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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EAAT2, excitatory amino acid transporter; FLIPR, fluorometric imaging plate reader; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; LY341495, (2S)-2-Amino-2-[(1S,2S)-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)-9H-fluoren-2-yl]-L-asparagine; WAY-211686, 3-{[(4'-chloro-2-methyl-1,1'-biphenyl-4-yl)carbonyl]amino}-L-alanine

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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 inhibition of human EAAT2 was WAY-213613 (N^4-[4-(2-bromo-4,5-difluorophenoxy)phenyl]-L-asparagine) (IC_{50}, 85 ± 5 nM) > WAY-213394 (N^4-(2'-methyl-1,1'-biphenyl-4-yl)-L-asparagine) (145 ± 22 nM) = WAY-212922 (N^4-[7-(trifluoromethyl)-9H-fluoren-2-yl]-L-asparagine) (157 ± 11 nM) = WAY-211686 (3-{[(4'-chloro-2-methyl-1,1'-biphenyl-4-yl)carbonyl]amino}-L-alanine) (190 ± 10 nM). WAY-213613 was the most selective of the compounds examined with IC_{50}s 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 non-substrates 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.

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Introduction

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, while paradoxically over-stimulation of the ionotropic receptors contributes to the excitotoxic actions attributed to glutamate. Consequently, synaptic glutamate levels must be tightly regulated in order 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 neurones and astroglia is responsible for the clearance of extracellular glutamate by mediating the cellular uptake of glutamate in a process driven largely by the energy of the transmembrane Na^+ gradient (see review by Danbolt, 1999). 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

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deletion of the EAAT2 transporter lose greater 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 utilized pharmacological tools such as the pyrollidine 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 TBOA (*threo*-β-benzyloxyaspartate) and the novel heptane dicarboxylate WAY-855 (3-amino-tricyclo[2.2.1.0$^{2.6}$]heptane-1,3-dicarboxylic acid) 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 lead to the identification of nanomolar potent EAAT2 inhibitors with the most selective agent CNB-TBOA ((2S,3S)-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.

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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. Based on our hypothetical binding site interaction model, generated based on 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 (N^4-[4-(2-bromo-4,5-difluorophenoxy)phenyl]-L-asparagine) 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.

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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 prior to 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-[³H]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 addition of Microscint 20 for the

determination of [³H] accumulation in the wells using a Packard TopCount. Uptake was

linear for incubation times up to 30 min (data not shown), thus data were analyzed as true

rates. Non-specific 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 ≤ 10% of total uptake and was subtracted prior to 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, 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

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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 HBS containing 50 µg 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 cold 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 Packard 96 well Unifilter (Whatman GF/B) 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 Packard TopCount. 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* oocytes (10ng/oocyte), and the membrane currents were recorded 3-7 days later. Recording solution ND96 contained 96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1mM $MgCl_2$, 5 mM HEPES, pH 7.4. Two electrode voltage clamp recordings were performed at room temperature with a NPI TURBO TEC-03X amplifier, EPC-9 and PULSE program for A/D interface and data acquisition. Microelectrodes were filled with 3 M KCl solution and had resistance around 1 mega Ohm. Cells were clamped at –40 mV, and depolarized to –80 mV for 100 msec where 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.

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The current was low-pass filtered at 200 Hz, and digitized at 5 kHz. Compounds were dissolved in DMSO as stock, and diluted to final concentration with less than 1% DMSO in the bath. Data were averaged from all oocytes tested and are expressed as mean ± 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 fluoresecent dye (FLIPR Membrane Potential Dye, FMP dye, Molecular Devices, Sunnyvale, CA). Minced hippocampi dissected from E18 embryos were incubated at 37°C in 0.01% papain (Worthington, Freehold, NJ), 0.1% dispase (Roche Products, Hertforshire, UK) and 0.01% DNase (Sigma) dissolved in Hank's Balanced Salt Solution (HBSS). Individual neurons were plated in 96-well format at a density of 100,000 cells/well. Neurons were cultured at 37°C, 5% CO_2 in Neurobasal Media (GIBCO Media #21103-049) supplemented with 2 mM glutamine and 1x B27 supplement (GIBCO # 17504-044). Assays were performed 2 weeks after plating. After removing the culture media, 200 µl FMP dye was added to each well and plates were incubated at room temperature for 0.5-1 hr. Agonist activity was evaluated by direct application of the compounds to the plate, following dye loading, after monitoring 15 s baseline fluorescence on-line. For antagonist assays compounds were included during the dye loading step with subsequent stimulation by addition of 50µl depolarizing solution containing 100 µM NMDA or AMPA added to each well during FLEX station runs after 15 s reading of base line. As positive controls, 100 µM NMDA or AMPA was added to

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confirm the agonist activity and 30 µM Ro-25,6981 or NBQX used to block NMDA- and AMPA-mediated responses, respectively.

Metabotropic Glutamate Receptor Selectivity Studies. Stable CHO cell lines expressing either the human mGlu receptors 1, 2 or 4 subtypes were used for functional studies employing the measurement of agonist-stimulated mobilization of intracellular calcium with the fluorometric imaging plate reader (FLIPR). For the Gαi-coupled mGlu receptors 2 and 4 functional calcium coupling was facilitated by stable co-expression 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 hours prior to the experiment in poly-D-lysine coated 96 well plates at a density of approximately 50,000 cells per well. In preparation of the assay, the confluent monolayer of cells was washed twice with HBSS supplemented with 20 mM Hepes and 2.5 mM probenecid (FLIPR buffer), then the cells were loaded by adding 4 µM Fluo-4 AM (Molecular Probes, Eugene, OR) in FLIPR buffer for 1 hour at 37°C. Following loading, the cells were then rinsed twice with FLIPR buffer and intracellular calcium increases following agonist application were detected by measuring increases in fluorescence with the FLIPR. For evaluation of antagonist activity LY341495 ((2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid) was included during the dye-loading step and subsequently stimulated by the addition of an approximate EC_{80} concentration of the agonists L-quisqualate, LY354740 ((+)-2-aminobicyclo[3.1.0.]hexane-2,6,-dicarboxylic acid) and L-AP4 (L-(+)-2-amino-4-phosphonobutyric acid) 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/mmole; 1 mCi/ml) for 1 hr at room temperature with constant shaking. 100 µl synaptosome suspension equilibrated with [^{3}H] label was diluted into 100 µl HBS containing compound at 100 µM final concentration immediately after the 1 hr 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. Consequently, substrate inhibitors, themselves translocated by the transport system, will favor [^{3}H] efflux via heteroexchange, while non-transportable inhibitors will either 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 dataset was assembled using literature reported EAAT inhibitors and the novel compounds disclosed in the present study (Figure 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 non-transportable, competitive inhibitors of high-affinity glutamate uptake. A 'common feature hypothesis' in Catalyst 4.6 (Acclerys, San Diego) was used to develop the pharmacophore model. The Catalyst pharmacophore model contains the location

