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Design, synthesis and structure-activity relationships of novel phenylalanine-based amino acids as kainate receptors ligands

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Keywords

Ionotropic glutamate receptors, kainate receptors, GluK1, GluK3, phenylalanine

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Abstract

A new series of carboxyaryl-substituted phenylalanines was designed, synthesized and pharmacologically characterized *in vitro* at native rat ionotropic glutamate receptors as well as at cloned homomeric kainate receptors GluK1-GluK3. Among them, six compounds bound to GluK1 receptor subtypes with reasonable affinity (K_i values in the range of 4.9-7.5 μM). A structure-activity relationship (SAR) for the obtained series, focused mainly on the pharmacological effect of structural modifications in the 4- and 5-position of the phenylalanine ring, was established. To illustrate the results, molecular docking of the synthesized series to the X-ray structure of GluK1 ligand binding core was performed. The influence of individual substituents at the phenylalanine ring for both the affinity and selectivity at AMPA, GluK1 and GluK3 receptors was analyzed, giving directions for future studies.

Kainate (KA) receptors are cation-selective ligand-gated ion channels that belong to the family of ionotropic glutamate receptors together with α-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors. They are widely expressed in the mammalian central nervous system, particularly in the hippocampus, where they act principally as the modulators of synaptic transmission and neuronal excitability.[1] Functional KA receptors are tetrameric structures formed by the combination of subunits GluK1–5, which are assembled into two dimers of two homo- or two heteromeric subunits. It has been shown that GluK1-3 may form functional homomeric or heteromeric receptors, whereas GluK4 and GluK5 only form functional receptors in combination with subunits GluK1, GluK2 or GluK3.[2,3]

In recent years, dynamic growth has been reported in the understanding of the biophysical properties and function of KA receptors in the brain. A particular problem in the studies on this type of receptors is the relative lack of specific pharmacological tools. Most known KA receptors agonists, including AMPA and kainate themselves, act on both AMPA and KA receptors,[4] similar to the early non-NMDA receptor antagonists (such as the isoxazol-based (*S*)-ATPO, Fig. 1A).[3-6] Despite an extensive effort, only a few antagonists (such as UBP302 and LY466195, Fig. 1A) with preference for GluK1 receptors compared to GluK3 receptors and recombinant AMPA receptors have been reported.[3,4,7-10] GluK1 antagonists have been shown to be highly effective in animal models of epilepsy,[11,12] neurodegeneration,[11] neuropathic pain[3,12,13] and migraine.[14]

Recently, we have reported two series of competitive non-NMDA receptor antagonists built on the phenylalanine scaffold.[6,15-18] 2-Carboxyethylphenylalanines **1** (Fig. 1B) were found to block AMPA, GluK1 and GluK3 receptors, but not GluK2 receptors.[15,16] The analysis of structure-activity relationship (SAR) within the series showed that both R^1 and R^2 positions are structural factors influencing AMPA/GluK1 and GluK3 selectivity. Introduction of a

4-nitro-5-chloro substitution pattern into the phenylalanine ring was beneficial for both AMPA and homomeric kainate receptors affinity, whereas a hydrogen atom or a polar amino group in the 4-position introduced several-fold selectivity for GluK1 and GluK3 receptors. Within the series of biaryl phenylalanine analogues based on the scaffold **2** (Fig. 1B) it was shown that only compounds containing a 3'-carboxylate, such as **2a** and **2b**, bound selectively to GluK1 receptors.[6] In this series, only the 4,5-dichloro substitution pattern of the phenylalanine ring has been studied.

