. The reciprocal value of the rate is plotted versus the DL-TBOA concentration. In both subtypes, straight-line plots are obtained, and they intersect at a common point ($-K_i$), indicating that DL-TBOA inhibited the glutamate uptake in a competitive manner. The Dixon plots shown are from a single representative experiment. The K_i values of other inhibitors were obtained in the same manner and are summarized in Table 2, where they are presented as the mean ± standard error of three independent experiments.

DL-TBOA is a competitive blocker of EAAT1 and EAAT2. For EAAT1, the apparent affinity for glutamate was shifted from 14 ± 1 to 27 ± 2 and 54 ± 3 μM in the presence of 10 and 30 μM DL-TBOA, respectively, without a significant modification of the maximum current (Fig. 5A). A similar parallel shift of the dose-response curve was obtained on EAAT2. The apparent affinity for glutamate was shifted from 31 ± 3 to 152 ± 4, 436 ± 18, and 1410 ± 74 μM in the presence of 1, 3, and 10 μM DL-TBOA, respectively (Fig. 5B). The agonist dose ratio (dr)

TABLE 2

Kinetic parameters of EAAT1 and EAAT2

Inhibition constants (K_i) were obtained from initial rates of glutamate uptake measured at various concentrations of glutamate and inhibitors (Fig. 3). Antagonist equilibrium dissociation constants (K_b) were obtained from Schild analysis (Fig. 5).

	K_i/K_b	
	EAAT1	EAAT2
	μM	
Uptake assay		
DL-TBOA	42 ± 19	5.7 ± 1.1
DL-TBzOAsp	149 ± 33	83 ± 7.7
DL-THA	58 ± 24	14 ± 4.6
DHKA	>1000	79 ± 19
L-CCG-III	7.5 ± 3.8	2.5 ± 0.4
Electrophysiology		
DL-TBOA	9.0	0.116

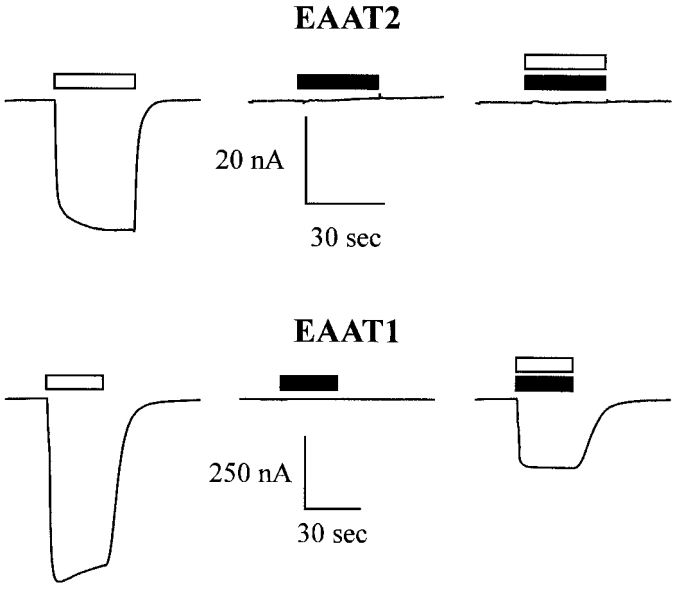

Fig. 4. DL-TBOA is a blocker of both EAAT1 and EAAT2. The application of 100 μM glutamate (□) to oocytes expressing EAAT1 or EAAT2 and voltage clamped at −70 mV induced an inward current. The application of 100 μM DL-TBOA (■) on the same oocytes did not induce any detectable current, but the coapplication of 100 μM DL-TBOA and 100 μM glutamate caused a reduction in the amplitude of the current compared with the application of glutamate alone. There is complete block in the case of EAAT2.

was calculated at each blocker concentration, and log(dr − 1) was plotted against log[DL-TBOA] (Fig. 5C). Antagonism equilibrium dissociation constants (K_b values) of 9.0 μM for EAAT1 and 116 nM for EAAT2 were obtained from a linear fit of the data with slopes of 1.01 and 1.06, respectively.