constraints (3-dimensional coordinates and their tolerance) of commonly used chemical

features such as hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), negative

ionizable (NI) groups such as carboxylic acid and positive ionizable (PI) groups such as

amino groups and ring aromatics. Prior to 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 4

parameter logistic curve fitting model using OriginLabs Origin 7.5 (OriginLabs,

Northampton , MA) using the following equation $y=((A1-A2)/1+(x/x0)^p)+A2$ where A1

is the maximum, A2 is the minimum, x0 is the IC_{50} and p is the Hill slope coefficient.

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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 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 (Figure 2, Table 1). WAY-213613 was the most potent of the compounds with an IC$_{50}$ 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 Figure 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 non-selective and widely utilized pharmacological agent L-*trans*-pyrollidine-2,4-dicarboxylate (*trans*-2,4-PDC). Concentration-response curves are illustrated in Figure 3. Glutamate uptake in the synaptosomal preparation in the absence of added ccompound (control) was 2-2.5 nmol/min/mg. Estimated IC$_{50}$ values for inhibition of synaptosomal L-[^{3}H]glutamate uptake (Table 1) and rank order of potency were essentially identical to those determined for inhibition of EAAT2 with WAY-213613 being the most potent (IC$_{50}$, 35 ± 7 nM) of

the compounds evaluated, while the potency of *trans*-PDC was identical to 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-Burke transformation) of the effect of multiple concentrations of WAY-

213613 on the saturable uptake of L-[^{3}H]glutamate is presented in Figure 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 IC_{50} determination above. Similar

experiments performed with WAY-213394, WAY-212922 and WAY-211686 also

indicated a competitive mode of inhibition for these compounds (data not shown).

Oocyte Studies. Based on 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 non-substrate (data not shown). Inward currents evoked by application

of glutamate to EAAT2 injected oocytes were blocked in a concentration-dependent

manner by WAY-213613 (Figure 5) with an estimated IC_{50} value of 130 nM. WAY-

213613 produced similar concentration-dependent block of glutamate-induced currents in

either EAAT1 or EAAT3 injected oocytes (Figure 5) with IC_{50} values of 48 and 4 µM,

respectively, reflecting 370- and 31-fold selectivity toward EAAT2.

Ionotropic Glutamate Receptor Assays. Using a membrane potential sensitive

fluorescent indicator dye we established that 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

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hippocampal neurons produced an increase in dye fluorescence indicative of

depolarization. Under these experimental conditions AMPA stimulation can result in

activation of both AMPA (directly) and kainate (by indirect release of glutamate)

receptors. The depolarizing responses to NMDA or AMPA could be selectivity

antagonized by either Ro-25,6981 or NBQX, respectively (Figure 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 (Figure 6A) or AMPA (Figure 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), while no antagonist effect was observed at 5 µM. Based on

these data, it can be concluded that WAY-213613 exhibits good selectivity over

ionotropic receptors; approximately 60- and 140-fold when compared to its effect on L-

[^{3}H]glutamate uptake by the cloned EAAT2 subtype or D-[^{3}H]aspartate 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, resepectively.

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 Figure 7, agonist responses were

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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

(Figure 7). In addition, agonist-stimulated responses were blocked by the mGlu receptor

antagonist LY341495, used at concentrations appropriate to each mGlu receptor studied,

while 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 non-substrate inhibitor, failing to activate

an inward transporter-like current when applied to EAAT2 injected oocytes yet blocking

the glutamate induced currents. A biochemical index which can be used for the

determination of substrate versus non-substrate activity is the capacity for drug-mediated

heteroexchange of accumulated D-[^{3}H]aspartate substrate (Dunlop, 2001; Griffiths et al.,

1994; Koch et al., 1999). Cortical synaptosomes were equilibrated with D-[^{3}H]aspartate

prior to the addition of exogenous unlabeled D-aspartate or WAY-213613 to assess drug-

mediated [^{3}H] efflux. As illustrated, addition of exogenous D-aspartate to D-[^{3}H]aspartate

loaded synaptosomes stimulated the efflux of [^{3}H] label (Fig. 8), however WAY-213613

failed to increase [^{3}H] efflux over control levels consistent with the property of a non-

substrate 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

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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. Interestingly, 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 as 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 (Figure 9), while 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 utilized 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. and Campiani et al. suggest 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.

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-

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TBOA was used to generate composite molecular volumes of the novel agents. The

superimpositions of the known and novel ligands are presented (Figure 10A and 10B,

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.

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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 while preventing the excessive accumulation of extracellular glutamate responsible for excitotoxic cell death. While this fundamental function of the EAATs has been well established a complete elucidation of the relative contribution of individual subtypes and 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 (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)-9H-fluoren-2-yl]-L-asparagine) and WAY-211686 (3-{[(4'-chloro-2-methyl-1,1'-biphenyl-4-yl)carbonyl]amino}-L-alanine). The aryl ether aspartic acid analog WAY-213613 was both the most potent and EAAT2 selective of the compounds studied exhibiting an IC_{50} 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. By 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). Utilizing 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

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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 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 to those determined in the cell line uptake studies, while a 10-fold weaker IC_{50} was observed in EAAT1 injected oocytes compared to EAAT1 expressing HEK cells. It is possible that such a discrepancy arises from the indirect index of glutamate

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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 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 exchange of accumulated D-[^{3}H]aspartate. Given the bulky nature of the

substituent group in WAY-213613 it is likely 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 non-

substrate inhibitor of EAAT2.