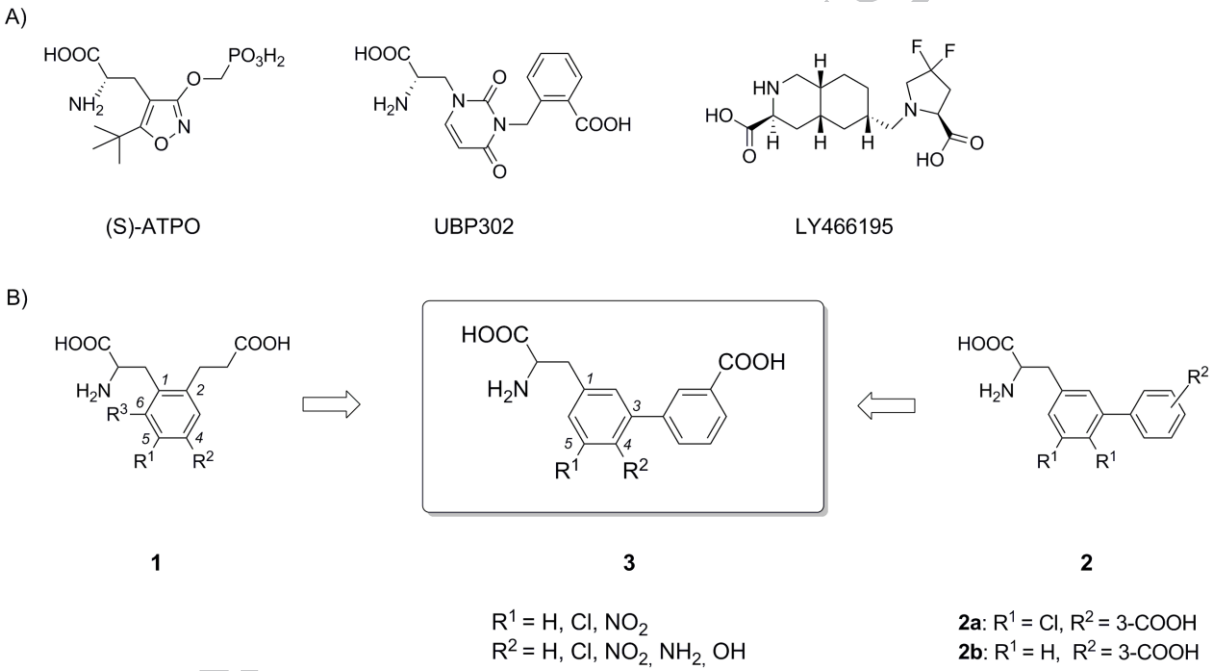


Figure 1. (A) Structures of selected AMPA and kainate receptor antagonists. (B) Design of the new phenylalanine-based ligands of AMPA and kainate receptors.

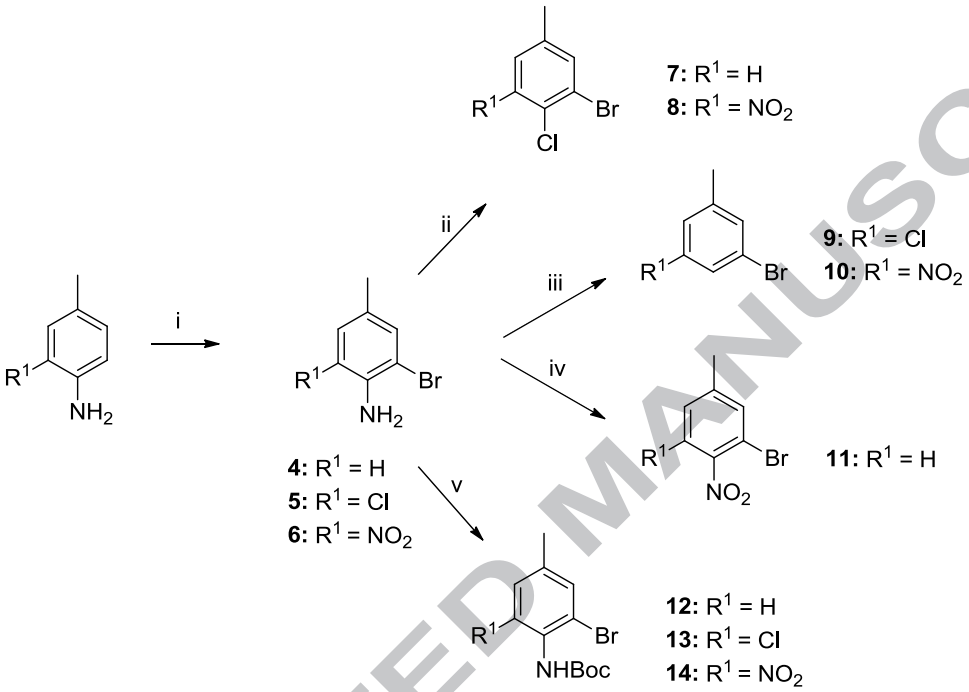
Here, we report the synthesis and biological evaluation of a new series of biaryl-based phenylalanine analogues, with a carboxyl-benzene (or a carboxyl-thiophene ring) as a distal aromatic function (Fig. 1B). To find the substitution pattern optimal for kainate receptors affinity, the structural modifications were focused on positions 5- and 4- of the phenylalanine ring. Various combinations of small-sized groups, both polar and lipophilic, hydrogen bond

