Synthesis and Biological Evaluation of Novel 9-Heteroaryl Substituted 7-Chloro-4,5-dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylates (TQX) as (*R*,*S*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic Acid (AMPA) Receptor Antagonists

Daniela CATARZI,*[,a] Vittoria COLOTTA,[a] Flavia VARANO,[a] Guido FILACCHIONI,[a] Paola GRATTERI,[a] Jacopo SGRIGNANI,[a] Alessandro GALLI,[b] and Chiara COSTAGLI[b]

[a] *Dipartimento di Scienze Farmaceutiche, Laboratorio di Progettazione, Sintesi e Studio di Eterocicli Biologicamente Attivi, Universita' degli Studi di Firenze; Polo Scientifico, Via U. Schiff, 6, 50019 Sesto Fiorentino (Firenze), Italy: and* [b] *Dipartimento di Farmacologia Preclinica e Clinica, Universita' degli Studi di Firenze; Viale G. Pieraccini, 6, 50134 Firenze, Italy.* Received December 19, 2007; accepted April 30, 2008; published online May 8, 2008

In this paper, we report a study on some new 4,5-dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylate derivatives (TQXs), bearing a nitrogen-containing heterocycle at position-9, and designed as (*R*,*S*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) receptor antagonists. These compounds ensue from the structural modification of previously reported 8-heteroaryl-TQXs which were endowed with high affinity and selectivity for the AMPA receptor. All the newly synthesized compounds were biologically evaluated for their binding at the AMPA receptor. Gly/*N*-methyl-D-aspartic acid (NMDA) and kainic acid (KA) high-affinity binding assays were performed to assess the selectivity of the reported derivatives toward the AMPA receptor. This study produced some new TQXs which are less potent than the reference compounds, and endowed with a mixed AMPA and Gly/NMDA receptor binding affinity. To rationalize the experimental findings, a molecular modeling study was performed by docking some TQX derivatives to the AMPA receptor model.

Key words ionotropic glutamate receptor; competitive antagonist; triazoloquinoxaline; (*R*,*S*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid

Glutamate (Glu) is the major excitatory neurotransmitter in the central nervous system (CNS), where it is involved in the physiological regulation of processes such as learning, memory and synaptic plasticity.[1—3]

Glu activates specific receptors which belong to the classes of metabotropic (mGluRs, coupled to G-protein) and ionotropic receptors (iGluRs, ligand-gated ion channel), the latter consisting of three major subclasses: *N*-methyl-D-aspartic acid (NMDA), kainic acid (KA) and (*R*,*S*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) receptors which are classified according to their preferential synthetic agonists.[2,4]

It is well known that many neurological disorders, such as cerebral ischemia, epilepsy, amiotrophic lateral sclerosis and Parkinson's diseases,[1] are caused by excessive release of Glu from presynaptic terminals which overstimulates postsynaptic GluRs, thus leading to neurotoxicity.[5—12]

An approach to antagonize the overstimulation of postsynaptic iGluRs by excessive endogenous Glu is represented by the use of AMPA receptor antagonists that, together with other Glu receptor antagonists, have been proposed as potential useful neuroprotectives for the prevention and treatment of the above mentioned neurological disorders.[10—20] The success of AMPA receptor antagonists as potential therapeutic agents is in part due to their greater clinical potential with respect to other pharmacologically well-characterized iGluR antagonists: for example, AMPA receptor antagonists do not produce the adverse psychotomimetic and cardiovascular effects observed for competitive NMDA receptor antagonists.[21] These data have consequently added great impetus for the development of AMPA receptor antagonists as research tools.

In the course of our efforts to find novel competitive AMPA receptor antagonists,[22—35] we have published works which report the synthesis and pharmacological studies on 4,5-dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylates (TQXs) bearing different substituents on the fused benzo moiety (Series A, Fig. 1).[26,27,29,31] These studies provide evidence on the structural requirements which are important for obtaining potent and selective AMPA receptor antagonists: i) a NH proton donor that binds to a proton acceptor of the receptor; ii) the 3-nitrogen atom and the oxygen atom of the 4-carbonyl group that are δ-negatively charged heteroatoms able to form a coulombic interaction with a positive site of the receptor; iii) a carboxylate function at position-2 able to engage a strong hydrogen-bond interaction with a cationic proton donor site of the receptor; iv) an electron-withdrawing substituent (EWG) at position-7; and v) a N^3-nitrogen-containing heterocycle at position-8 of the TQX framework, which is an essential feature for selective AMPA receptor antagonists. These structural requirements are in accordance with those emphasized in the pharmacophore models of the AMPA receptor reported in the literature for the binding of quinoxalinedione and heterocyclic-fused quinoxalinone antagonists.[10,16—18,36]