In order to assess the potential utility 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.

In comparison with other known glutamate transporter inhibitors WAY-213613 would

appear to be among the most potent 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 utility as pharmacological tools

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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) pyrollidine 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 PMB-TBOA

and CNB-TBOA (Shimamoto et al., 2004) as potent EAAT2 inhibitors (IC_{50} values of 12

and 36 nM, respectively). However, while 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 these agents 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

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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 non-substrate 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.

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Footnotes

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Legends for figures

Figure 1. Chemical structures of 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)-9H-fluoren-2-yl]-L-asparagine) and WAY-211686 (3-{[(4'-chloro-2-methyl-1,1'-biphenyl-4-yl)carbonyl]amino}-L-alanine), kainate, dihydrokainate and L-TBOA (L-threo-b-benzyloxyaspartate).

Figure 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 (closed triangles), EAAT2 (closed circles) and EAAT3 (closed squares) transporter subtypes. L-[^{3}H]glutamate uptake was measured in the absence or presence of the indicated drug concentrations. Data are expressed as percent control uptake measured in the absence of added compound and represent the mean $\pm$ SEM from 3 independent experiments.

Figure 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 percent control uptake measured in the absence of added compound and represent the mean $\pm$ SEM from 3 independent experiments.

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Figure 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 (closed squares) and presence of 3 (open circles), 30 (open squares) or 300 (closed circles) 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.

Figure 5. Log-concentration response curves for the effect of WAY-213613 on L-glutamate-induced currents in oocytes expressing the human EAAT1 (closed triangles), EAAT2 (closed circles) and EAAT3 (closed squares) transporter subtypes. 10 µM L-glutamate-induced currents were measured in the absence or presence of the indicated drug concentrations. Data are expressed as percent control current measured in the absence of added compound and represent the mean $\pm$ SEM from 3 independent experiments.

Figure 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) following 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 ($\pm$ SEM). Insets; antagonism of NMDA-

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mediated responses by Ro-25,6981 (A, inset) and AMPA-mediated responses by NBQX

(B, inset).

Figure 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-quis, LY354740 and L-AP4 at mGlu receptors 1,2 and 4, respectively. An approximate

EC_{50} concentration of LY341495 for each subtype was utilized 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 $\pm$ SEM.

Figure 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 hr prior to

dilution into buffer with or without compounds at 2x 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 $\pm$ SEM from 3 independent experiments.

Figure 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

in yellow. HBA, hydrogen bond acceptor (note white arrow indicates 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.

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Figure 10. Composite molecular volumes generated by overlay of known and newly identified WAY EAAT inhibitors. The non-selective 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).

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Table 1.

Estimated IC_{50} values for the inhibition of L-[^{3}H]glutamate uptake by human EAAT

subtypes and D-[^{3}H]aspartate uptake into rat cortical synaptosome fractions. Data are the