donors and acceptors, were investigated. All the target amino acids were pharmacologically characterized by radioligand binding at native AMPA, KA and NMDA receptors in rat brain membranes. All the compounds were further evaluated in radioligand binding assays at homomeric recombinant rat GluK1-3 receptors, expressed in *Sf9* insect cell membranes.

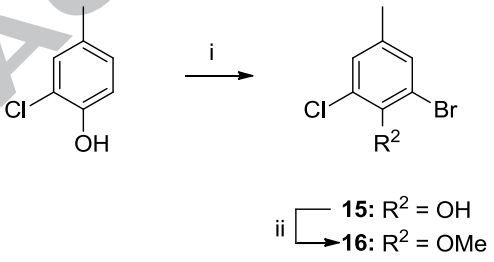
The general procedure for the synthesis of the target amino acids is outlined in Schemes 1-3. Modified literature methods[6,19-23] were applied to obtain the suitable substitution pattern of bromophenyl intermediates **7-14** and **16**, starting from commercially available substituted 4-methylanilines (Scheme 1) or 2-chloro-4-methylphenol (Scheme 2). Oxidative bromination with $NaClO_3$ and HBr followed by Sandmayer reaction using $CuCl_2$ afforded **7** and **8**. The removal of an amine group performed through diazotization yielded compounds **9** and **10**. Oxidation of amine **4** to 2-bromo-4-methylnitrobenzene **11** was performed by means of 3-chloroperbenzoic acid (*m*CPBA). BOC-protection of the amino group, carried out in the mixture of dioxane and water in the presence of Na_2CO_3 was successful in the case of amine **4**, but these conditions were not sufficient for **5** and **6**. To obtain **13** and **14**, a catalytic amount of 4-dimethylaminopyridine (DMAP) was used in addition to *tert*-butoxycarbonyl anhydride. The reaction was carried out in tetrahydrofuran (THF) for 72 hours at room temperature, in the presence of triethylamine (TEA) as the base. To yield **16**, bromination of 2-chloro-4-methylphenol followed by alkylation of the hydroxy group was applied.

The treatment of bromotoluene precursors **7-14** and **16** with N-bromosuccinimide (NBS) under free radical conditions and the following substitution with the sodium salt of diethyl acetamidomalonate were performed in one step, without the isolation of benzyl bromides (Scheme 3). The target amino acids **3a-i** were prepared as a result of a number of Suzuki coupling reactions of intermediates **17-25** with 3-cyanophenylboronic acid, followed by deprotection in acidic environment. In most cases, the product of the cross-coupling reaction was refluxed in the mixture of concentrated hydrochloric acid, acetic acid and water for 24

