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Characterization of Novel Aryl-Ether, Biaryl, and Fluorene Aspartic Acid and Diaminopropionic Acid Analogs as Potent Inhibitors of the High-Affinity Glutamate Transporter EAAT2

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ABSTRACT

In this study, we describe the pharmacological characterization of novel aryl-ether, biaryl, and fluorene aspartic acid and diaminopropionic acid analogs as potent inhibitors of EAAT2, the predominant glutamate transporter in forebrain regions. The rank order of potency determined for the inhibition of human EAAT2 was N^4-[4-(2-bromo-4,5-difluorophenoxy)phenyl]-L-asparagine (WAY-213613) (IC_{50} = 85 ± 5 nM) > N^4-(2′-methyl-1,1′-biphenyl-4-yl)-L-asparagine (WAY-213394) (IC_{50} = 145 ± 22 nM) = N^4-[7-(trifluoromethyl)-9*H*-fluoren-2-yl]-L-asparagine (WAY-212922) (IC_{50} = 157 ± 11 nM) = 3-{[(4′-chloro-2-methyl-1,1′-biphenyl-4-yl)carbonyl]amino}-L-alanine (WAY-211686) (IC_{50} = 190 ± 10 nM). WAY-213613 was the most selective of the compounds examined, with IC_{50} values for inhibition of EAAT1 and EAAT3 of 5 and 3.8 μM, respectively, corresponding to a 59- and 45-fold selectivity toward EAAT2. An identical rank order of potency [WAY-213613 (35 ± 7 nM) > WAY-213394 (92 ± 13 nM) = WAY-212922 (95 ± 8 nM) = WAY-211686 (101 ± 20 nM)] was observed for the inhibition of glutamate uptake in rat cortical synaptosomes, consistent with the predominant contribution of EAAT2 to this activity. Kinetic studies with each of the compounds in synaptosomes revealed a competitive mechanism of inhibition. All compounds were determined to be nonsubstrates by evaluating both the stimulation of currents in EAAT2-injected oocytes and the heteroexchange of D-[^{3}H]aspartate from cortical synaptosomes. WAY-213613 represents the most potent and selective inhibitor of EAAT2 identified to date. Taken in combination with its selectivity over ionotropic and metabotropic glutamate receptors, this compound represents a potential tool for the further elucidation of EAAT2 function.

Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system. Glutamate transmission is mediated via interaction with the ligand-gated ion channel receptors, termed the ionotropic receptors, and the seven-transmembrane domain G-protein–coupled receptors, termed metabotropic glutamate receptors (Barnard, 1997; Schoepp et al., 1999). Activation of these receptors is responsible for the physiological actions of glutamate, whereas paradoxically, overstimulation of the ionotropic receptors contributes to the excitotoxic actions attributed to glutamate. Therefore, synaptic glutamate levels must be tightly regulated to maintain the integrity of synaptic transmission and to limit or prevent the pathophysiological activity of this excitatory neurotransmitter.

A family of high-affinity Na^+-dependent glutamate transporters expressed in the plasma membranes of both neurons and astroglia is responsible for the clearance of extracellular glutamate by mediating the cellular uptake of glutamate in a

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ABBREVIATIONS: EAAT, excitatory amino acid transporter; FLIPR, fluorometric imaging plate reader; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; LY341495, (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; LY354740, (+)-2-aminobicyclo[3.1.0.]hexane-2,6,-dicarboxylic acid; WAY-213613, N^4-[4-(2-bromo-4,5-difluorophenoxy)phenyl]-L-asparagine; WAY-213394, N^4-(2′-methyl-1,1′-biphenyl-4-yl)-L-asparagine; WAY-212922, N^4-[7-(trifluoromethyl)-9*H*-fluoren-2-yl]-L-asparagine; WAY-211686, 3-{[(4′-chloro-2-methyl-1,1′-biphenyl-4-yl)carbonyl]amino}-L-alanine; WAY-855, 3-amino-tricyclo[2.2.1.$0^{2,6}$]heptane-1,3-dicarboxylic acid; TBOA, *threo*-β-benzyloxyaspartate; HEK, human embryonic kidney; D-PBS, Dulbecco's phosphate-buffered saline; HBS, HEPES-buffered saline; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Ro 25-6981, (±)-(*R**,*S**)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidine propanol; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline; mGlu, metabotropic glutamate; *trans*-2,4-PDC, L-*trans*-pyrollidine-2,4-dicarboxylate.

process driven largely by the energy of the transmembrane Na^+ gradient (Danbolt, 2001). Five members of this transporter family have been identified by molecular cloning and are designated EAAT1/GLAST, EAAT2/GLT-1, EAAT3/EAAC1, EAAT4, and EAAT5 (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992; Arriza et al., 1994, 1997; Fairman et al., 1995). Among the transporter subtypes, EAAT2 accounts for the bulk of transport activity measured in forebrain preparations. The pharmacological profile of glutamate transport measured in rat forebrain preparations is well correlated with the pharmacology of the cloned rat GLT-1 and human EAAT2 subtypes expressed in mammalian cell lines (Dunlop et al., 1999; Tan et al., 1999). Moreover, transgenic mice with a targeted deletion of the EAAT2 transporter lose more than 90% of the forebrain glutamate transport capacity and exhibit increased vulnerability to an experimental head trauma insult (Tanaka et al., 1997). Experiments using antisense oligonucleotides to knock down levels of EAAT2 resulted in reduced glutamate transport capacity, elevated extracellular glutamate, and increased neurodegeneration (Rothstein et al., 1996), providing another line of evidence in support of a major role of EAAT2 in glutamate clearance.

A pharmacological elucidation of the functional role of EAAT2 has been hampered by the lack of potent and selective transport inhibitors. Widely used pharmacological tools such as the pyrrolidine dicarboxylates (Bridges et al., 1991), aminocyclobutane dicarboxylates (Fletcher et al., 1991), and carboxycyclopropyl glycines (Nakamura et al., 1993) suffer from combinations of poor selectivity across EAAT subtypes, rather modest potencies in the micromolar range, and cross-reactivity with glutamate receptor targets. More recently, the *threo*-β-hydroxyaspartate derivative *threo*-β-benzyloxyaspartate (TBOA) and the novel heptane dicarboxylate WAY-855 have been reported as novel EAAT inhibitors (Lebrun et al., 1997; Shimamoto et al., 1998; Dunlop et al., 2003), and these agents provide some improvement over the earlier compounds. Subsequent structure activity relationship studies with TBOA have led to the identification of nanomolar potent EAAT2 inhibitors, with the most selective agent, (2*S*,3*S*)-3-[3-(4-methoxybenzoylamino)benzyloxy]aspartate), exhibiting 39-fold selectivity over EAAT3 (Shimamoto et al., 1998). Such agents are likely to be better tools for the characterization of transporter function.