Activity on ionotropic glutamate receptors and mGluRs. A key requirement for blockers of EAATs to be valuable pharmacological tools is selectivity toward EAATs versus glutamate receptors. We analyzed the activity of our derivatives on the ionotropic glutamate receptors by binding assays and on the mGluRs by calcium influx measurements (mGluR1) or cAMP formation monitoring (mGluR2 and mGluR4). DL-TBOA showed only a weak affinity toward ionotropic glutamate receptors. In binding competition with [^{3}H]CGS 19755 (*N*-methyl-D-aspartate-type antagonist), [^{3}H]KA, and [^{3}H]AMPA on rat brain synaptic membranes, the IC_{50} values of DL-TBOA were 472 ± 139 μM, 550 ± 250 μM, and > 1 mM, respectively (Table 3). DL-TBOA did not show any agonist or antagonist activity on mGluRs (mGluR1, mGluR2, and mGluR4) at a concentration of 100 μM (data not shown).

Discussion

The results of this study demonstrated that DL-TBOA is a potent competitive blocker of EAAT1 and EAAT2. Furthermore, it showed no activity on the metabotropic receptors tested so far (mGluR1, mGluR2, and mGluR4) at a concentration of 100 μM and was only weakly active on the ionotropic receptors. Thus, this compound proved to be highly selective for EAATs versus the glutamate receptors. The previously described blockers of EAAT2 lacked such a selectivity. KA and 2S4R4MG are high affinity ligands for KA receptors, and DHKA is active on both KA receptors and EAAT2 within the same concentration range; therefore, DL-TBOA will be the blocker of choice for studying the physiological roles of EAATs.

We determined the kinetic parameters of DL-TBOA on EAAT1 and EAAT2 in two different assay systems: the uptake in COS-1 cells and the voltage-clamp study in *X. laevis* oocytes. It should be noted that the K_i value for DL-TBOA obtained from the uptake assay was significantly higher than the K_b value from the electrophysiological assay. The discrepancy was lower in the case of EAAT1 (K_i = 42 μM, K_b = 9 μM) than in the case of EAAT2 (K_i = 5.7 μM, K_b = 0.12 μM). The K_b values for DHKA and 2S4R4MG on EAAT2 were determined previously (9 and 3.4 μM, respectively) with a electrophysiological study using *X. laevis* oocytes (Arriza *et al.*, 1994; Vandenberg *et al.*, 1997). Their affinity in COS-1 cells were also tested and proved to be underestimated. System differences might be attributed to differences in lipid composition, post-translational modification, and other factors (Arriza *et al.*, 1994). Of importance could be that COS-1 cells are not voltage-clamped as opposed to *X. laevis* oocytes. Presumably, transport over a 12-min interval would cause a significant depolarization and, as such, would slow the transport rate. The blocker could suppress such a depolarization and restore the transport rate. The control cells that take up glutamate would be more depolarized than the blocked ones; therefore, the relative measurement would lead to underestimation of the apparent activity of blockers. EAAT1 displays a stronger substrate-dependent chloride conductance than EAAT2 (Wadiche *et al.*, 1995). This chloride conductance may reduce the membrane depolarization and therefore limit the underestimation of the affinity for EAAT1. We nevertheless acknowledge that Arriza *et al.* (1994) observed a lower discrepancy in the affinity of DHKA on EAAT2 expressed in COS-7 cells (K_i = 23 μM) versus in *X. laevis* oocytes (K_b = 9 μM).

