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Discovery of novel small-molecule antagonists for GluK2

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ABSTRACT

KA receptors have shown to be potential therapeutic targets in CNS diseases such as schizophrenia, depression, neuropathic pain and epilepsy. Through the use of our docking tool Fitted, we investigated the relationship between ligand activity towards GluK2 and the conformational state induced at the receptor level. By focusing our rational design on the interaction between the ligand and a tyrosine residue in the binding site, we synthesized a series of molecules based on a glutamate scaffold, and carried out electrophysiological recordings. The observed ability of some of these molecules to inhibit receptor activation shows the potential of our design for the development of effective antagonists with a molecular size comparable to that of the endogenous neurotransmitter L-glutamate.

Kainate-selective ionotropic glutamate receptors (KA-type iGluRs) have received substantial attention as potential drug targets due to their modulatory role in several neuropathological disease states.[1] For example, KARs are involved in frontal lobe epilepsy, neuropathic pain, neurodegeneration, and migraines.[2] Despite showing promise in animal models of these conditions, no KA receptor antagonist has yet been approved for therapeutic intervention in humans.[3] This failure is partially attributable to the difficult development of KA receptor subunit-specific antagonists,[4] however, a more general obstacle is a limited knowledge concerning how iGluR structure relates to function.[5] In particular, it is difficult to predict how compounds rationally designed to target KA receptors will modulate channel activity.

An early structural model describing iGluR activation proposed that the degree of closure of the extracellular agonist binding cleft around agonist molecules correlated with increased agonist efficacy.[6] In this sense, the dimeric agonist binding domain (ABD) can be thought of as a clamshell, comprised of an upper lobe (D1) and lower lobe (D2) that both form the agonist cleft (Fig. 1). When an agonist binds to the ABD, it is thought that the D2 lobes are pulled upward, closing the agonist binding cleft and generating tension on residues linking the ABD to the transmembrane pore. This tension has been shown to correlate with channel opening, whereby cations pass through the cell membrane to generate a physiological response.[7] For KA receptors, channel opening is very transient, occurring for just milliseconds before the agonist-bound receptor enters a desensitized state characterized by long duration channel closures.[8,9] Alternatively, deactivation can produce

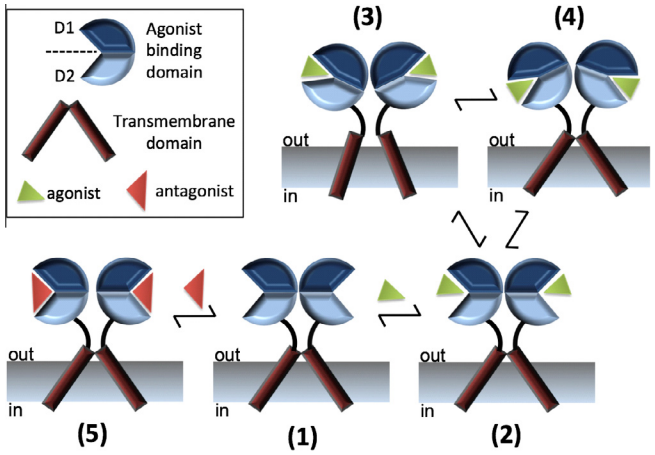

Figure 1. In its resting state (1), the receptor ion channel is closed and the ABD is ready to accept ligands. Binding of agonist molecules (2) favors closure of the ABD, which is correlated with increased frequency of channel opening (3). Receptor activation is in equilibrium with desensitization, where the ABD adopts a different conformation that inhibits opening of the ion channel (4). When binding, an antagonist interferes with closure of the ABD and consequently prevents activation of the receptor (5).