In this study, we present the in vitro pharmacological characterization of novel chemotypes possessing potent and EAAT2-preferring inhibitory activity. Compounds were designed by appending a lipophilic load on a relatively polar aspartic acid or aspartic acid-like (diaminopropionic acid) headpiece. On the basis of our hypothetical binding site interaction model, generated from a set of literature and in-house EAAT inhibitors, additional aromatic centers observed in these novel compounds are implicated in the enhancement of EAAT2 potency and concomitant improvement in selectivity over EAAT1 and EAAT3. The synthesis and structure-activity relationship of these series of compounds will be presented elsewhere. These novel compounds offer advantages over existing EAAT2 inhibitors in terms of potency, selectivity, and ease of synthesis. WAY-213613 was found to be the most potent and selective of the compounds described herein, inhibiting EAAT2 with an IC_{50} of 85 nM and exhibiting 59- and 45-fold selectivity over EAAT1 and EAAT3, respectively. Moreover, WAY-213613 was devoid of ionotropic and metabotropic glutamate receptor activity, indicating it to be a highly EAAT2-preferring transporter ligand.

Materials and Methods

Uptake in Stable Cell Lines. Stable HEK cell lines expressing each of the human glutamate transporter subtypes EAAT1–3 were maintained in T175 flasks at 37°C in a humidified atmosphere with 5% CO_2. Cells were detached and replated at 50,000 cells/well in 96-well culture plates the day before measurements of glutamate uptake. Uptake assays were performed in Dulbecco's phosphate-buffered saline (D-PBS) in the presence of 1 μM glutamate and 0.2 μCi/ml L-[^{3}H]glutamate (specific activity, 20–30 Ci/mmol; 1 mCi/ml) in a final volume of 100 μl. Cells were washed with D-PBS before incubation with substrate in the absence or presence of compounds for 20 min at room temperature. Assays were stopped by aspiration followed by two ice-cold D-PBS washes. Cells were solubilized with 0.5 N NaOH before the addition of Microscint 20 for the determination of ^{3}H accumulation in the wells using a TopCount scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Uptake was linear for incubation times up to 30 min (data not shown); thus, data were analyzed as true rates. Nonspecific uptake was corrected for by performing all experiments in the absence and presence of sodium (replacement of sodium chloride with choline chloride). Sodium-independent uptake accounted for $\leq$10% of the total uptake and was subtracted before any further data calculation.

Uptake in Rat Cortical Synaptosomes. P2 synaptosomal fractions isolated from rat cerebral cortex were prepared by homogenization of tissue in ice-cold isolation medium (310 mM sucrose and 10 mM HEPES, pH 7.4) followed by centrifugation at 1000*g* for 5 min. The resulting supernatant was collected and centrifuged at 20,000*g* for 20 min to obtain the crude synaptosomal P2 pellet, which was used for uptake studies at a protein concentration of 1 mg/ml in HEPES-buffered saline (HBS). L-[^{3}H]Glutamate (specific activity, 20–30 Ci/mmol; 1 mCi/ml) uptake was assayed in a final volume of 300 μl of HBS containing 50 μg of synaptosomal protein, 1 μM L-glutamate, and 0.25 μCi/assay L-[^{3}H]glutamate in the absence and presence of drug for the determination of IC_{50} values. Kinetic experiments were performed in the presence of 0.25 μCi/assay L-[^{3}H]glutamate over a range of nonradioactive L-glutamate concentrations in both the absence and presence of compounds. Uptake assays were incubated for 5 min at room temperature and were terminated by filtration on a 96-well Unifilter (Whatman GF/B; Whatman, Clifton, NJ) plate followed by two washes with ice-cold buffer. Microscint 20 was added directly to filter plates for the determination of ^{3}H retained on the filters using a TopCount scintillation counter. Uptake was linear over the 5-min incubation period, and data were corrected to represent sodium-dependent uptake.

Oocyte Studies. EAAT mRNAs were injected into defolliculated stage IV or V *Xenopus laevis* oocytes (10 ng/oocyte), and the membrane currents were recorded 3 to 7 days later. Recording solution contained 96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4. Two-electrode voltage-clamp recordings were performed at room temperature with an NPI TURBO TEC-03X amplifier, EPC-9, and PULSE program for analog-to-digital interface and data acquisition. Microelectrodes were filled with 3 M KCl solution and had resistance of approximately 1 MÙ. Cells were clamped at -40 mV and depolarized to -80 mV for 100 ms, at which the current was measured at the end of the step protocol. EAAT current amplitude was determined by measuring the difference of the step currents before and after the substrate (10 μM glutamate) applied to the bath. The current was low-pass–filtered at 200 Hz and digitized at 5 kHz. Compounds were dissolved in dimethyl sulfoxide as stock and diluted to final concentration with less than 1% dimethyl sulfoxide in the bath. Data were averaged from all oocytes tested and are expressed as mean $\pm$ S.E.

Ionotropic Glutamate Receptor Assays. Evaluation of the compounds for effects on ionotropic glutamate receptors was performed by measuring their effects on NMDA and AMPA-induced depolarization of primary cultures of rat hippocampal neurons using a membrane potential-sensitive fluorescent dye [fluorometric imaging plate reader (FLIPR) membrane potential dye; Molecular Devices, Sunnyvale, CA]. Minced hippocampi dissected from embryonic day 18 embryos were incubated at 37°C in 0.01% papain (Worthington Biochemicals, Freehold, NJ), 0.1% dispase (Roche Products, Hertfordshire, UK), and 0.01% DNase (Sigma Chemical, St. Louis, MO) dissolved in Hanks' balanced salt solution. Individual neurons were plated in a 96-well format at a density of 100,000 cells/well. Neurons were cultured at 37°C in 5% CO_2 in Neurobasal Media (Invitrogen, Carlsbad, CA) supplemented with 2 mM glutamine and 1× B27 supplement (Invitrogen). Assays were performed 2 weeks after plating. After removing the culture media, 200 μl of FLIPR membrane potential dye was added to each well, and plates were incubated at room temperature for 0.5 to 1 h. Agonist activity was evaluated by direct application of the compounds to the plate, after dye loading, and after monitoring 15-s baseline fluorescence online. For antagonist assays, compounds were included during the dye loading step with subsequent stimulation by the addition of 50 μl of depolarizing solution containing 100 μM NMDA or AMPA added to each well during FLEX station runs after a 15-s reading of baseline. As a positive control, 100 μM NMDA or AMPA was added to confirm the agonist activity, and 30 μM Ro 25-6981 or NBQX was used to block NMDA- and AMPA-mediated responses, respectively.

Metabotropic Glutamate Receptor Selectivity Studies. Stable Chinese hamster ovary cell lines expressing either the human mGlu receptors 1, 2, or 4 subtypes were used for functional studies using the measurement of agonist-stimulated mobilization of intracellular calcium with the FLIPR. For the Gαi-coupled mGlu receptors 2 and 4, functional calcium coupling was facilitated by stable coexpression of a G-protein chimera comprising Gαq with the C-terminal five amino acids replaced with those from Gαi (Kowal et al., 2003). Cells were maintained and passaged upon reaching approximately 80% confluence. Cells were plated 24 h before the experiment in poly-D-lysine–coated 96-well plates at a density of approximately 50,000 cells/well. In preparation of the assay, the confluent monolayer of cells was washed twice with Hanks' balanced salt solution supplemented with 20 mM HEPES and 2.5 mM probenecid (FLIPR buffer), and then the cells were loaded by adding 4 μM Fluo-4 AM (Molecular Probes, Eugene, OR) in FLIPR buffer for 1 h at 37°C. After loading, the cells were rinsed twice with FLIPR buffer, and intracellular calcium increases after agonist application were detected by measuring increases in fluorescence with the FLIPR. For evaluation of antagonist activity, LY341495 was included during the dye-loading step and was stimulated subsequently by the addition of an approximate EC_{80} concentration of the agonists L-quisqualate, LY354740, and L-AP4 for mGlu receptors 1, 2, and 4.