It would be plausible that a particular conformation of glutamate or aspartate can be recognized by the substrate-binding sites of transporters. TBOA is a flexible molecule that can adopt several conformations. For the development of selective blockers, it is to elucidate the active conformation for binding to EAATs. In recent studies, assessment was attempted of the conformational requirements for glutamate binding to EAATs (Shimamoto *et al.*, 1991; Bridges *et al.*, 1991, 1993, 1994; Kawai *et al.*, 1992; Nakamura *et al.*, 1993; Yamashita *et al.*, 1995; Vandenberg *et al.*, 1997). Bridges *et al.* (1991, 1993, 1994) proposed that a folded form of glutamate binds to EAATs while an extended form of aspartate is

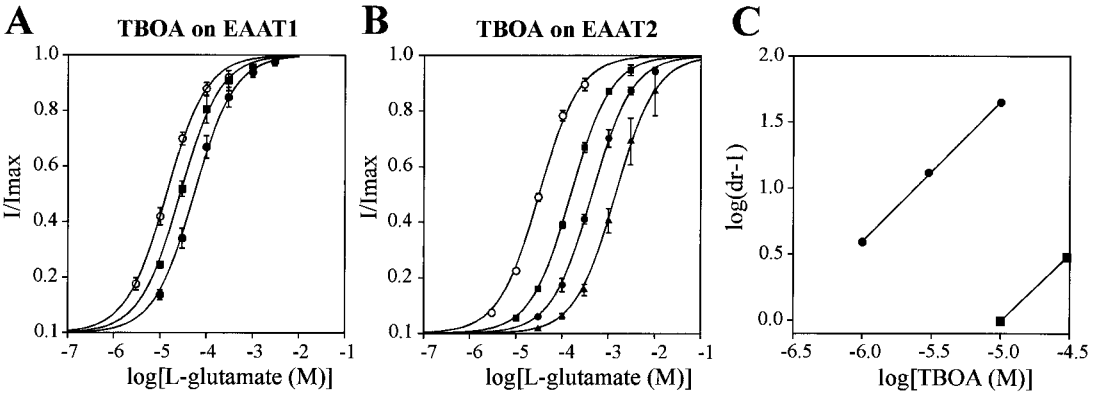

Fig. 5. DL-TBOA is a competitive blocker of EAAT1 and EAAT2. Glutamate dose responses of oocytes expressing EAAT1 or EAAT2 and voltage-clamped at −70 mV were measured as described in Experimental Procedures in the presence of increase dose of DL-TBOA (all doses tested for each cell). The concentrations of DL-TBOA were 0 (○), 10 (□), and 30 (●) μM for the experiments with EAAT1 and 0 (○), 1 (■), 3 (●), and 10 (▲) μM on the experiments with EAAT2. Values are given as mean ± standard deviation from three cells. The agonist dose ratio (dr) was calculated for each DL-TBOA concentration, and log(dr − 1) was plotted against log[DL-TBOA]. The linear fit to the data has a slope of 1.01 (■) for EAAT1 and 1.06 (●) for EAAT2, which indicates that DL-TBOA is a competitive blocker of EAAT1 and EAAT2.

TABLE 3

IC_{50} values of ^{3}H-labeled ligand binding to ionotropic glutamate receptors on rat brain synaptic membranes

	Binding IC_{50}		
	[^{3}H]CGS19755	[^{3}H]KA	[^{3}H]AMPA
		μM	
Transporter inhibitor			
DL-TBOA	472 ± 139	550 ± 250	>1000
DL-TBzOAsp	476 ± 137	>1000	>1000
DL-THA	34 ± 9.5	139 ± 71	267 ± 29
t-2,4-PDC	>1000	>1000	700 ± 173
DHKA	350 ± 50	6.0 ± 2.6	1100 ± 58
Receptor agonist			
L-Glutamate	131 ± 27 (nM)	118 ± 16 (nM)	118 ± 25 (nM)
L-CCG-IV	8.0 ± 2.4(nM)		
NMDA	5.5 ± 0.52		
KA		2.2 ± 0.8(nM)	
AMPA			20 ± 0.5(nM)