mean $\pm$ SEM from 3 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

WAY-213613

WAY-213394

WAY-212922

WAY-211686

Kainic acid

Dihydrokainic acid

L-TBOA
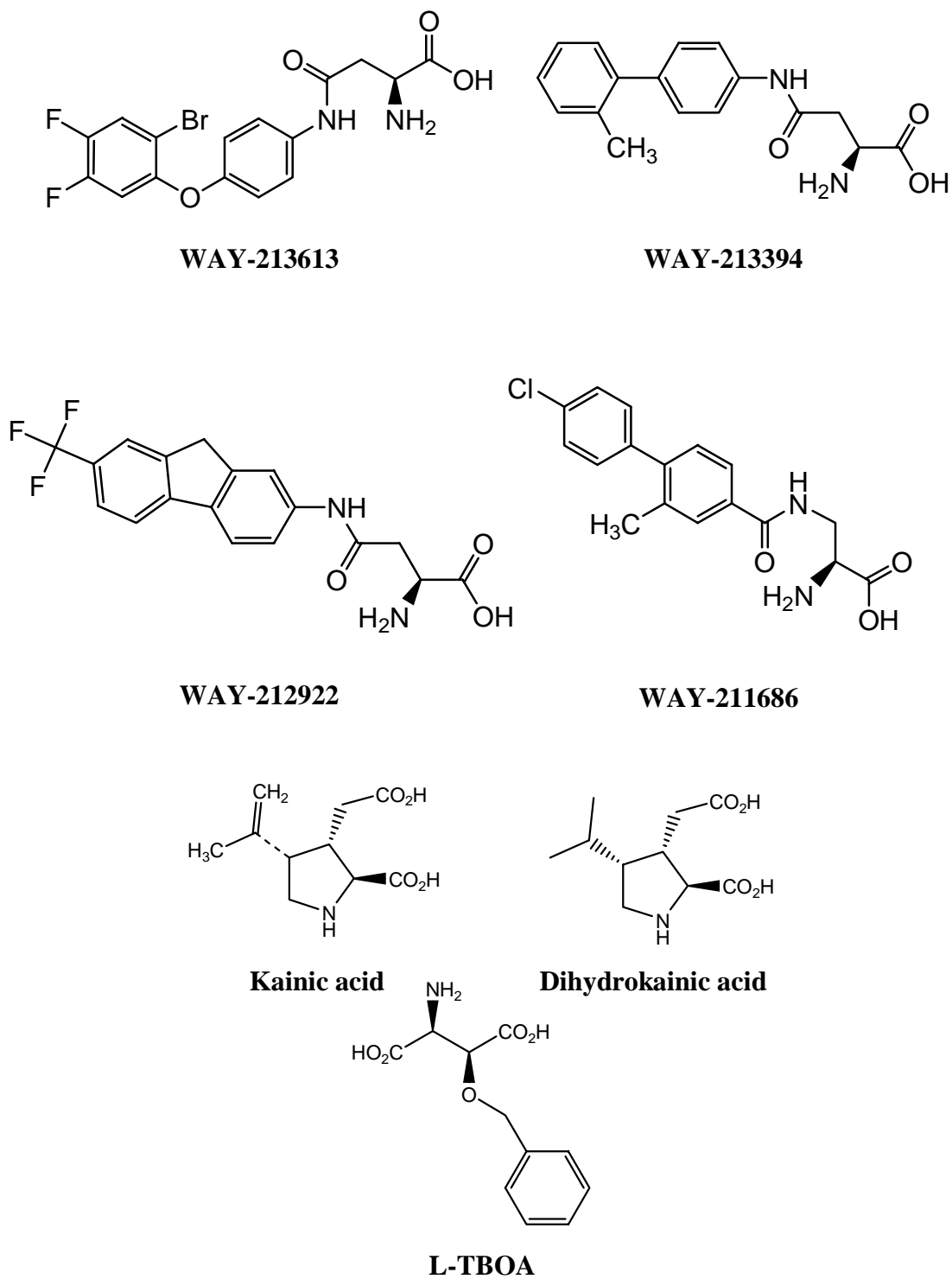