Synaptosomal Exchange. Rat cortical P2 synaptosome fractions were resuspended in HBS at a protein concentration of 1 mg/ml and incubated in the presence of 1 μCi/ml D-[^{3}H]aspartate (10–15 Ci/mmol; 1 mCi/ml) for 1 h at room temperature with constant shaking. Synaptosome suspension (100 μl) equilibrated with ^{3}H-label was diluted into 100 μl of HBS containing compound at final concentration of 100 μM immediately after the 1-h loading period. Under this dilution paradigm, the equilibrium established between internal and external ^{3}H label is disrupted such that efflux of the label is now favored as the system tends toward re-establishing equilibrium. Therefore, substrate inhibitors, themselves translocated by the transport system, will favor ^{3}H efflux via heteroexchange, whereas nontransportable inhibitors either will have no effect if efflux is limited or will block efflux. Synaptosomes were incubated with drug for 5 min at room temperature, followed by centrifugation to separate pellet and supernatant. An aliquot of supernatant was subsequently removed for the determination of ^{3}H efflux, a direct index of drug-mediated exchange.

Molecular Modeling. A data set was assembled using literature-reported EAAT inhibitors and the novel compounds disclosed in the present study (Fig. 1). It was assumed that the compounds interact with the transporter(s) in a similar manner, a reasonable assumption given that the compounds have similar mechanism of action as nontransportable, competitive inhibitors of high-affinity glutamate uptake. A "common feature hypothesis" in Catalyst 4.6 (Accelrys, San Diego, CA) was used to develop the pharmacophore model. The Catalyst pharmacophore model contains the location constraints (three-dimensional coordinates and their tolerance) of commonly used chemical features such as hydrogen bond acceptor, hydrogen bond donor, negative ionizable groups such as carboxylic, acid and positive ionizable groups such as amino groups and ring aromatics. Before importing structures into Catalyst, each molecule was fully minimized using MMFF94 force field (Halgren, 1996), and the conformational expansion was performed using the optimal algorithm implemented in Catalyst for each molecule, with a maximum of 250 conformers per molecule and an energy range of 15 kcal/mol.

Data Analysis. IC_{50} values for the dose-response curves were generated using a four-parameter logistic curve-fitting model using Origin 7.5 (OriginLab Corp, Northampton, MA) using the following equation: $y = [(A1 - A2)/1 + (x/x0)^{nH}] + A2$, where $A1$ is the maximum, $A2$ is the minimum, $x0$ is the IC_{50} value, and n_H is the Hill slope coefficient.

Results

Inhibition of Human EAAT Subtypes. Figure 1 illustrates the structures of WAY-213613, WAY-213394, WAY-212922, and WAY-211686. Potent EAAT2 inhibitory activity

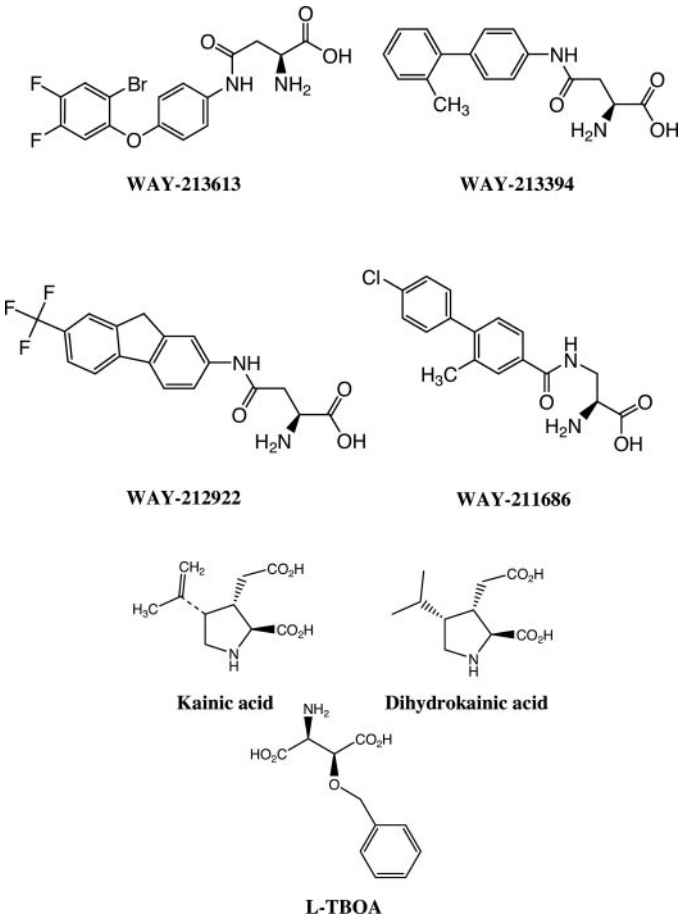

Fig. 1. Chemical structures of WAY-213613, WAY-213394, WAY-212922, and WAY-211686, kainate, dihydrokainate, and L-TBOA.

was observed for all four compounds as determined by the inhibition of L-[^{3}H]glutamate uptake in a stable HEK cell line expressing the human EAAT2 subtype (Fig. 2 and Table 1). WAY-213613 was the most potent of the compounds, with an IC_{50} value of 85 ± 5 nM. Primary selectivity determinations for the compounds using HEK cells stably expressing either the human EAAT1 or EAAT3 subtype are also illustrated in Fig. 2 with estimated IC_{50} values presented in Table 1. In addition to being the most potent compound, WAY-213613 was the most selective of the four, exhibiting 59- and 45-fold selectivity over EAAT1 and EAAT3, respectively. The uptake of L-glutamate measured in the EAAT-expressing HEK cell lines in the absence of drug was 10.7, 158.6, and 8.0 pmol/min/mg for EAAT1, -2, and -3, respectively.

Synaptosomal Uptake Studies. Additional pharmacological characterization in the form of inhibition of L-[^{3}H]glutamate uptake into rat brain cortical synaptosome fractions, a preparation in which EAAT2 contributes the bulk of the measured substrate accumulation, was performed for the compounds in addition to the nonselective and widely used pharmacological agent L-*trans*-pyrollidine-2,4-dicarboxylate (*trans*-2,4-PDC). Concentration-response curves are illus-

TABLE 1

Estimated IC_{50} values for the inhibition of L-[^{3}H]glutamate uptake by human EAAT subtypes and L-[^{3}H]glutamate uptake into rat cortical synaptosome fractions

Data are presented as mean ± S.E.M. from three independent experiments.