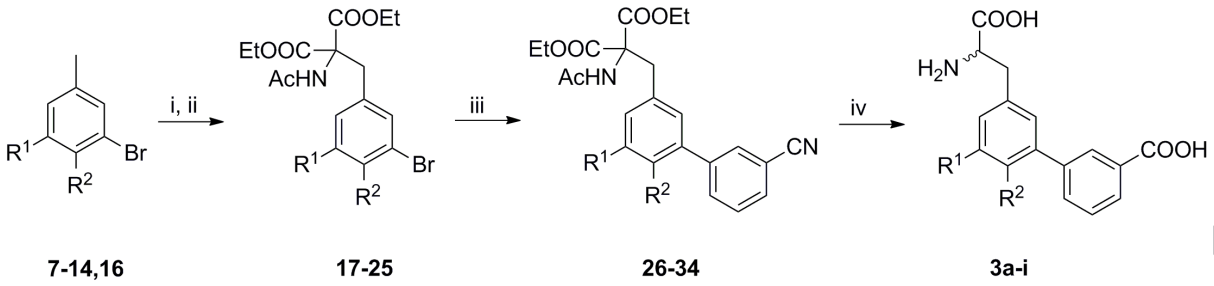
hours, except for the methoxy-substituted compound **31**, for which aqueous hydrobromic acid was used. The racemic mixtures of final products were purified by evaporation of the acid(s), neutralization of pH followed by reverse-phase liquid chromatography. All final amino acids were obtained in their zwitterionic form.



Scheme 1. Synthesis of compounds **5**-**14**. Reagents and conditions: (i) 48% HBr, $NaClO_3$, acetic acid, 5→45 °C; (ii) 1. $CuCl_2$, *tert*-$BuNO_2$, acetonitrile, 65 °C, 2. 20% HCl; (iii) 50% H_3PO_2, $NaNO_2$, acetic acid/water/HCl, 0 °C→ r.t.; (iv) 77% *m*CPBA, toluene, reflux; (v) for R^1 = H: BOC anhydride, Na_2CO_3, dioxane/water, r.t.; for R^1 = Cl, NO_2: BOC anhydride, DMAP, TEA, tetrahyfrofuran, r.t.



Scheme 2. Synthesis of compounds 1**5** and **16**. Reagents and conditions: (i) HBr, $NaClO_3$, acetic acid, 5→30 °C; (ii) CH_3J, K_2CO_3, dimethylformamide, r.t.



7-14,16 **17-25** **26-34** **3a-i**

Scheme 3. Synthesis of compounds **17**-**34** and **3a-i**. Reagents and conditions: (i) NBS, azobisisobutyronitrile, carbon tetrachloride, reflux; (ii) NaH, diethyl acetamidomalonate, dimethylformamide, r.t.; (iii) 3-cyanophenylboronic acid, $PdCl_2(PPh_3)_2$, triethylamine, DME/water, 50 °C; (iv) HCl or HBr, reflux.

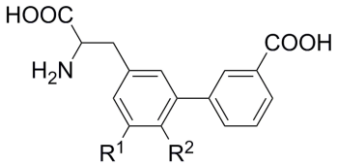
All final amino acids **3a-i** were characterized pharmacologically in the radioligand binding assays at native ionotropic glutamate receptors (rat membrane preparations) as well as cloned rat homomeric subtypes, GluK1-3.[24-30] The binding data are shown in Table 1, together with those previously reported for **2a** and **2b,** cited here as reference compounds.[6] In addition to the previous binding data, the present work also examined analogues **2a** and **2b** for their affinity at the GluK2 and GluK3 receptors.

All compounds were found to be inactive at native NMDA receptors as well as homomeric GluK2 receptors (K_i > 100 μM, data not shown). Within the series investigated, **3h** was the only analogue that demonstrated micromolar affinity for native kainate receptors (K_i = 32 μM, Table 1). Weak AMPA binding was observed for some of phenylalanines for which K_i values varied in the range of 38-65 μM. This effect seems to be associated with the presence of a chloro atom or nitro group in the 5-position of the phenylalanine ring with concomitant unsubstituted 4-position (**3d**, **3g**), while a polar hydroxyl or amine group in position 4 markedly reduced AMPA affinity.