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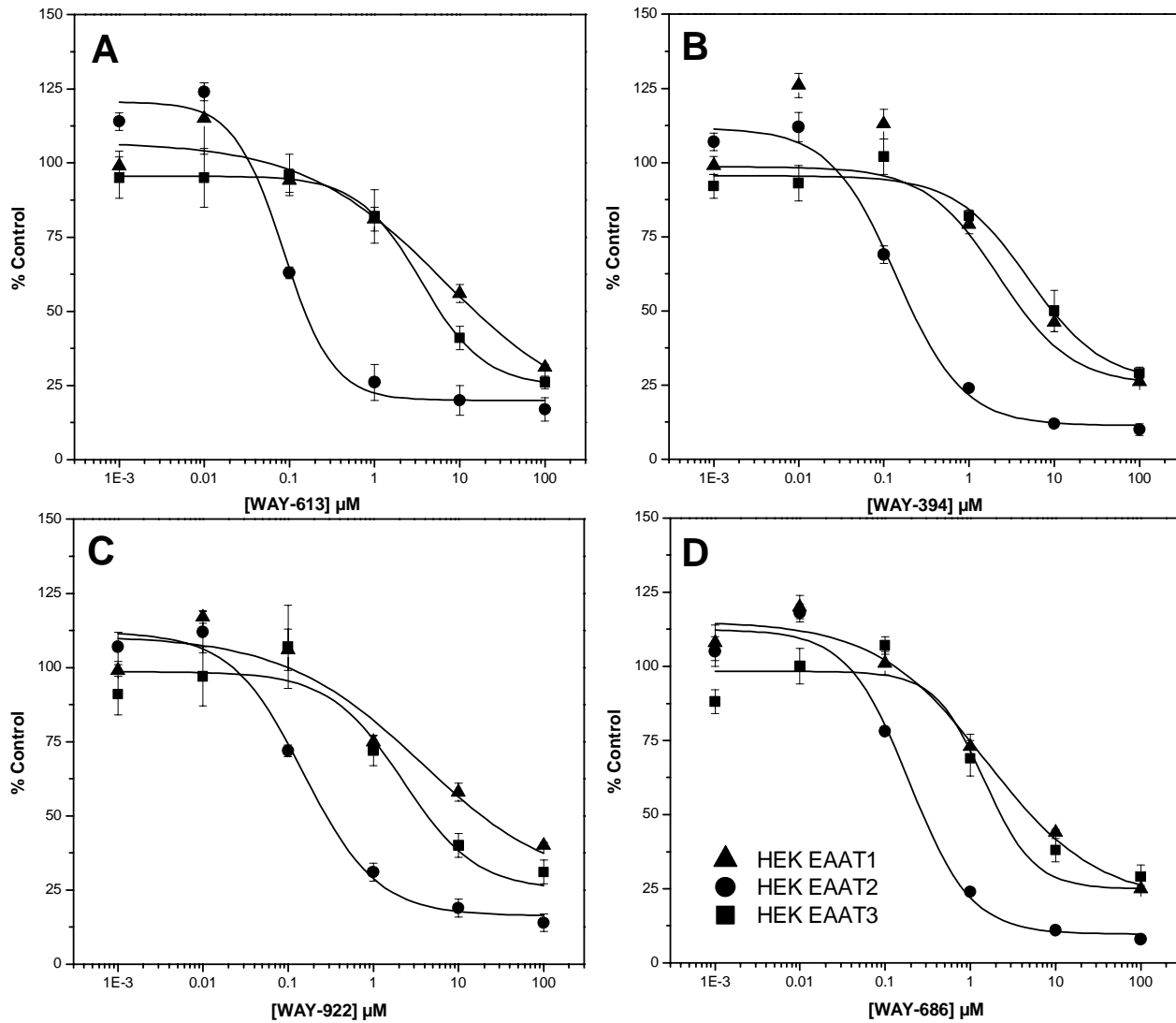
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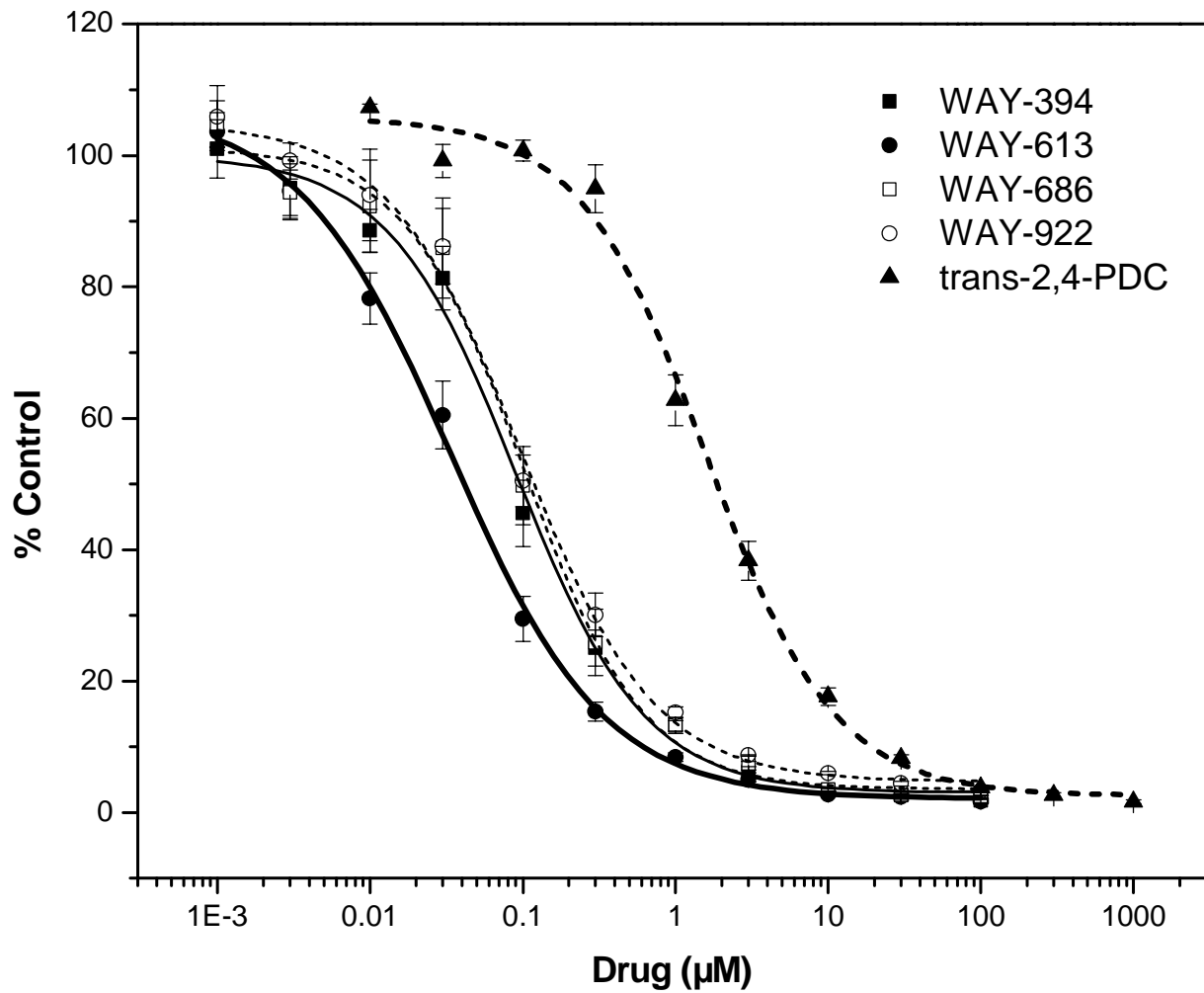
Figure 3
MOLPHARM/2005/012005