	EAAT2	EAAT1	EAAT3	Synaptosomes
		nM		
WAY-213613	85 ± 5	5004 ± 1904	3787 ± 1927	35 ± 7
WAY-213394	145 ± 22	1971 ± 162	6561 ± 2690	92 ± 13
WAY-212922	157 ± 11	3426 ± 815	1837 ± 654	95 ± 8
WAY-211686	190 ± 10	1681 ± 243	1453 ± 282	101 ± 20

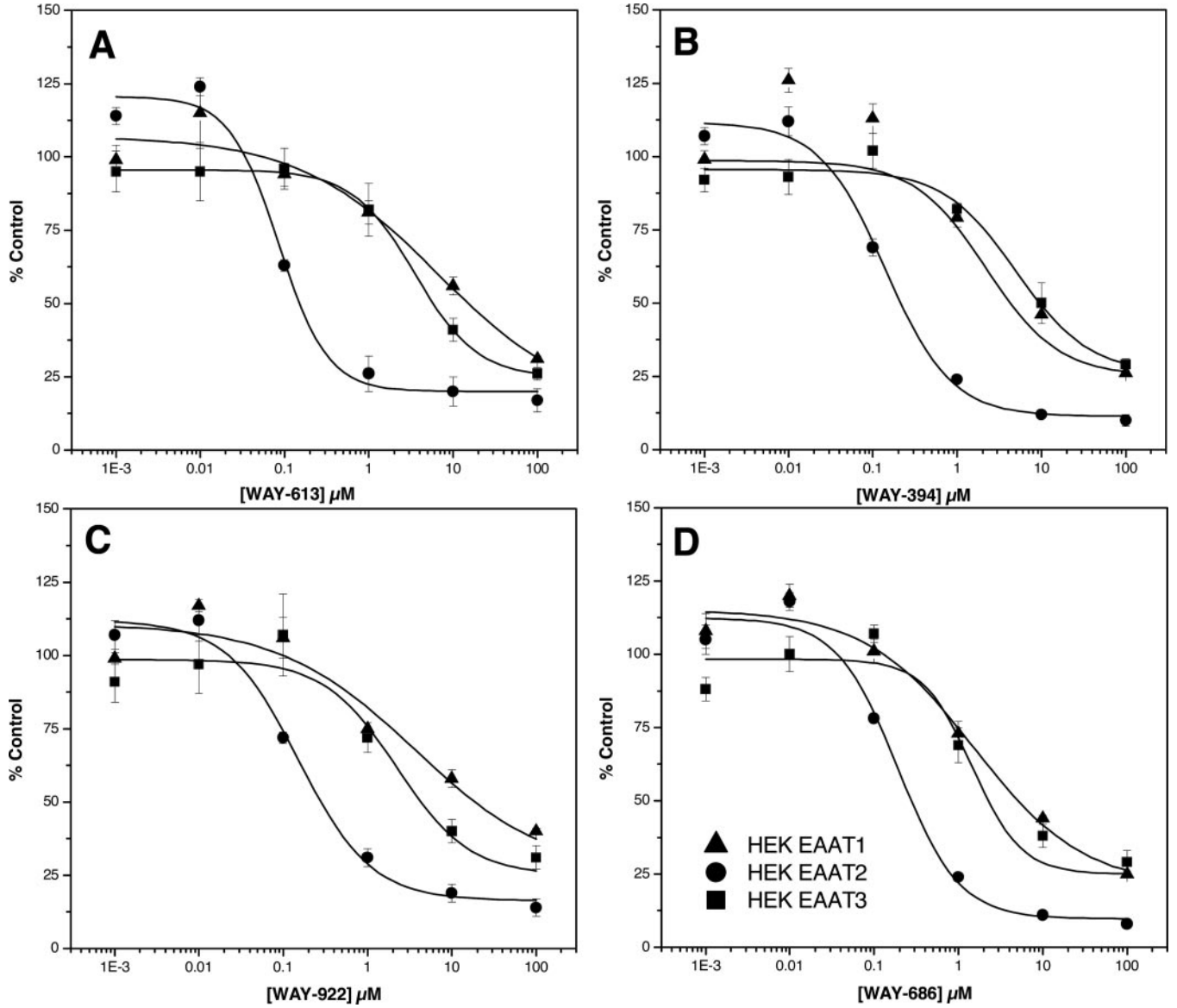

Fig. 2. Log concentration-response curves for the effects of WAY-213613 (A), WAY-213394 (B), WAY-212922 (C), and WAY-211686 (D) on L-[^{3}H]glutamate uptake in stable HEK cells expressing the human EAAT1 (▲), EAAT2 (●), and EAAT3 (■) transporter subtypes. L-[^{3}H]Glutamate uptake was measured in the absence or presence of the indicated drug concentrations. Data are expressed as the percentage of control uptake measured in the absence of added compound and represent the mean ± S.E.M. from three independent experiments.

trated in Fig. 3. Glutamate uptake in the synaptosomal preparation in the absence of added compound (control) was 2 to 2.5 nmol/min/mg. Estimated IC_{50} values for the inhibition of synaptosomal L-[^{3}H]glutamate uptake (Table 1) and rank order of potency were essentially identical with those determined for inhibition of EAAT2, with WAY-213613 being the most potent (IC_{50} = 35 ± 7 nM) of the compounds evaluated, whereas the potency of *trans*-PDC was identical with that reported by others (IC_{50} = 1.6 ± 0.2 μM). Kinetic studies were also performed with the synaptosomal preparation to establish mode of inhibition. A double-reciprocal plot (Lineweaver-Burk transformation) of the effect of multiple concentrations of WAY-213613 on the saturable uptake of L-[^{3}H]glutamate is presented in Fig. 4, indicative of a competitive mechanism of inhibition. Calculated K_I values in the presence of 3, 30, and 300 nM WAY-213613 were 15, 41, and 55 nM, respectively, consistent with the inhibitory potency determined for WAY-213613 in the determination of IC_{50} values described above. Similar experiments performed with WAY-213394, WAY-212922, and WAY-211686 indicated a competitive mode of inhibition for these compounds (data not shown).

Oocyte Studies. On the basis of its superior potency and EAAT2 selectivity, the effect of WAY-213613 on glutamate-induced currents in oocytes was examined. Application of 30 μM WAY-213613 (or indeed the other three compounds described in this study) to EAAT2 injected oocytes failed to induce a transporter-like current, indicating the compound to be a nonsubstrate (data not shown). Inward currents evoked by the application of glutamate to EAAT2-injected oocytes were blocked in a concentration-dependent manner by WAY-213613 (Fig. 5) with an estimated IC_{50} value of 130 nM. WAY-213613 produced a similar concentration-dependent block of glutamate-induced currents in either EAAT1- or EAAT3-injected oocytes (Fig. 5), with IC_{50} values of 48 and 4 μM, respectively, reflecting 370- and 31-fold selectivity toward EAAT2.