At KA receptors, a structural modification in the 4- and 5-position of the phenylalanine ring affected both affinity and selectivity at homomeric GluK1 and GluK3 receptors. The

introduction of a 4-nitro group to the unsubstituted **2b** induced affinity at GluK3 receptors and did not affect GluK1 binding (**3b**), while 4-chloro or 4-amino groups (**3a** and **3c**, respectively) led to a noticeable reduction in binding affinity at GluK1 receptor. A different effect was observed in the case of substituents in 5-position. The 5-chloro analogue (**3d**) was found to be equipotent at GluK1 receptors compared to **2b** and showed micromolar affinity at GluK3 receptors, whereas the 5-nitro group (**3g**) induced 4-fold reduction in GluK1 affinity. The addition of the second substituent to 5-chloro or 5-nitro structures resulted in the active GluK1 ligands **3e**, **3f**, **3h** and **3i** with micromolar range of affinity (K_i = 4.9-7.5 μM). An interesting effect was observed in the case of compounds possessing a polar group in the 4-position of the phenylalanine ring. The 4-amino group seemed to substantially diminish the affinity at GluK3 receptor (**3c**, **3e**, **3i**), while 4-hydroxy group together with the 5-chloro substituent of **3f** was found to be a substitution pattern beneficial for GluK3 receptors. Thus, the 4-amino-5-chloro-substituted biphenylalanine **3e**, the most potent compound at GluK1 within the present series, presented over 20-fold selectivity vs. GluK3 (and native AMPA) receptors, while its close (and equipotent at GluK1 receptors) analogue **3f** showed only 2-fold difference between GluK1 and GluK3 subtypes affinities.

Table 1. Binding pharmacology at native AMPA and KA receptors (rat brain membranes) as well as homomeric recombinant GluK1 and GluK3 receptors.[a]



Compound	R^1	R^2	cloned homomeric receptors		native receptors	
			GluK1	GluK3	[^{3}H]AMPA	[^{3}H]KA
			K_i (μM)	K_i (μM)	K_i (μM)	K_i (μM)

(*S*)-ATPO			2.9[b]	nd.	16[c]	> 100[c]
UBP302			0.6[d]	4.0[e]	nd.	nd.
LY466195			0.05[f]	8.9[f]	nd.	nd.
2a	Cl	Cl	2.8[b]	> 100	> 100[g]	> 100[g]
2b	H	H	7.9[b]	> 100	> 100[g]	> 100[g]
3a	H	Cl	35 [4.46 ± 0.02]	> 100	> 100	> 100
3b	H	NO_2	7.2 [5.15 ± 0.03]	23 [4.64 ± 0.02]	65 [4.19 ± 0.04]	> 100
3c	H	NH_2	26 [4.58 ± 0.01]	> 100	> 100	> 100
3d	Cl	H	5.7 [5.26 ± 0.09]	25 [4.60 ± 0.02]	51 [4.31 ± 0.09]	> 100
3e	Cl	NH_2	4.9 [5.33 ± 0.08]	> 100	≈ 100	> 100
3f	Cl	OH	5.5 [5.31 ± 0.13]	11 [4.95 ± 0.04]	> 100	> 100
3g	NO_2	H	32 [4.50 ± 0.06]	> 100	38 [4.43 ±0.03]	> 100
3h	NO_2	Cl	6.1 [5.23 ± 0.07]	> 100	62 [4.21 ± 0.05]	32 [4.50 ± 0.02]
3i	NO_2	NH_2	7.5 [5.15 ± 0.08]	> 100	> 100	> 100

[a] Data are given as mean of at least three separate experiments conducted in triplicate. Mean pK_i ± SEM (M) are indicated in brackets. [b] Data from ref.[6]. [c] IC_{50} values, data from ref.[5]. [d] Data from ref.[8]: K_i value for inhibition of glutamate-evoked currents in HEK293 cells expressing homomeric KA receptors. [e] Data from ref.[9]: IC_{50} values in an *Xenopus* oocyte assay. [f] Data from ref.[10]. [g] IC_{50} values, data from ref.[6]. nd. – not determined.