WAY-613

Legend:
- ● 300 nM
- □ 30 nM
- ○ 3 nM
- ■ control

Y-axis: **1/Velocity**

X-axis: **1/[Glutamate] µM**
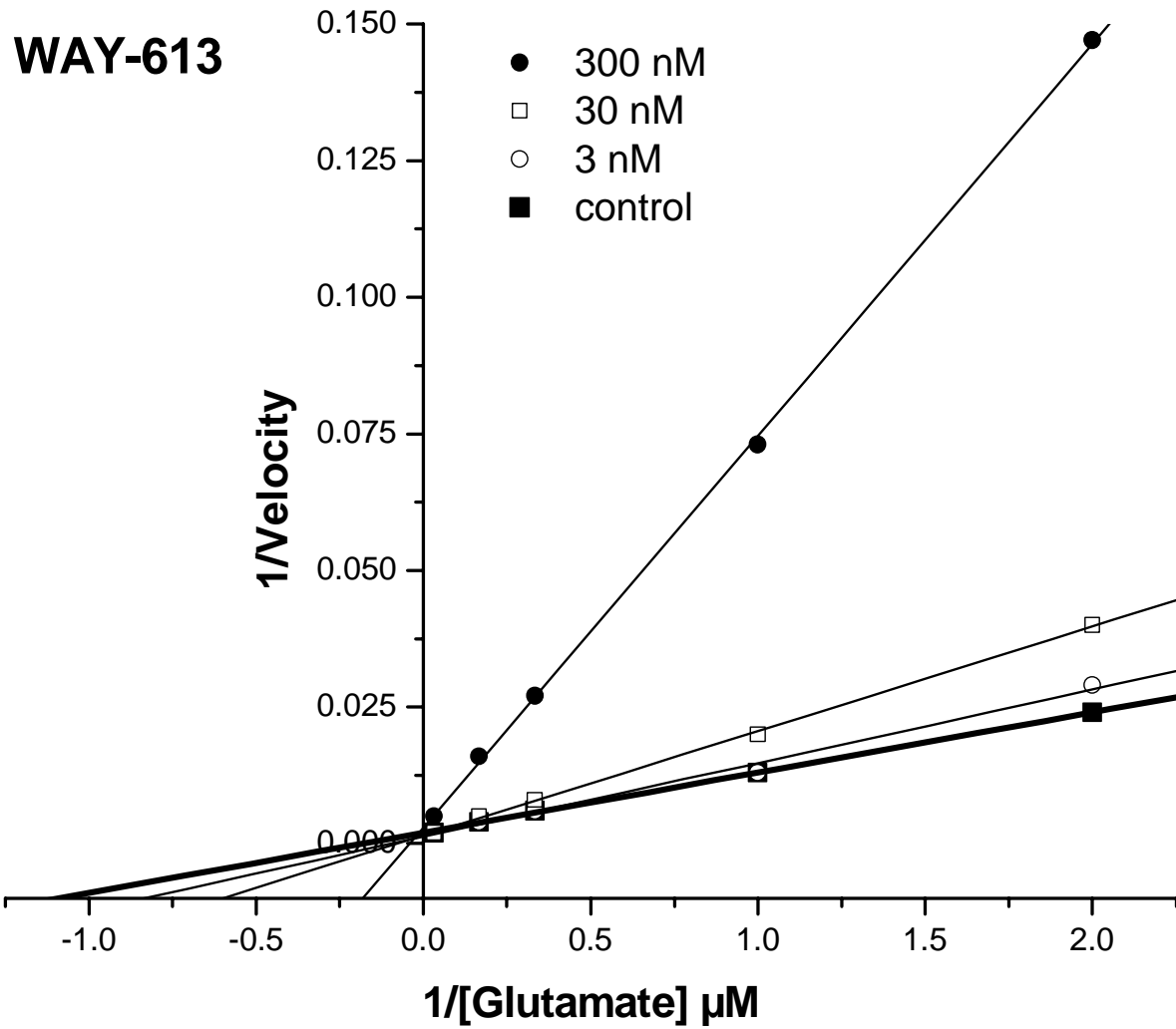
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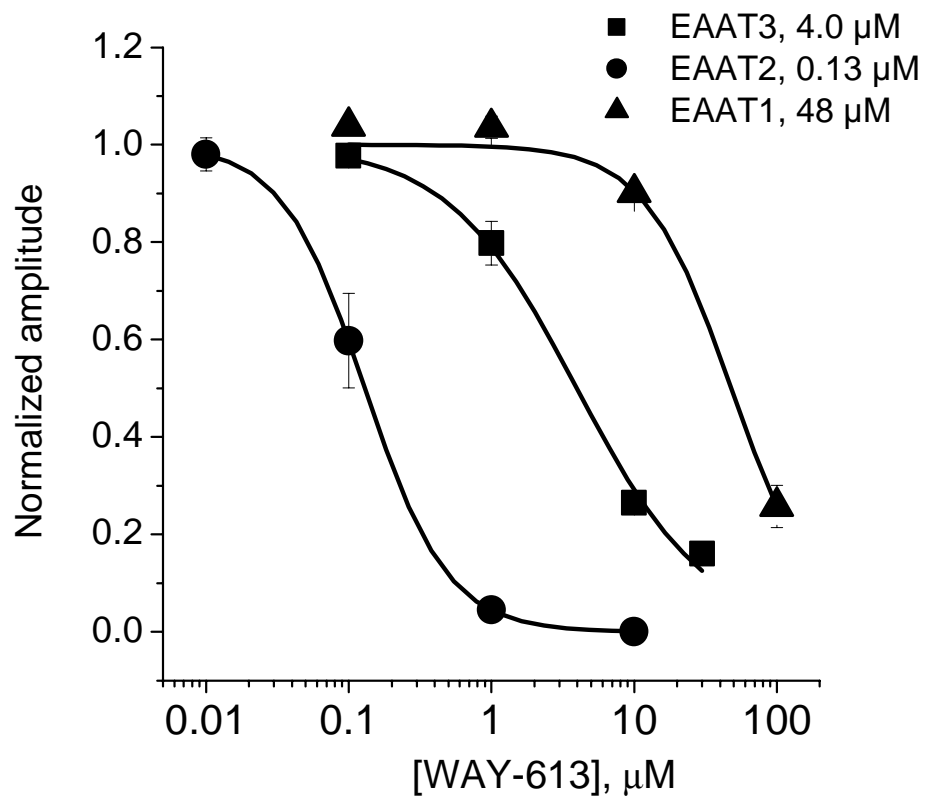