Among the previously reported TQX series, the 7-chloro-4,5-dihydro-4-oxo-8-(1,2,4-triazol-4-yl)-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylic acid **TQX-173** and its corresponding ethyl ester (**TQX-197**) (Fig. 1), both bearing a (1,2,4-triazol-4-yl) moiety at the crucial 8-position, emerged as two of the most active and selective TQX compounds toward the AMPA receptor.[27,29] Thus, it can be hypothesized that the 8-heteroaryl group on the TQX framework interacts with a hydrophobic receptor region which also contains a hydrogen bond donor site.[10,16—18,36]

Pursuing our studies on the SAR of the TQX series, the 8-

* To whom correspondence should be addressed. e-mail: daniela.catarzi@unifi.it © 2008 Pharmaceutical Society of Japan

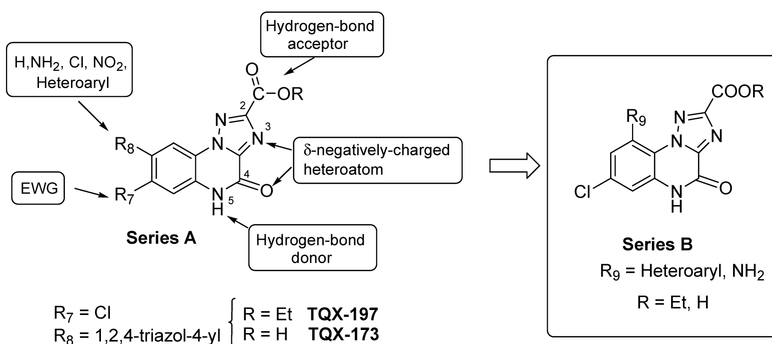
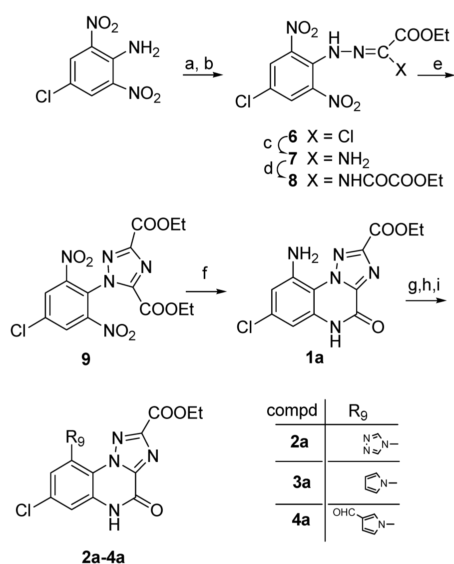
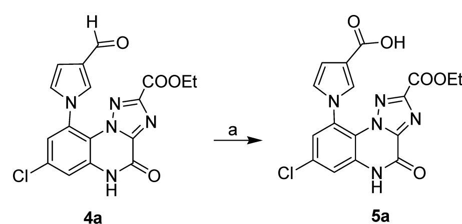


Fig. 1. Previously (Series A) and Currently (Series B) Reported TQX Derivatives



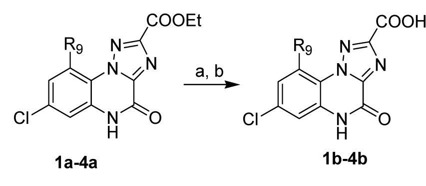
Reagent: (a) i) $NaNO_2$, conc. H_2SO_4; ii) $NaBF_4$, H_2O; (b) $CH_3COCHClCOOEt$, MeOH; (c) NH_3 (gas), anhydrous dioxane; (d) ClCOCOOEt, anhydrous diethyl ether and toluene; e) conc.H_2SO_4 (f) iron powder, glacial AcOH; (g) $(OHCNH)_2$, anhydrous pyridine, Me_3SiCl, Et_3N; (h) 2,5-dimethoxy-3-tetrahydrofurancarboxyaldehyde, glacial AcOH; (i) 2,5-di-ethoxytetrahydrofuran, glacial AcOH.