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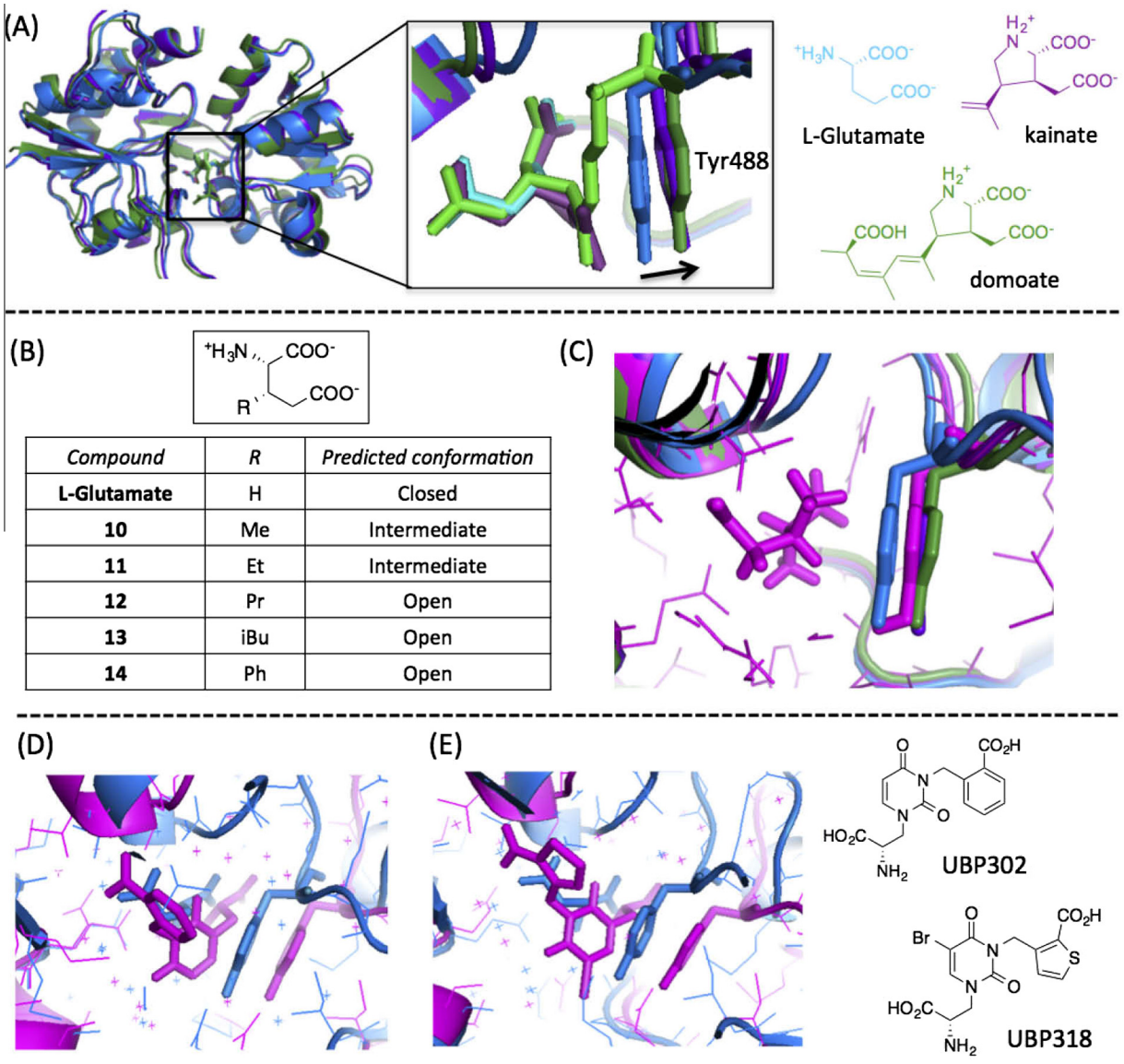
channel closure when agonists unbind from receptor, leading the protein to revert to an unliganded apo state.

Over the last decade, the two most studied KA subunits, GluK1 and GluK2, have been crystallized with many agonists and antagonists. This has permitted extensive investigation of the connections between structural changes and pharmacological responses induced by various ligands.