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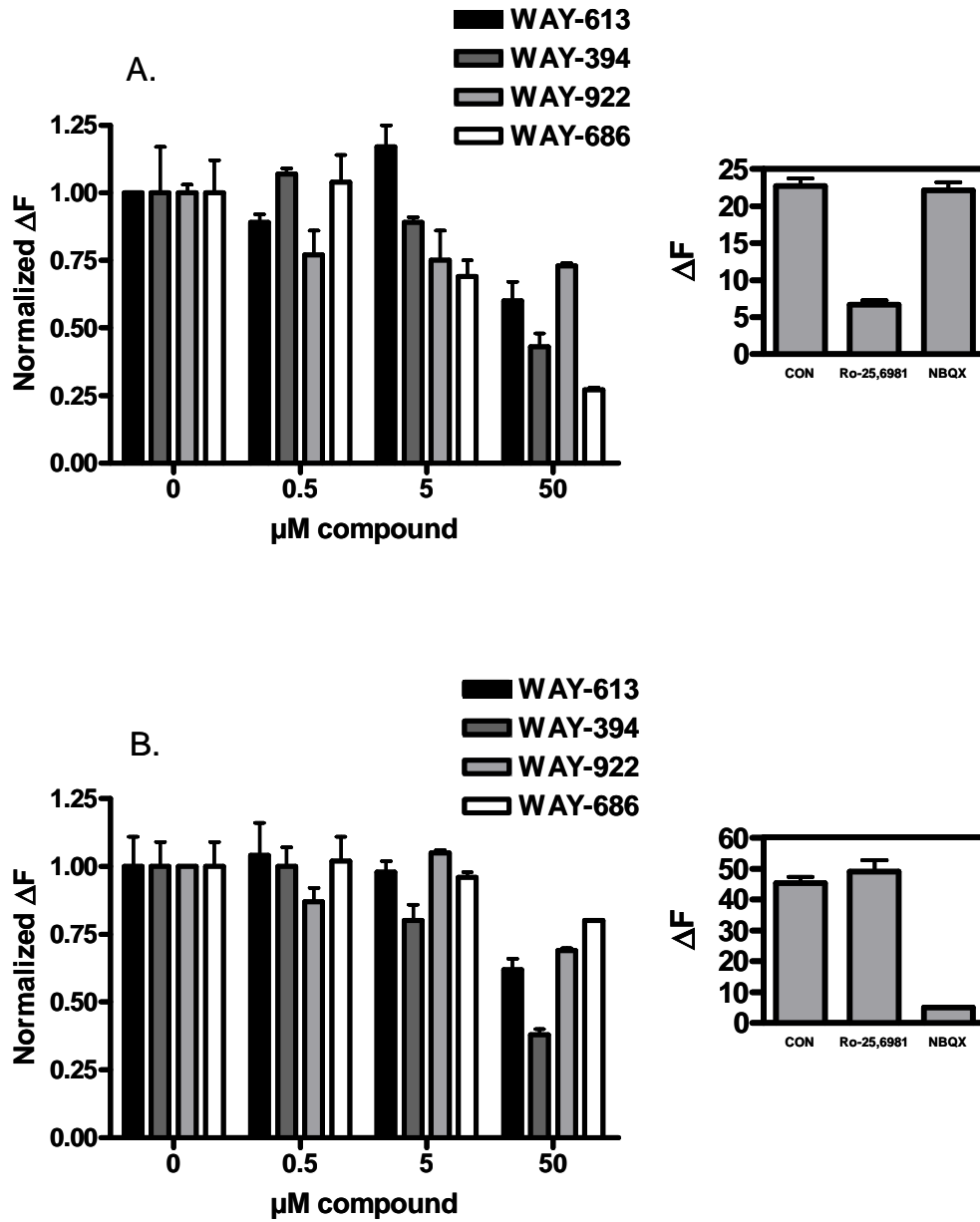
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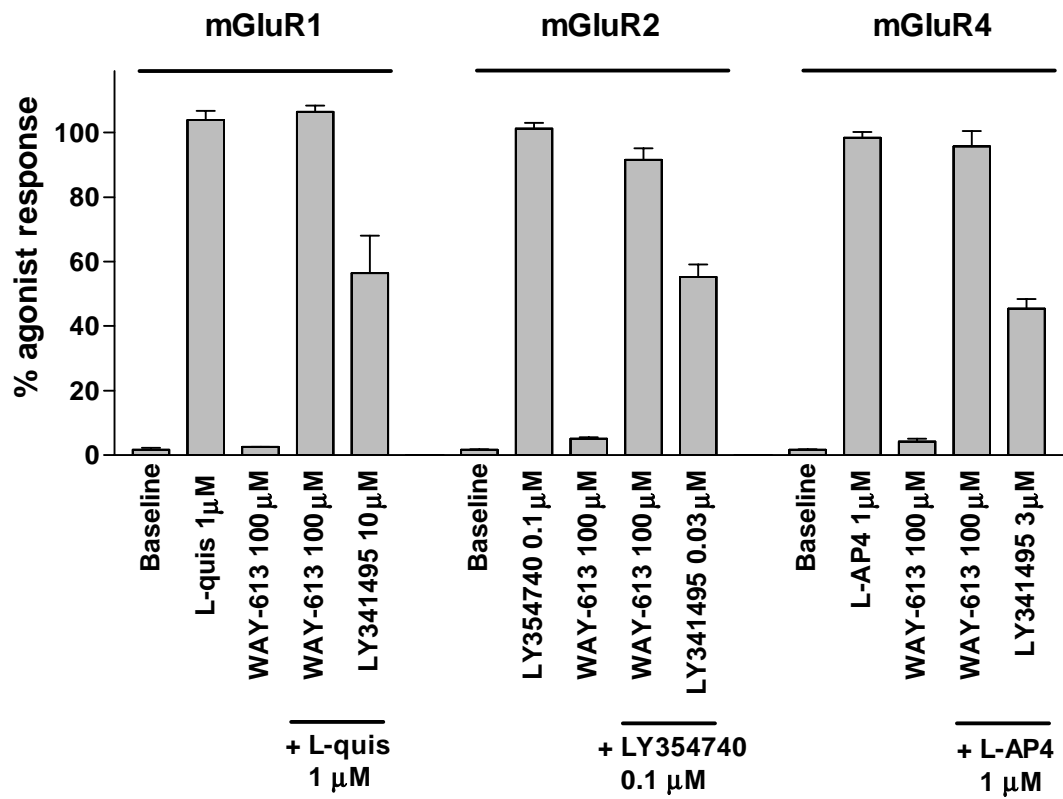
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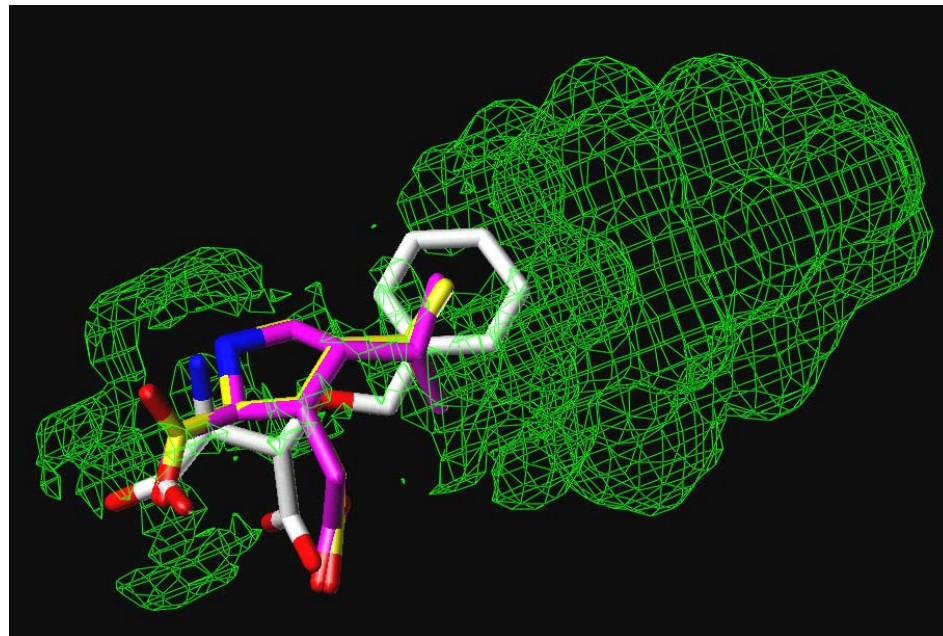**MOLPHARM/2005/012005**