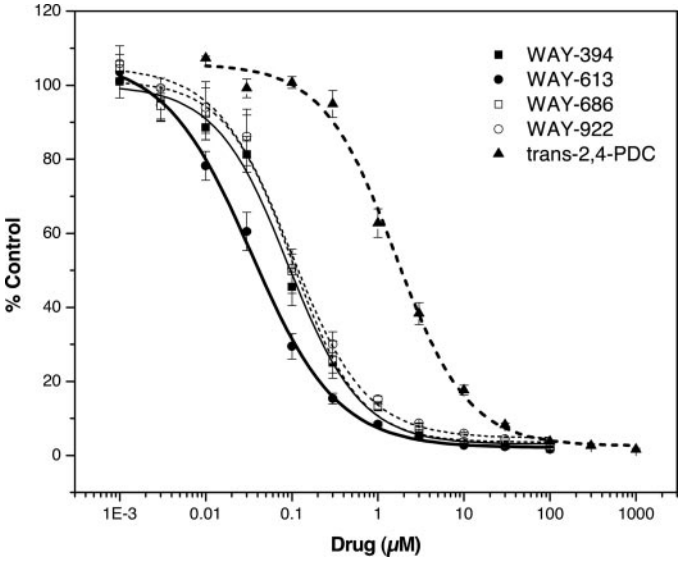

Fig. 3. Log concentration-response curves for the effects of WAY compounds and *trans*-2,4-PDC on L-[^{3}H]glutamate uptake into rat cortical synaptosome fractions. Data are expressed as the percentage of control uptake measured in the absence of added compound and represent the mean ± S.E.M. from three independent experiments.

Ionotropic Glutamate Receptor Assays. Using a membrane potential-sensitive fluorescent indicator dye, we established that the addition of either NMDA, in the absence of Mg^{2+}, or AMPA, in the presence of 250 μg/ml concanavalin A, to primary cultures of hippocampal neurons produced an increase in dye fluorescence indicative of depolarization. Under these experimental conditions, AMPA stimulation can result in the activation of both AMPA (directly) and kainate (by indirect release of glutamate) receptors. The depolarizing responses to NMDA or AMPA could be selectivity antago-

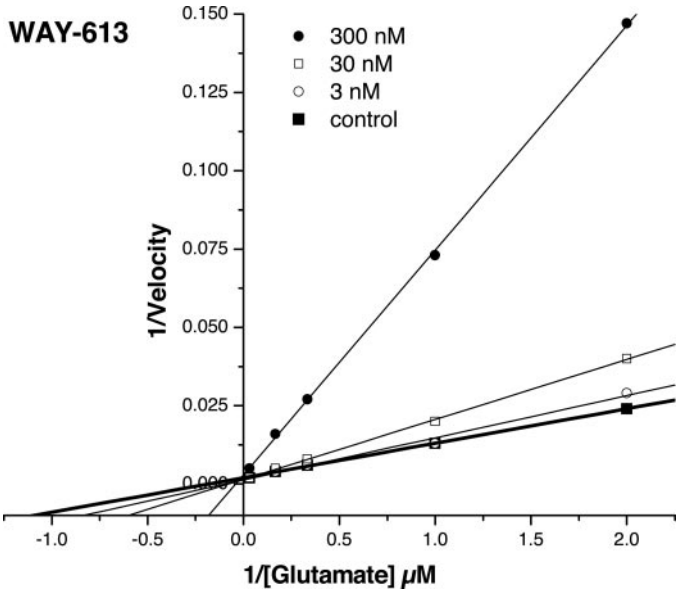

Fig. 4. Kinetic characterization of the inhibitory effect of WAY-213613 on synaptosomal L-[^{3}H]glutamate uptake. L-Glutamate uptake was measured in the presence of increasing concentrations of substrate with or without the addition of WAY-213613. Lineweaver-Burk transformations of the saturable uptake data obtained in the absence (■) and presence of 3 (○), 30 (□), or 300 (●) nM WAY-213613 obtained in a representative experiment are depicted. The profile is consistent with a competitive mode of inhibition and the K_I values estimated in the presence of 3, 30, and 300 nM WAY-213613 were 15, 41, and 55 nM, respectively.

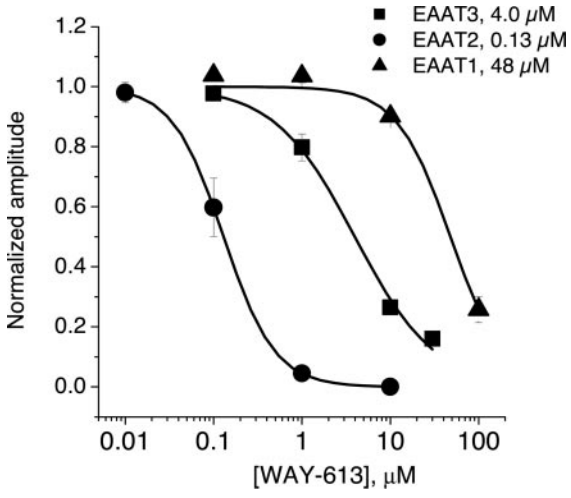

Fig. 5. Log concentration-response curves for the effect of WAY-213613 on L-glutamate–induced currents in oocytes expressing the human EAAT1 (▲), EAAT2 (●), and EAAT3 (■) transporter subtypes. L-Glutamate (10 μM)-induced currents were measured in the absence or presence of the indicated drug concentrations. Data are expressed as the percentage of control current measured in the absence of added compound and represent the mean ± S.E.M. from three independent experiments.

nized by either Ro 25-6981 or NBQX, respectively (Fig. 6, insets). In contrast, addition of 50 μM WAY-213613, WAY-213394, WAY-212922, or WAY-211686 failed to produce a depolarization in these neurons indicating that the compounds failed to activate either NMDA or non-NMDA receptors (data not shown). In addition, the compounds were evaluated for their ability to antagonize depolarizing responses to either NMDA (Fig. 6A) or AMPA (Fig. 6B) in cultured hippocampal neurons. In the case of WAY-213613, the compound exhibiting the best EAAT2 selectivity, <40% inhibition of depolarizing response to either NMDA or AMPA was observed at the highest concentration tested (50 μM), whereas no antagonist effect was observed at 5 μM. From these data, it can be concluded that WAY-213613 exhibits good selectivity over ionotropic receptors; approximately 60- and 140-fold compared with its effect on L-[^{3}H]glutamate uptake by the cloned EAAT2 subtype or L-[^{3}H]glutamate uptake in rat cortical synaptosome fractions (EAAT2 predominant), respectively. WAY-211686 exhibited the most activity toward blocking NMDA-stimulated responses with 31 and 73% inhibition observed at 5 and 50 μM, respectively. In the case of AMPA-stimulated responses, WAY-213394 exhibited 20 and 62% inhibition at 5 and 50 μM, respectively.