We previously studied the relationship between closure of the agonist binding cleft and agonist efficacy in GluK2 type KA receptors, by relying on our computational tool Fitted.[10] This software, which is part of our platform Forecaster, can dock ligands to flexible proteins and can therefore predict not only the binding mode of the ligand but also the most favored conformation of the protein.[11,12] In particular, a number of known conformations are given as input files, and the software ranks the likelihood of those conformations induced by a given ligand. In our previous work we identified a series of structurally related amino acids that exhibit the entire range of agonist behavior at GluK2. Then, we applied Fitted to predict the most likely conformation adopted by those ligands. Starting from three input conformations, induced by L-glutamate, kainate and domoate, all new ligands tested were predicted to bind to the same conformation induced by the natural agonist L-glutamate. This particular conformation had been identified as a 'closed' state, as opposed to the 'intermediate' and 'open' state induced by partial agonists kainate and domoate. This observation supported the hypothesis that agonist efficacy might have other structural bases apart from the degree of cleft closure. Instead, it was argued that agonist efficacy was related to the stability of the closed conformation, in other words to the number of favorable interactions in that particular conformational state.

In the same study we could correlate the degree of opening of GluK2 agonist binding cleft to the translational movement of a



Compound	R	Predicted conformation
L-Glutamate	H	Closed
10	Me	Intermediate
11	Et	Intermediate
12	Pr	Open
13	iBu	Open
14	Ph	Open

Figure 2. (A) Superimposition of crystal structures of GluK2 ABD bound to glutamate (PDB 1S7Y), kainate (PDB 1TT1) and domoate (PDB 1YAE). The zoom shows the translational movement of Tyr488 in going from a closed state (blue), to intermediate (purple) and open state (green) of GluK2 ABD. Respective ligands are indicated in lighter colors and their structures shown on the right. (B) Series of compounds designed, synthesized and tested. All compounds are predicted by Fitted to induce either an intermediate or an open state of GluK2 ABD. (C) In magenta, predicted conformation induced by **10**, compared to the closed, intermediate and open conformations described in panel A (same color scheme). (D) Superimposition of crystal structures of GluK1 ABD bound to glutamate (in blue, PDB 1TXF) and to antagonist UBP302 (in magenta, PDB 2F35); (E) Superimposition of crystal structures of GluK1 ABD bound to glutamate (in blue) and to antagonist UBP318 (in magenta, PDB 2QS2); On the right, chemical structures of UBP302 and UBP318.

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Tyrosine residue (Tyr488) in the orthosteric site.[10] (Fig. 2A). The role of this residue has been also emphasized by Nayeem et al.[13] We hypothesized that the clash between this residue and a bulky group on the nearby ligand could be responsible for hindering full closure of the ABD.

In the study described herein, we wanted to investigate further the effect of ligands that are specifically designed to constrain GluK2 ABD in an intermediate or open state (similar to each other but significantly different from the closed state) through a predicted interaction with the Tyr488. A combination of docking, synthesis and electrophysiological recordings allowed us to collect interesting data for the future design of drugs targeting KA receptors.

In line with traditional structure-based drug design, we started from the visual inspection of the available crystal structures of GluK2. When GluK2 ABD is bound to L-glutamate, it appears that a substituent at the β position of the amino acid would point directly towards Tyr488 (Fig. 2E). To probe this hypothesis, and at the same time minimize the synthetic challenge, we considered the introduction of alkyl groups only. We then relied on our software FITTED to predict which conformational state was favored upon binding of each new potential ligand. Interestingly, the stereochemistry at the β carbon appeared to play an important role in the outcome of the docking. In was found, in fact, that all (3*S*)-β-substituted-glutamates, regardless of the size of the β-substituent, were predicted to induce an intermediate or open state. On the contrary, (3*R*)-analogues showed a less consistent pattern, with some poses being more similar to the closed state. Following our initial interest, only compounds with a (3*S*) stereochemistry were carried forward. Among them, five were successfully synthesized (Fig. 3).