Incubation conditions were (1) for *N*-methyl-D-aspartate receptors, [^{3}H]CGS-19755, 10 nM, 4° for 1 hr, 50 mM Tris · HCl buffer, pH 8.0; (2) for KA receptors, [^{3}H]KA, 1 nM, 4° for 1 hr, 100 mM Tris · HCl buffer, pH 7.1; and (3) for AMPA receptors, [^{3}H]AMPA, 5 nM, 4° for 1 hr, 50 mM Tris · HCl buffer, pH 7.4, containing 100 mM KSCN.

adopted. This hypothesis seemed to be reasonable because the functional groups (two carboxylates and an amino group) of these conformers and those of the active compounds can be well superimposed (Bridges *et al.*, 1991, 1993, 1994). Thus, we assumed that the active conformation of TBOA would be an extended one.

Besides the conformational requirements described above, the diastereomeric properties of the compounds are recognized by transporters. L-Glutamate is a high affinity substrate, whereas D-glutamate is poorly transported. On the other hand, both enantiomers of aspartate are known to be excellent substrates of EAATs. Moreover, four diastereomers of β-hydroxyaspartate (L- and D-, and *threo*- and *erythro*-) were nearly equal in activity (Robinson *et al.*, 1993). We suggest that such a permissiveness in structure could be lost in TBOA, in which the small hydroxyl group of THA has been replaced by a bulky benzyl group. We propose that the bulky substituent in TBOA plays a very important role in conferring blocking activity, whereas the hydroxyl of THA is simply tolerated in the pharmacophore (Lebrun *et al.*, 1997). Therefore, the orientation of this substituent relative to the aspartate framework should be very important. The synthesis of the pure stereoisomers of TBOA and *erythro*-isomers is under way. The elucidation of the binding conformation and blockade mechanism might make it possible to develop more potent, and perhaps more selective, EAAT blockers.

So far, DL-TBOA has proved to be the most potent blocker for EAAT1 and EAAT2, with high selectivity toward EAATs versus the glutamate receptors. Moreover, our preliminary results in this electrophysiological study indicated that DL-TBOA also was a blocker for the mouse EAAC1 (EAAT3 subtype). Therefore, this compound opens the way for the mechanistic and kinetic studies of EAAT1 and EAAT3. It should prove useful in determination of the physiological roles of EAATs in various preparations.

Acknowledgments

We wish to thank Prof. Y. Kanai (Kyorin University, Tokyo, Japan) for providing the cDNA of mouse EAAC1, and Prof. S. Nakanishi (Kyoto University, Kyoto, Japan) for supplying the CHO cells expressing mGluRs. We also thank Dr. M. Fujimoto and Dr. T. Nakano (Shionogi Co., Osaka, Japan) for assistance with the mGluR assay.

References

Arriza JL, Eliasof S, Kavanaugh MP, and Amara SG (1997) Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. *Proc Natl Acad Sci USA* **94:**4155–4160.

Arriza JL, Fairman WA, Wadiche JI, Murdoch GH, Kavanaugh MP, and Amara SG (1994) Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. *J Neurosci* **14:**5559–5569.

Bridges RJ, Lovering FE, Humphrey JM, Stanley MS, Blakely TN, Cristofaro MF, and Chamberlin AR (1993) Conformationally restricted inhibitors of the high affinity L-glutamate transporter. *Bioorg Med Chem Lett* **3:**115–121.

Bridges RJ, Lovering FE, Koch H, Cotman CW, and Chamberlin AR (1994) A conformationally constrained competitive inhibitor of the sodium-dependent glutamate transporter in forebrain synaptosomes: L-anti-endo-3,4-methanopyrrolidine dicarboxylate. *Neurosci Lett* **174:**193–197.