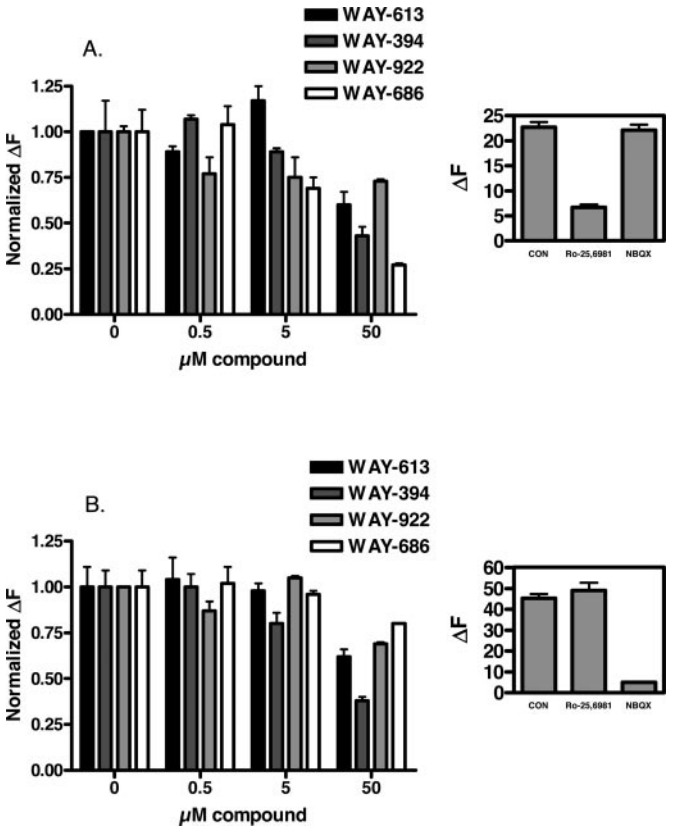

Fig. 6. Functional evaluation of WAY-213613, WAY-213394, WAY-212922, and WAY-211686 in cultured hippocampal neurons as measured by FLIPR membrane potential sensitive dye. The ability of compounds (0.5–50 μM) to antagonize depolarizing responses to either NMDA (A) or AMPA (B) was examined. Increase in fluorescence (ΔF) after NMDA or AMPA application was normalized to 1.0, and data are expressed relative to ΔF observed in the absence of added compound. Data are presented from triplicate determinations ($\pm$S.E.M.). Insets, antagonism of NMDA-mediated responses by Ro 25-6981 (A) and AMPA-mediated responses by NBQX (B).

Metabotropic Glutamate Receptor Assays. WAY-213613 was evaluated for agonist and antagonist activity at human mGlu receptors 1, 2, and 4, examples of group I, II, and III mGlu receptors, respectively. As illustrated in Fig. 7, agonist responses were elicited at each receptor subtype by appropriate ligands; 1 μM L-quisqualate for mGlu receptor 1, 0.1 μM LY354740 for mGlu receptor 2, and 1 μM L-AP4 for mGlu receptor 4. In contrast, 100 μM WAY-213613 failed to activate any of the mGlu receptors examined (Fig. 7). In addition, agonist-stimulated responses were blocked by the mGlu receptor antagonist LY341495, used at concentrations appropriate to each mGlu receptor studied, whereas 100 μM WAY-213613 failed to exhibit antagonist activity at any of the subtypes.

Synaptosomal Exchange. Electrophysiological studies in oocytes indicated that WAY-213613 behaved in a manner consistent with a nonsubstrate inhibitor, failing to activate an inward transporter-like current when applied to EAAT2-injected oocytes yet blocking the glutamate-induced currents. A biochemical index that can be used for the determination of

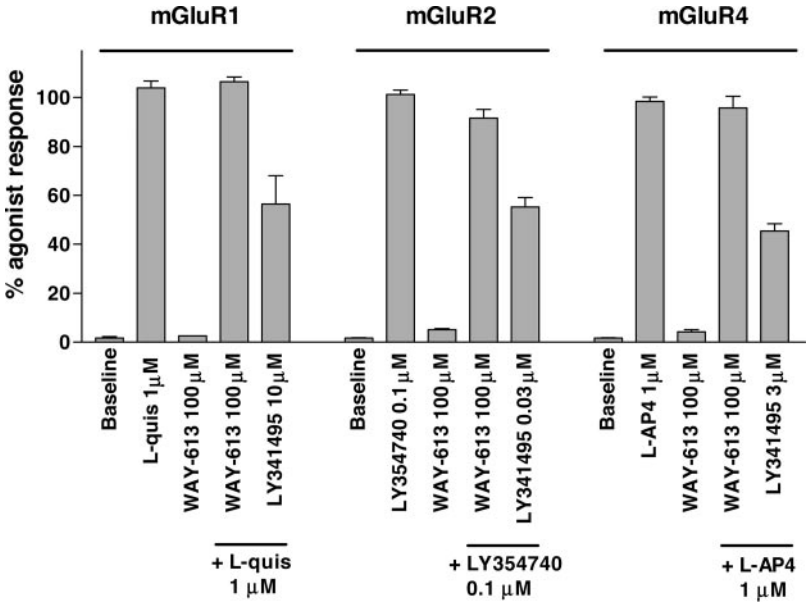

Fig. 7. Functional evaluation of WAY-213613 at representative group I, II, and III mGlu receptor subtypes as measured by the calcium indicator dye Fluo-4 AM. WAY-213613 was examined in the absence and presence of EC_{80} concentrations of the agonists L-quisqualate, LY354740, and L-AP4 at mGlu receptors 1, 2, and 4, respectively. An approximate EC_{50} concentration of LY341495 for each subtype was used as a positive control for antagonist determinations. Data are expressed as a percentage of the agonist EC_{80} response observed at each receptor. Calcium responses are presented in triplicate $\pm$ S.E.M.

substrate versus nonsubstrate activity is the capacity for drug-mediated heteroexchange of accumulated D-[^{3}H]aspartate substrate (Griffiths et al., 1994; Koch et al., 1999; Dunlop, 2001). Cortical synaptosomes were equilibrated with D-[^{3}H]aspartate before the addition of exogenous unlabeled D-aspartate or WAY-213613 to assess drug-mediated ^{3}H efflux. As illustrated, the addition of exogenous D-aspartate to D-[^{3}H]aspartate-loaded synaptosomes stimulated the efflux of the ^{3}H-label (Fig. 8); however, WAY-213613 failed to increase ^{3}H efflux over control levels, consistent with the property of a nonsubstrate inhibitor.

Molecular Modeling. A pharmacophore model containing a number of key features—hydrogen bond acceptor sites, positive ionizable (protonatable nitrogen), negative ionizable (acidic function), and two hydrophobic centers—was developed using both literature-reported EAAT inhibitors and the novel compounds presented in this study (Fig. 9). Figure 9 illustrates the fit of L-TBOA and WAY-213613 to the identified pharmacophore, indicating both overlapping and distinct conformity to the model. A major distinction between L-TBOA and WAY-213613 is the conformity of the former to all features with the exception of the hydrophobic center 4.11, whereas WAY-213613 fits all features with the exception of the hydrogen bond acceptor. We find that the newly described inhibitors presented in the current study lack a distal acidic group identified by others (Campiani et al., 2001, 2003) as an important pharmacophoric site corresponding to the hydrogen bond acceptor feature in our model. Moreover, the compounds reported in the current study share in common the distal hydrophobic center 4.11. These two distinctive features are candidates to be considered important in conferring EAAT2 selectivity of our newly identified agents. It is important to note that in the current model, the folded form of the aspartate framework in TBOA is well-superimposed with the folded form of glutamate (Fig. 9), whereas others have proposed the extended form for the aspartate framework in their model (Koch et al., 1999; Campiani et al., 2001, 2003). Each of these approaches has used different modeling scenarios, and although we have predicted the folded form of the aspartate framework, the other features of the molecule present a very similar configuration in all modeling studies. Moreover, close inspection of the models predicted by Koch et al. (1999) and Campiani et al. (2001, 2003) suggests that these do not predict exactly the same configuration of the aspartate framework. Our model provides a plausible alternative hypothesis for EAAT binding and should be considered in the rational design of new compounds.