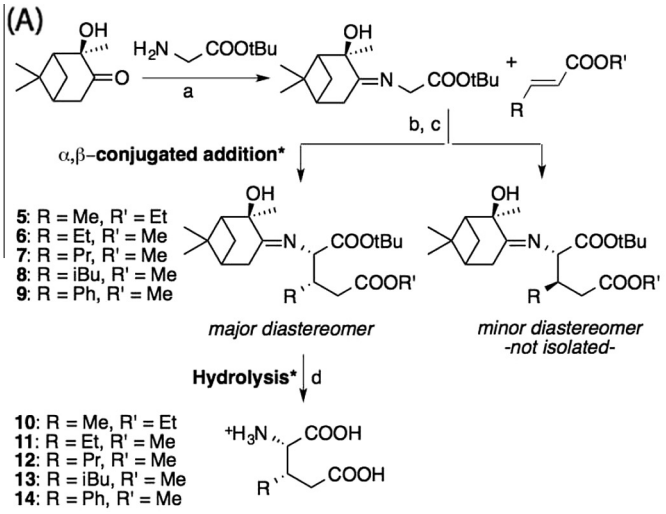
For the synthesis we relied on a procedure that was developed by Wehbe et al. to test β-methylglutamates as EAAT (excitatory amino acid transporters) blockers.[14] In this synthetic methodology the glutamate scaffold is built through a conjugated addition and a chiral auxiliary is used to induce high stereoselectivity at the α carbon (Fig. 3B). The stereoselectivity is not equally high for the β carbon, however we were able to isolate and purify the desired diastereomer. Interestingly, our attempts to introduce secondary or tertiary carbons at the β position (e.g., isopropyl, cyclopropyl, *tert*-butyl) were unproductive. We believe this can be attributed to the high tendency of these β-glutamates to cyclize to pyroglutamic acids in the conditions used for the experiment, a phenomenon reported by Wehbe for methyl analogues. (Fig. 3C).

The five synthesized compounds were tested in a functional assay to determine whether they could competitively bind to GluK2, thereby interfering with L-glutamate binding and the ability of the receptor to activate. Recording pipettes were rapidly switched between background and agonist-containing (typically L-glutamate) external solutions (Fig. 4H), using a piezo-stack driven double-barrelled glass application pipette (details in the Supporting information). The antagonist behaviour of compounds **10**–**14** was assessed by measuring the reduction in GluK2 peak current response. We observed that the first two members of the series, **10** and **11**, when used at 1 mM concentration, inhibited the response of GluK2 to saturating (10 mM) L-Glutamate to 38 ± 3% ($n = 4$) and 46 ± 6% ($n = 4$) of its maximum amplitude (Fig. 4A–B). Compound **12**, when used at the same concentration, slightly inhibited L-glutamate responses to 95 ± 3% of their original amplitude ($n = 4$) (Fig. 4C). The last two bulkier compounds, **13** and **14**, did not affect the current response to glutamate under the same conditions (Fig. 4E–F), indicative of reduced (or minimal) affinity for receptors, relative to their counterparts with smaller substituent groups. Because other trials indicated that the compounds lacked detectable agonist activity at GluK2 receptors (Fig. 4G) our results suggest that the β-methyl, ethyl and propyl glutamates were acting as antagonists.

We also performed an additional experiment to check the absence of artifacts associated with our functional assay set-up. In particular, we wanted to verify that the inhibitor does not have time to unbind from the receptor during the solution exchange (time scale of 100 us). This possibility was indeed ruled out: when compound **11** was present at 1 mM in both the glutamate solution and the background solution, no increase in the inhibition effect was observed. Instead, the response to L-glutamate was reduced to 53 ± 6% ($n = 4$) (data not shown).

Although no experiment definitively confirmed that the molecules under study act as competitive antagonists, their high structural similarity to the natural agonist strongly suggests this is the case. This hypothesis is further supported by the observation that the inhibitory activity decreases with a clear trend in going from more-alike to less-alike analogues of glutamate. Therefore, our assays suggest that compounds **11**, **12** and **13** can compete with the natural ligand for occupancy of the agonist binding cleft.

It also appears that increasing the size of the side chain induces significant clashes of the ligand that is no longer able to compete with the natural ligand under the conditions of the experiments.



(B)

α,β-conjugated addition*				Hydrolysis*
R	d.r.**	yield (major)		% yield
Me	6:4	34		89
Et	6:4	40		85
Pr	6:4	40		75
iBu	6:4	41		87
Ph	6:4	42		96

** diastereomeric ratio based on ^{1}H NMR analysis

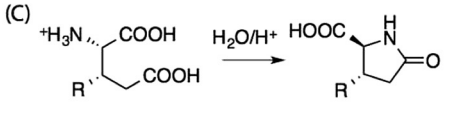


Figure 3. (A) Synthesis of designed ligands: (a) BF_3, benzene, reflux; (b) MeMgBr, THF, −30 °C; (c) DBU, THF, −30 °C; (d) HCl 3 M, reflux; (B) diastereomeric ratios and chemical yields; (C) Proposed reaction of cyclization for β-substituted glutamic acids.