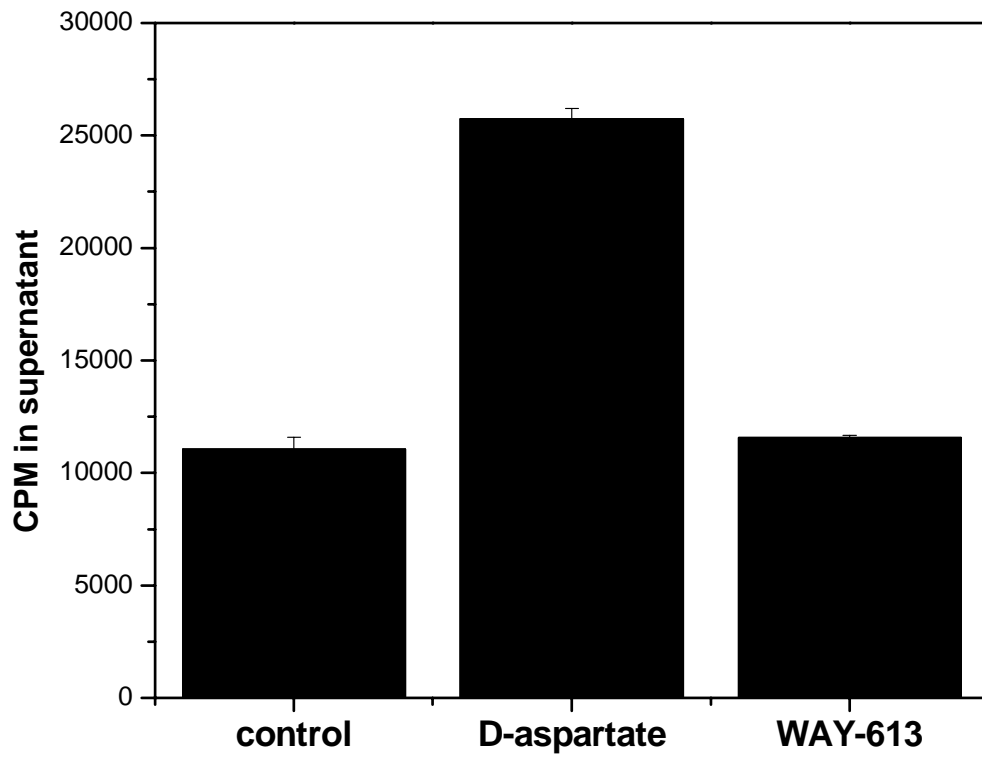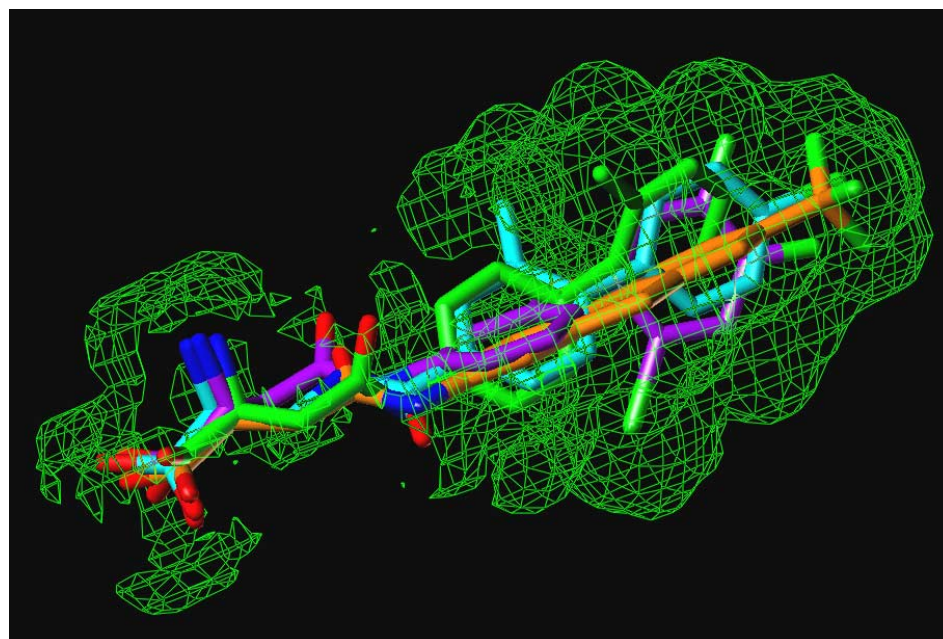
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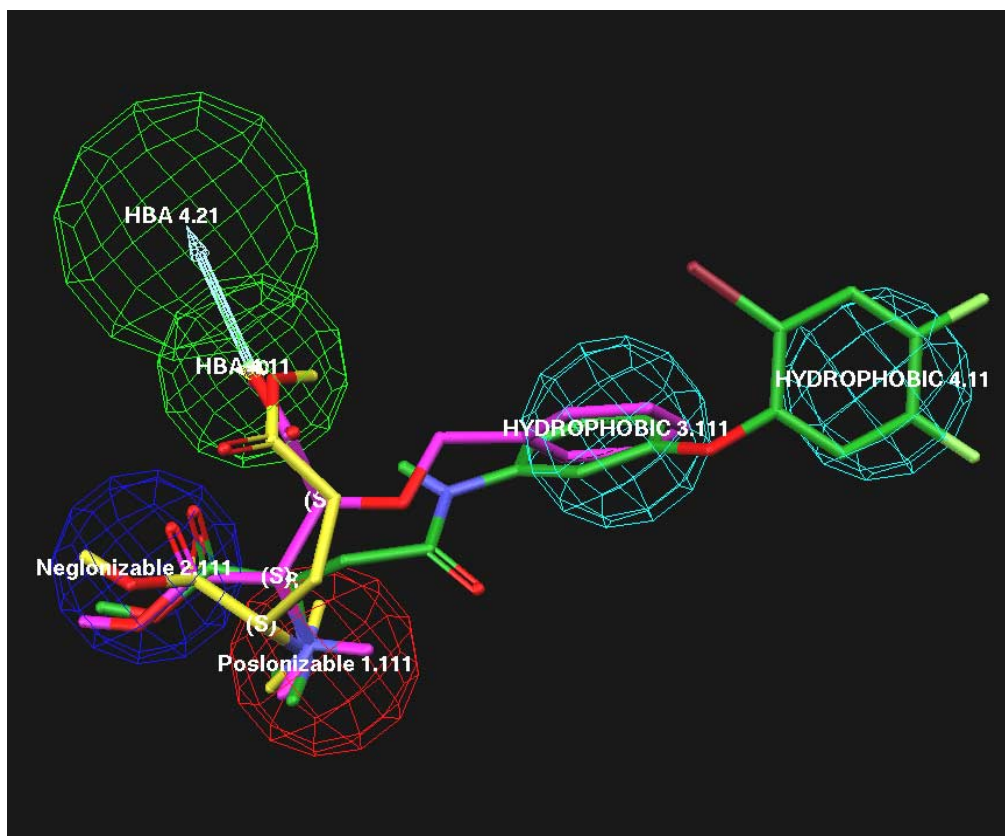
Figure 9
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A

B

Figure 10
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