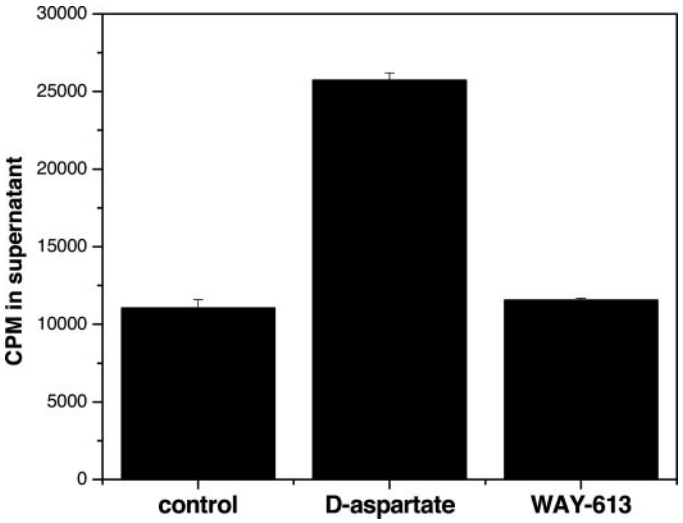

Fig. 8. Effect of WAY-213613 on synaptosomal exchange of accumulated D-[^{3}H]aspartate. Synaptosomes were pre-equilibrated with D-[^{3}H]aspartate for 1 h before dilution into buffer with or without compounds at 2× concentration for 5 min followed by centrifugation to separate pellet and supernatant. An aliquot of supernatant was removed for the determination of ^{3}H efflux, and data are expressed as cpm in the supernatant fraction representing mean values ± S.E.M. from three independent experiments.

To gain further insight into the molecular basis contributing to the pharmacological specificity toward EAAT2 inhibition, the superimposition of the compounds presented in this study with other known EAAT inhibitors, including kainate, dihydrokainate, and L-TBOA, was used to generate composite molecular volumes of the novel agents. The superimpositions of the known and novel ligands are presented (Fig. 10, A and B, respectively), indicating significant lipophilic volume occupied by the novel compounds. Access to this distal lipophilic pocket is achieved consistently with all four novel compounds represented in this study.

Discussion

High-affinity glutamate transporters termed EAATs, play a critical role in maintaining the fine balance between the physiological actions of glutamate as an excitatory neurotransmitter and preventing the excessive accumulation of extracellular glutamate responsible for excitotoxic cell death. Although this fundamental function of the EAATs has been well established, a complete elucidation of the relative contribution of individual subtypes and a better understanding of their physiological roles has been hampered by the unavailability of potent and selective pharmacological tools.

In the present study, we provide a pharmacological characterization of novel aryl-ether, biaryl, and fluorene aspartic acid and diaminopropionic acid analogs as nanomolar potent inhibitors of the high-affinity glutamate transporter EAAT2. Four compounds are described (WAY-213613, WAY-213394, WAY-212922, and WAY-211686). The aryl-ether aspartic acid analog WAY-213613 was both the most potent and EAAT2-selective of the compounds studied, exhibiting an IC_{50} value for inhibition of glutamate uptake by the cloned EAAT2 subtype of 85 nM and a 59- and 45-fold separation in potency for EAAT1 and EAAT3, respectively. In comparison, the biaryl substituted aspartic acid analog WAY-213394, although exhibiting similar EAAT2 potency (145 nM) and retaining selectivity toward EAAT3 (45-fold), exhibited reduced selectivity toward EAAT1 (14-fold). Using a fluorene ring scaffold in the aspartic acid analog WAY-212922 resulted in a compound with potent EAAT2 inhibitory activity accompanied by reduced selectivity over EAAT1 and EAAT3 of 22- and 12-fold, respectively. Finally, the biaryl-substituted diaminopropionic acid analog WAY-211686 also exhibited potent EAAT2 inhibitory activity but suffered from the poorest selectivity of the compounds examined (9- and 8-fold for EAAT1 and EAAT3, respectively).

The inhibitory potency of the compounds was also confirmed in a native preparation of rat cortical synaptosomes, a model proposed on the basis of pharmacological studies to

reflect predominantly EAAT2 activity (Dunlop et al., 1999; Tan et al., 1999). Each of the four compounds exhibited similar inhibitory potencies in the synaptosomal preparation and an identical rank order of potency, confirming their property as potent glutamate transport inhibitors and providing further evidence for the identity between EAAT2-mediated uptake and uptake by the transport system expressed in rat cortical synaptosomes. Additional studies in the synaptosomal preparation were undertaken to determine that the compounds behaved in a manner consistent with competitive inhibitors, suggesting that they overlap with the glutamate recognition site on the transporter. Given that the compounds retain the carboxylic acid moiety, it is perhaps not surprising that they would exhibit significant overlap with the glutamate-binding site.

A third index used to estimate the inhibitory activity of the most potent and selective compound WAY-213613 was to evaluate its effect on glutamate-induced currents in oocytes expressing each of the transporters. It was observed that the inhibitory potencies for blocking glutamate-induced currents in both EAAT2- and EAAT3-injected oocytes were identical with those determined in the cell line uptake studies, whereas a 10-fold weaker IC_{50} value was observed in EAAT1-injected oocytes compared with EAAT1-expressing HEK cells. It is possible that such a discrepancy arises from the indirect index of glutamate translocation provided by current measurements in oocytes reflecting both ion conductances coupled to glutamate translocation and uncoupled ion flux. Studies in oocytes also allowed for the determination that WAY-213613 and the other compounds failed to activate a transporter-like current when applied to oocytes, indicating that they were not substrates for the transporter. Such an observation was confirmed with the independent biochemical index of ^{3}H-substrate exchange with WAY-213613 failing to promote the exchange of accumulated D-[^{3}H]aspartate. Given the bulky nature of the substituent group in WAY-213613, it is likely that this impedes substrate translocation despite significant overlap with the glutamate recognition site. Taken together, the above observations indicate WAY-213613 to be a potent, EAAT2-preferring, competitive nonsubstrate inhibitor of EAAT2.

To assess the potential usefulness of WAY-213613 as a pharmacological tool to study EAAT2, it is of importance to provide an assessment of the compound at other principal sites of glutamate action, specifically the ionotropic and metabotropic glutamate receptors. Our studies demonstrate that WAY-213613 was devoid of either agonist or antagonist activity at the ionotropic NMDA and AMPA receptors expressed natively in cultured hippocampal neurons, or at the human mGlu receptors 1, 2, and 4 heterologously expressed in stable cell lines. These results confirm the specificity of WAY-213613 for the glutamate transport system.