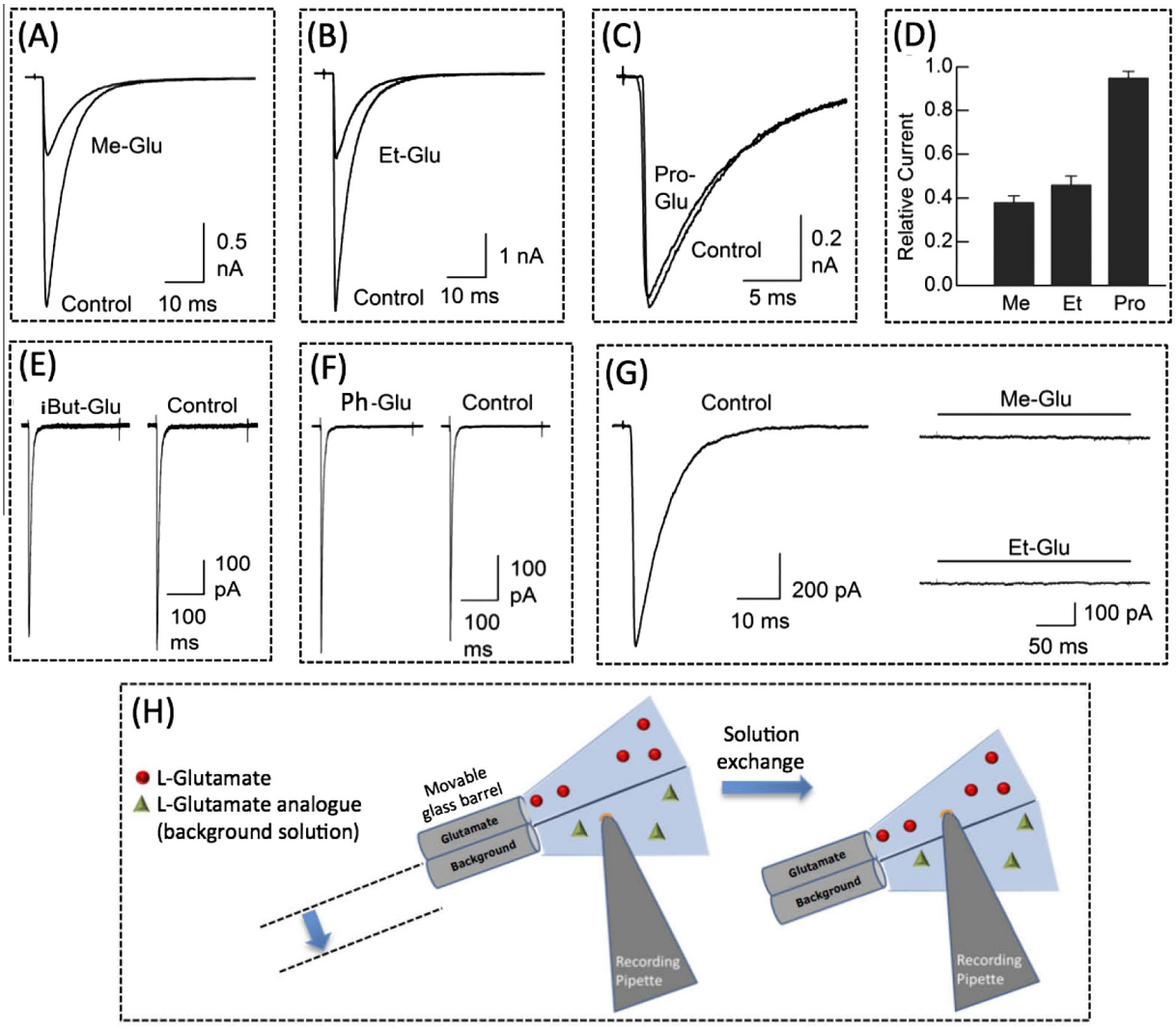


Figure 4. Me-Glu (compound **10**), (B) Et-Glu (compound **11**), and (C) Pro-Glu (compound **12**) inhibition of GluK2 current responses to 10 mM L-glutamate. (D) Summary of inhibition produced by compounds **10**–**12**, shown relative to the L-glutamate induced current measured following a return to the control background solution. (E) In contrast, iBut-Glu (Compound **13**) and (F) Ph-Glu (Compound **14**) did not appear to inhibit responses to 10 mM L-glutamate. (G) When GluK2-expressing patches were exposed to 1 mM Me-Glu ($n = 5$) and Et-Glu ($n = 4$), no current responses were detected, despite the same patches having displayed robust responses to 10 mM L-Glutamate (control). (H) Schematic representation of the functional assay set-up: a membrane patch containing the receptor of interest is first placed on the tip of the recording pipette, and exposed to the antagonist in the background solution prior to L-glutamate application.

The literature contains several reports that can be helpful to rationalize the data. Mayer and colleagues provided insight into the structural basis of KA receptor competitive antagonism by crystallizing GluK1 ABD complexes with novel antagonists. The authors showed that UBP302, UBP310, UBP315, UBP318 and LY466195 induced a similar, generally large opening of the GluK1 agonist binding cleft in comparison to the closed conformation elicited by the full agonist glutamate.[15,16] Although no structural work is available for GluK2 bound to the same or other antagonists, it is reasonable to assume that it behaves similarly to GluK1 from a structural point of view. The two subunits share ~80% peptide sequence, with the most conserved region being around the glutamate binding site. When we look more closely at the available structures of GluK1 ABD bound to L-glutamate and UBP antagonists, and we align the structures, we notice that the tyrosine residue in the ligand proximity undergoes a significant shift (Fig. 2B and C). This indicates that there may be a direct relationship between antagonist behavior and an open conformational state induced at the ABD level.