Bridges RJ, Stanley MS, Anderson MW, Cotman CW, and Chamberlin AR (1991) Conformationally defined neurotransmitter analogues: selective inhibition of glutamate uptake by one pyrrolidine-2,4-dicarboxylate diastereomer. *J Med Chem* **34:**717–725.

Enna SJ and Snyder SH (1977) Influence of ions, enzymes, and detergents on γ-aminobutyric acid receptor binding in synaptic membranes of rat brain. *Mol Pharmacol* **13:**442–453.

Fairman WA, Vandenberg RJ, Arriza JL, Kavanaugh MP, and Amara SG (1995) An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. *Nature (Lond)* **375:**599–603.

Ferkany J and Coyle JT (1986) Heterogeneity of sodium-dependent excitatory amino acid uptake mechanisms in rat brain. *J Neurosci Res* **16:**491–503.

Gegelashvili G and Schousboe A (1997) High affinity glutamate transporters: regulation of expression and activity. *Mol Pharmacol* **52:**6–15.

Gu ZQ, Hesson DP, Pelletier JC, Maccecchini ML, Zhou LM, and Skolnick P (1995) Synthesis, resolution, and biological evaluation of the four stereoisomers of

4-methylglutamic acid: selective probes of kainate receptors. *J Med Chem* **38:** 2518–2520.

Hayashi Y, Tanabe Y, Aramori A, Masu M, Shimamoto K, Ohfune Y, and Nakanishi S (1992) Agonist analysis of 2-(carboxycyclopropyl)glycine isomers for cloned metabotropic glutamate receptor subtypes expressed in Chinese hamster ovary cells. *Br J Pharmacol* **107:**539–543.

Inoue K, Sakaitani M, Shimada S, and Tohyama M (1995) Cloning and expression of a bovine glutamate transporter. *Mol Brain Res* **28:**343–348.

Jane DE, Olverman HJ, and Watkins JC (1994) Agonist and competitive antagonists: structure-activity and molecular modelling studies, in *The NMDA Receptor* (Collingridge GL and Watkins JC, eds) pp 31–104, Oxford University Press, Oxford.

Kawakami H, Tanaka K, Nakayama T, Inoue K, and Nakamura S (1994) Cloning and expression of a human glutamate transporter. *Biochem Biophys Res Commun* **199:**171–176.

Kanai Y (1997) Family of neutral and acidic amino acid transporters: molecular biology, physiology and medical implications. *Curr Opin Cell Biol* **9:**565–572.

Kanai Y and Hediger MA (1992) Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature (Lond)* **360:**467–471.

Kawabata S, Tsutsumi R, Kohara A, Yamaguchi T, Nakanishi S, and Okada M (1996) Control of calcium oscillations by phosphorylation of metabotropic glutamate receptors. *Nature (Lond)* **383:**89–92.

Kawai M, Horikawa Y, Ishihara T, Shimamoto K, and Ohfune Y (1992) 2-(Carboxycyclopropyl)glycines: binding, neurotoxicity and induction of intracellular free Ca^{2+} increase. *Eur J Pharmacol* **211:**195–202.

Kitano K, Fukuda Y, Nagahira K, Nasu T, Izumi R, Kawashima K, and Nakanishi T (1995) Production and characterization of monoclonal antibodies against human natriuretic peptide receptor-A or -B. *Immunol Lett* **47:**215–222.

Lebrun B, Sakaitani M, Shimamoto K, Yasuda-Kamatani Y, and Nakajima T (1997) New β-hydroxyaspartate derivatives are competitive antagonists for bovine glutamate/aspartate transporter. *J Biol Chem* **272:**20336–20339.

London ED and Coyle JT (1979) Specific binding of [^{3}H]kainic acid to receptor sites in rat brain. *Mol Pharmacol* **15:**492–505.

Manfras BJ, Rubert WA, Trucco M, and Boehm BO (1994) Cloning and characterization of a glutamate transporter cDNA from human brain and pancreas. *Biochim Biophys Acta* **1195:**185–188.