Compared with other known glutamate transporter inhibitors, WAY-213613 would seem to be among the most potent

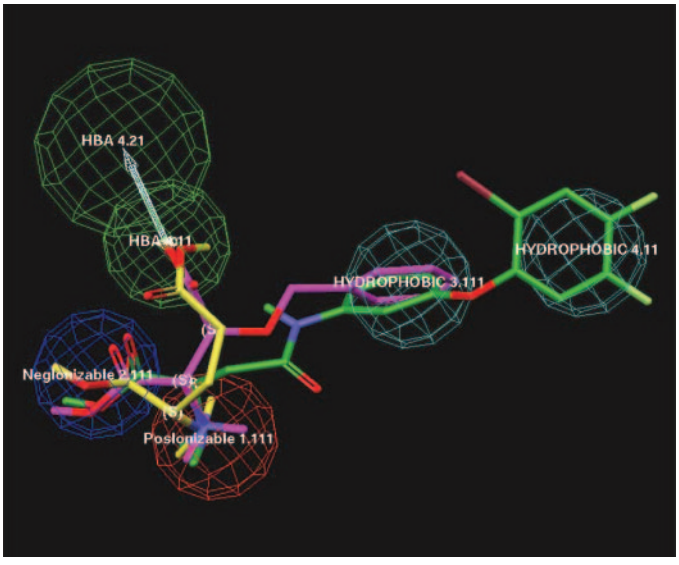

Fig. 9. Pharmacophore model constructed using literature reported and novel WAY EAAT inhibitors. Key features of the model are indicated with the carbon atoms of L-TBOA highlighted in magenta, those from WAY-213613 shown in green, and glutamate shown in yellow. HBA, hydrogen bond acceptor (note that the white arrow indicates the direction of hydrogen bonding); NegIonizable, negative ionizable; PosIonizable, positive ionizable. Numbers assigned to each of the chemical features (e.g., 4.21) arbitrarily in Catalyst.

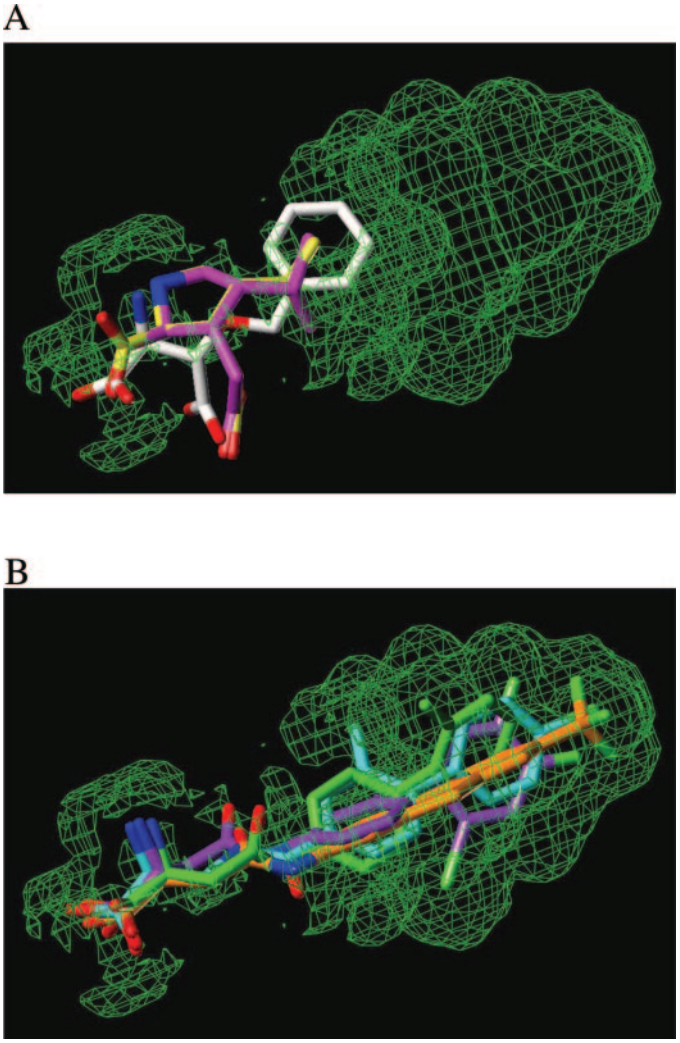

Fig. 10. Composite molecular volumes generated by overlay of known and newly identified WAY EAAT inhibitors. The nonselective EAAT inhibitors kainate (carbon atoms in yellow), dihydrokainate (magenta), and L-TBOA (white) are superimposed in A compared with the extra volume occupied only by the newly identified compounds shown in B. WAY-213613 (purple), WAY-213394 (green), WAY-212922 (orange), and WAY-211686 (cyan).

and selective of agents reported in the literature. Although kainate and dihydrokainate are known to be selective for EAAT2 compared with the other EAAT subtypes (Arriza et al., 1994), their usefulness as pharmacological tools are hampered by affinity for ionotropic and metabotropic glutamate receptors, respectively, in addition to their weak (20–50 μM) potency. Compounds such as the carboxycycloproyl glycines (Nakamura et al., 1993), aminocyclobutane dicarboxylates (Fletcher et al., 1991), pyrrolidine dicarboxylates (Bridges et al., 1991), and the *threo*-hydroxyaspartate derivative *threo*-β-benzyloxyaspartate (TBOA) (Shimamoto et al., 1998; Shigera et al., 2001) have low micromolar potency and do not discriminate between the different EAAT subtypes. More recently, we described WAY-855 as a novel EAAT2 inhibitor with an IC_{50} value of 1 μM, although this compound only exhibited approximately 10-fold selectivity over EAAT3 (Dunlop et al., 2003). Recent structure-activity relationship studies with TBOA have resulted in the discovery of (2*S*,3*S*)-3-[3-(4-cyanobenzoylamino)benzyloxy]aspartate and (2*S*,3*S*)-3-[3-(4-methoxybenzoylamino)-benzyloxy]aspartate (Shimamoto et al., 2004) as potent EAAT2 inhibitors (IC_{50} values of 12 and 36 nM, respectively). However, although these compounds exhibited 22- and 39-fold selectivity over EAAT3, their inhibitory potencies for EAAT1 were essentially the same as those determined for EAAT2. Although WAY-213613 was slightly less potent than either of the TBOA analogs, the compound was more selective over both EAAT1 and EAAT3. By generating a pharmacophore model of known EAAT inhibitors and the novel agents reported here, we have identified a number of features associated with the newly identified compounds distinct from known EAAT inhibitors. These include the absence of a distal acidic group acting as a hydrogen bond acceptor identified in other known EAAT inhibitors and the presence of a hydrophobic center conferring significant lipophilic volume to the novel agents. Although by no means definitive, these observations may provide insight into structural features for selective EAAT2 binding and provide a working model for further validation toward rational design of selective EAAT inhibitors.

In conclusion, we have identified a number of structurally novel EAAT inhibitors exemplified by WAY-213613, a potent, EAAT2-preferring and nonsubstrate inhibitor of EAAT2. These newly discovered EAAT2 inhibitors will be important tools to further study EAAT2 function in addition to continuing to refine the structural requirements for binding to different transporter subtypes.

Acknowledgments

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