It must be emphasized that the extent of the movement of the aforementioned tyrosine is based on how structural alignments are performed. In our case, MATCH-UP, a piece of our computational platform FITTED, was used to superimpose the available structures of the ABD without specific structural constraints. Even though current models of iGlu activation predict that the ABD lower domain D2 swings upward relative to D1 when agonists bind, no definitive parallels can be made for GluK1 or GluK2 in the absence of full-length crystal structures of the receptor bound to an antagonist. It is possible that in the receptor physiological state the positions of some binding site residues are more or less constrained than in the isolated ABD, therefore causing unpredicted movements.

Furthermore, based on our observations, it is perplexing that kainate and domoate do not act as GluK2 antagonists, since they induce an intermediate and open conformation, respectively. Even though for most structures the trend remains 'greater closure, greater efficacy', no definitive connection can be made. Indeed, there are examples of antagonists inducing full closure in KAR structures,[17] and of partial agonists inducing varying degrees of closure.[18] One must remember that crystal structures are only snapshots of the receptor at one point in time and do not display other conformations that may be entered less frequently but that are critical for functional behavior. KAR receptors are thought to

be very dynamic and one must take into account the weighted time the receptor spends in closed cleft versus open cleft conformations in regulating efficacy.

Despite the uncertainty that is always correlated with docking studies based on crystal structures, this study refines the existing knowledge about structure-activity relationships for KA inhibition. Most of the antagonists reported so far have a molecular weight significantly higher than glutamate, which agrees with the theory that an effective antagonist must hinder full closure of the ABD acting like a jamming object. Our study reveals that smaller molecules could be as effective, if properly designed. In particular, we showed that targeting Tyr488 in the ABD could be important in the development of effective antagonists. Ensuring that this interaction takes place, while focusing on decreasing the molecular weight, could represent a new potential approach to develop molecules that are able to efficiently reach KA receptors across the blood brain barrier and eventually be useful in the treatment of neuropathological disease states.

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Supplementary data

Supplementary data (chemicals and methods used, as well as characterization of compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.04.008.

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