Murphy DE, Hutchison AJ, Hurt SD, Williams M, and Sills MA (1988) Characterization of the binding of [^{3}H]-CGS 19755: a novel N-methyl-D-aspartate antagonist with nanomolar affinity in rat brain. *Br J Pharmacol* **95:**932–938.

Murphy DE, Snowhill EW, and Williams M (1987) Characterization of quisqualate recognition sites in rat brain tissue using DL-[^{3}H]α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and a filtration assay. *Neurochem Res* **12:**775–782.

Nakamura Y, Kataoka K, Ishida M, and Shinozaki H (1993) (2S,3S,4R)-2-(Carboxycyclopropyl)glycine, a potent and competitive inhibitor of both glial and neuronal uptake of glutamate. *Neuropharmacology* **32:**833–837.

Nicholls D and Attwell D (1990) The release and uptake of excitatory amino acids. *Trend Pharmacol Sci* **11:**462–468.

Pines G, Danbolt NC, Bjoras M, Zhang Y, Bendahan A, Eide L, Koepsell, H, Storm-Mathisen J, Seeberg E, and Kanner BI (1992) Cloning and expression of a rat brain L-glutamate transporter. *Nature (Lond)* **360:**464–467.

Robinson MB, Sinor JD, Dowd LA, and Kerwin Jr JF (1993) Subtypes of sodium-dependent high-affinity L-[^{3}H]glutamate transport activity: pharmacologic specificity and regulation by sodium and potassium. *J Neurochem* **60:**167–179.

Rothstein J D, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Scielke JP, and Welty DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* **16:**675–686.

Shashidharan P and Plaitakis A (1993) Cloning and characterization of a glutamate transporter cDNA from human cerebellum. *Biochim Biophys Acta* **1216:**161–164.

Shashidharan P, Wittenberg I, and Paltakis A (1994) Molecular cloning of human brain glutamate/aspartate transporter II. *Biochim Biophys Acta* **1191:**393–396.

Shimamoto K, Ishida M, Shinozaki H, and Ohfune Y (1991) Synthesis of four diastereomeric L-2-(carboxycyclopropyl)glycines: conformationally constrained L-glutamate analogs. *J Org Chem* **56:**4167–4176.

Storck T, Schulte S, Hofmann K, and Stoffel W (1992) Structure, expression and functional analysis of a Na^+-dependent glutamate/aspartate transporter from rat brain. *Proc Natl Acad Sci USA* **89:**10955–10959.

Tanaka K (1993) Expression cloning of a rat glutamate transporter. *Neurosci Res* **16:**149–153.

Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Kikuchi T, Okuyama S, Kawashima N, Hori S, Takimoto M, and Wada K (1997) Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science (Washington DC)* **276:**1699–1702.

Vandenberg RJ, Mitrovic AD, Chebib M, Balcar VW, and Johnston GAR (1997) Contrasting modes of action of methylglutamate derivatives on the excitatory amino acid transporters, EAAT1 and EAAT2. *Mol Pharmacol* **51:**809–815.

Wadiche JI, Arriza JL, Amara SG, and Kavanaugh MP (1995) Kinetics of a human glutamate transporter. *Neuron* **14:**1019–1027.

Yamashita H, Kawakami H, Zhang Y, Hagiwara T, Tanaka K, and Nakamura S (1995) Inhibition by folded isomers of L-2-(carboxycyclopropyl)glycine of glutamate uptake via the human glutamate transporter hGluT-1. *Eur J Pharmacol* **289:**387–390.

Zerangue N and Kavanaugh MP (1996) Flux coupling in a neuronal glutamate transporter. *Nature (Lond)* **383:**634–637.

Send reprint requests to: Dr. Keiko Shimamoto, Suntory Institute for Bioorganic Research, 1–1-1, Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618, Japan. E-mail: ldf01705@niftyserve.or.jp