To analyze the structure-activity relationship of the new target compounds in more details, molecular docking studies to the GluK1 ligand binding core (LBD) were undertaken. Due to a size of designed ligands, an X-ray structure of GluK1 LBD with bound antagonist: (*S*)-1-(2'-amino-2'-carboxyethyl)-3-[(2-carboxythien-3-yl)methyl]thieno[3,4-*d*]pyrimidin-2,4-dione (PDB code: 3S2V)[31] has been chosen as a template. The results of docking showed, that inside the binding pocket biphenylalanines adopt the binding mode similar to that observed for most of α-amino acid-based competitive antagonists co-crystallized with the GluK1 and GluA2 LBDs (Fig. 2). Detailed analysis of high scored docking poses of (*S*)-**3e** and (*S*)-**3f** revealed the presence of two pockets surrounding the substituents in R^1 and R^2 positions of the phenylalanine ring. The one formed by Glu456, Tyr459, Pro531, Thr755, Ser756 and Tyr779 (full-length numbering including the signal peptide of isoform GluR5-1, UniProtKB identifier P22756-1) seems to accommodate equally well chlorine atom and a nitro group, however, lack of a substituent in this position is connected with a significant decrease of a docking score. Despite a high hydrogen bonding potential in this region of the receptor, no such an interaction is observed for docked ligands. The substituent in R^2 position is surrounded by residues Ser736, Ser756 and Met752. Among them Ser756 seems to exert the strong influence on GluK1 affinity of the compounds, probably interacting through a hydrogen bond with a hydroxy or amino group of ligands (Fig. 2). Furthermore, both residues Ser736 and Ser756 of GluK1 receptor are non-conserved. In GluK3 receptor in these positions there are asparagine and threonine, and in the AMPA subunit GluA2: threonine and methionine, respectively. Those differences may contribute to the observed AMPA/GluK1/GluK3 selectivity of the target biphenylalanines.

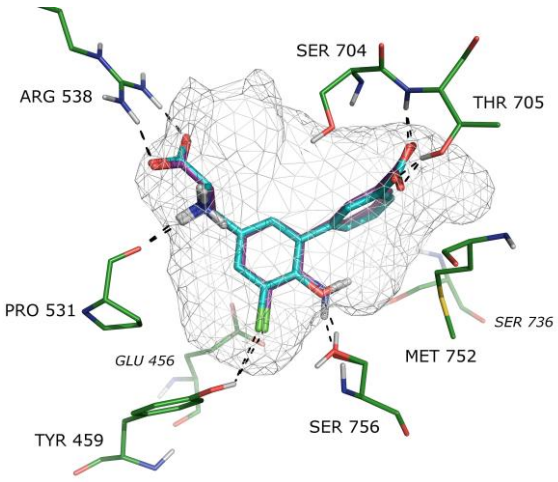


Figure 2. The highest ranked docking poses of (*S*)-**3e** (purple) and (*S*)-**3f** (cyan) at the GluK1 LBD (3S2V template). Depending on the rotameric position of the side chain, Ser756 may interact with either amino of **3e** or hydroxy group of **3f**. The shape of the GluK1 binding site (in mesh) was calculated for 3S2V.

In summary, the studies described in the present work have clarified the structure-activity relationship of the new series of phenylalanine derivatives based on the [1,1'-biphenyl]-3-carboxylic acid scaffold and acting as ligands of non-NMDA receptors. The influence of substituents in the 4- and 5-position of the phenylalanine on binding affinity to native AMPA and cloned homomeric GluK1 and GluK3 receptors was the main object of the study. The results clearly point at the significance of the nitro or hydroxyl group in the 4-position and the chloro atom in the 5-position for affinity at both GluK1 and GluK3 receptor subtypes. Furthermore, the analysis of the present results and the SAR established for the phenylalanines previously described based on the 2-carboxyethylphenylalanine scaffold (**1**)[15,17] shows 2-6 fold higher GluK1 receptor affinity of biphenyl analogues **3** compared to phenylalanines with structure **1** with the same substitution pattern in the phenylalanine ring. As the biphenylalanine core seems to be optimal for GluK1 ligands, a 5-chloro-4-nitro biphenyl-based analogue is expected to possess high GluK1 and GluK3 affinity and this compound will be included in our future studies. On the other hand, as the amino group in the

4-position of **3** reduces binding at both native AMPA and homomeric GluK3 receptors, 4-amino substitution should be taken into account in the design of selective GluK1 ligands based on the biphenylalanine core.

Acknowledgements

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Supplementary data

Supplementary data (full experimental details, compound characterization data, ^{1}H-NMR and ^{13}C-NMR spectra as well as pharmacology and molecular modeling protocols) associated with this article can be found in the online version.

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Figure 1. (A) Structures of selected AMPA and kainate receptor antagonists. (B) Design of the new phenylalanine-based ligands of AMPA and kainate receptors.

Figure 2. The highest ranked docking poses of (*S*)-**3e** (purple) and (*S*)-**3f** (cyan) at the GluK1 LBD (3S2V template). Depending on the rotameric position of the side chain, Ser756 may interact with either amino of **3e** or hydroxy group of **3f**. The shape of the GluK1 binding site (in mesh) was calculated for 3S2V.

Scheme 1. Synthesis of compounds **5-14**. Reagents and conditions: (i) 48% HBr, $NaClO_3$, acetic acid, 5→45 °C; (ii) 1. $CuCl_2$, *tert*-$BuNO_2$, acetonitrile, 65 °C, 2. 20% HCl; (iii) 50% H_3PO_2, $NaNO_2$, acetic acid/water/HCl, 0 °C→ r.t.; (iv) 77% *m*CPBA, toluene, reflux; (v) for R^1 = H: BOC anhydride, Na_2CO_3, dioxane/water, r.t.; for R^1 = Cl, NO_2: BOC anhydride, DMAP, TEA, tetrahyfrofuran, r.t.

Scheme 2. Synthesis of compounds 1**5** and **16**. Reagents and conditions: (i) HBr, $NaClO_3$, acetic acid, 5→30 °C; (ii) CH_3J, K_2CO_3, dimethylformamide, r.t.

Scheme 3. Synthesis of compounds **17-34** and **3a-i**. Reagents and conditions: (i) NBS, azobisisobutyronitrile, carbon tetrachloride, reflux; (ii) NaH, diethyl acetamidomalonate, dimethylformamide, r.t.; (iii) substituted phenyl- or thienylboronic acid, $PdCl_2(PPh_3)_2$, triethylamine, DME/water, 50 °C; (iv) HCl or HBr, reflux.

Table 1. Binding pharmacology at native AMPA and KA receptors (rat brain membranes) as well as homomeric recombinant GluK1 and GluK3 receptors.[a]

Footnote:

[a] Data are given as mean of at least three separate experiments conducted in triplicate. Mean pK_i ± SEM (M) are indicated in brackets. [b] Data from ref [6]. [c] IC_{50} values, data from ref [5]. [d] Data from ref [8]: K_i value for inhibition of glutamate-evoked currents in HEK293 cells expressing homomeric KA receptors. [e] Data from ref [9]: IC_{50} values in an *Xenopus* oocyte assay. [f] Data from ref [10]. [g] IC_{50} values, data from ref [6]. nd. – not determined.

Graphical abstract

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Design, synthesis and structure-activity relationships of novel phenylalanine-based amino acids as kainate receptors ligands

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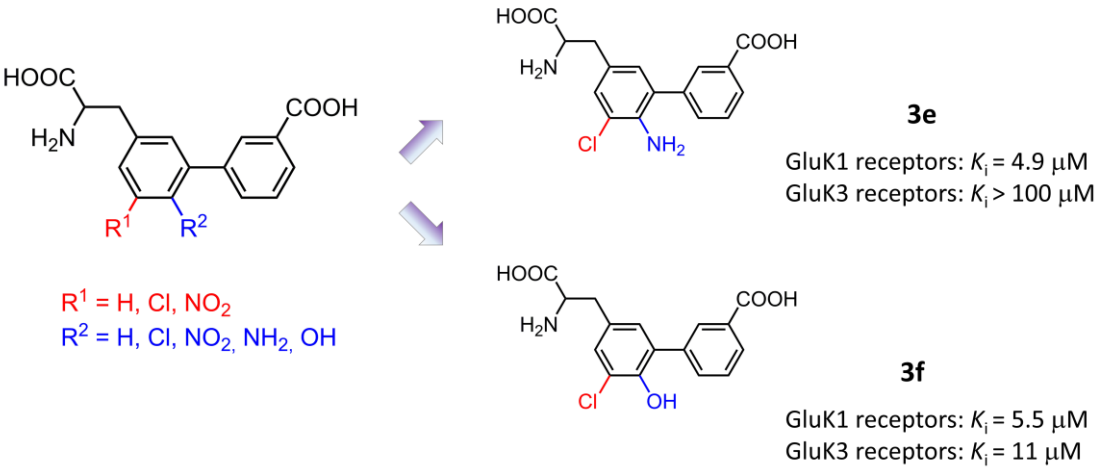

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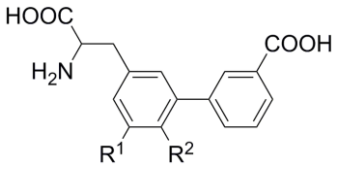

			cloned homomeric receptors		native receptors	
Compound	**R^1**	**R^2**	**GluK1 K_i (μM)**	**GluK3 K_i (μM)**	**[^{3}H]AMPA K_i (μM)**	**[^{3}H]KA K_i (μM)**
(*S*)-ATPO			2.9[b]	nd.	16[c]	> 100[c]
UBP302			0.6[d]	4.0[e]	nd.	nd.
LY466195			0.05[f]	8.9[f]	nd.	nd.
2a	Cl	Cl	2.8[b]	> 100	> 100[g]	> 100[g]
2b	H	H	7.9[b]	> 100	> 100[g]	> 100[g]
3a	H	Cl	35 [4.46 ± 0.02]	> 100	> 100	> 100
3b	H	NO_2	7.2 [5.15 ± 0.03]	23 [4.64 ± 0.02]	65 [4.19 ± 0.04]	> 100
3c	H	NH_2	26 [4.58 ± 0.01]	> 100	> 100	> 100

3d	Cl	H	5.7 [5.26 ± 0.09]	25 [4.60 ± 0.02]	51 [4.31 ± 0.09]	> 100
3e	Cl	NH_2	4.9 [5.33 ± 0.08]	> 100	≈ 100	> 100
3f	Cl	OH	5.5 [5.31 ± 0.13]	11 [4.95 ± 0.04]	> 100	> 100
3g	NO_2	H	32 [4.50 ± 0.06]	> 100	38 [4.43 ±0.03]	> 100
3h	NO_2	Cl	6.1 [5.23 ± 0.07]	> 100	62 [4.21 ± 0.05]	32 [4.50 ± 0.02]
3i	NO_2	NH_2	7.5 [5.15 ± 0.08]	> 100	> 100	> 100

[a] Data are given as mean of at least three separate experiments conducted in triplicate. Mean pK_i ± SEM (M) are indicated in brackets. [b] Data from ref.[6]. [c] IC_{50} values, data from ref.[5]. [d] Data from ref.[8]: K_i value for inhibition of glutamate-evoked currents in HEK293 cells expressing homomeric KA receptors. [e] Data from ref.[9]: IC_{50} values in an *Xenopus* oocyte assay. [f] Data from ref.[10]. [g] IC_{50} values, data from ref.[6]. nd. – not determined.