Chart 1



Reagents: (a) $KMnO_4$, acetone/ water 1:1.

Chart 2



compd	R_9
1a,b	NH_2
2a,b	
3a,b	
4a,b	

Reagents: (a) 0.8 N NaOH/EtOH; (b) 6 N HCl .

Chart 3

heteroaryl substituent of previously reported TQX derivatives was moved to position-9 of the fused benzo ring yielding compounds of Series B (Fig. 1). In fact, on the basis of the above mentioned AMPA receptor pharmacophore models reported in the literature,[10,17,18] the hydrophobic task which binds the R_8 substituent should be large enough to well accommodate also bulky groups at position-9 of the TQX framework.

Moreover, the effect of the presence of the electron-donating NH_2 group (Series B, Fig. 1) was evaluated.

All of the newly synthesized compounds were biologically evaluated for their binding at the AMPA receptor. Gly/NMDA and KA high-affinity binding assays were performed to assess the selectivity of the reported compounds toward the AMPA receptor. A selected compound was also tested for its functional antagonist activity at the AMPA receptor.

Chemistry The syntheses of the 9-substituted-4,5-dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylates **1a**—**4a**, **1b**—**4b**; **5a** are illustrated in Charts 1—3. The key intermediate, represented by the 9-amino-substituted derivative **1a**, was synthesized as reported in Chart 1.

Diazotization with $NaNO_2$ in conc. H_2SO_4 of the commercially available 4-chloro-2,6-dinitroaniline, followed by reaction with sodium tetrafluoborate, yielded the not isolated diazonium tetrafluoborate. The diazonium salt was directly reacted with ethyl 2-chloro-3-oxobutanoate to afford the N^2-chloroacetate **6**, which was transformed with ammonia into its corresponding N^2-oxamidrazonate **7**. By reacting **7** with ethyloxalyl chloride, the N^3-ethoxalyl derivative **8** was obtained, which was transformed into the diethyl 1-(4-chloro-2,6-dinitrophenyl)-1,2,4-triazole-3,5-dicarboxylate **9** by dehydration with concentrated H_2SO_4. Reduction of both the nitro groups of **9** and contemporary cyclization of the intermediate afforded the ethyl 9-amino-4,5-dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylate **1a**. By reacting **1a** with diformylhydrazine, the 9-(1,2,4-triazol-4-yl) ester **2a** was prepared. The esters 9-(pyrrol-1-yl)- and 9-(3-

formylpyrrol-1-yl)-substituted **3a** and **4a** were obtained by reacting **1a** with 2,5-diethoxytetrahydrofuran and 2,5-dimethoxy-3-tetrahydrofurancarboxaldehyde, respectively, in glacial AcOH.

Oxidation of the 9-(3-formylpyrrol-1-yl) derivative **4a** with potassium permanganate yielded the 9-(3-carboxypyrrol-1-yl) ester **5a** in very low yield (Chart 2).

Finally, the hydrolysis of the esters **1a**—**4a** to afford the corresponding 2-carboxylic acids **1b**—**4b** was performed (Chart 3). Due to the small amount of compound **5a**, its hydrolysis was not attempted.

Results and Discussion

The triazoloquinoxalines **1a**—**4a**, **1b**—**4b**, **5a**, together with **NBQX** (2,3-dihydroxy-6-nitro-7-sulphamoylbenzo[*f*]-quinoxaline) and **DCKA** (5,7-dichlorokinurenic acid) as standard compounds, were tested for their ability to displace tritiated AMPA and Gly from their specific binding in rat cortical membranes. High-affinity KA binding assays were also performed. The binding results are shown in Table 1 together with those of previously reported TQX derivatives included as reference compounds.[27,29)]

The binding results indicated that movement of the heteroaryl substituent from position-8 of the TQX framework to position-9 yielded some new AMPA receptor antagonists. In fact, compounds **2b** and **3b** are endowed with AMPA receptor affinities in the low micromolar range. It has to be noted that, also in this 9-substituted series, the most active compound is that bearing, on the fused benzo ring, the 1,2,4-triazol-4-yl moiety, *i.e.*, compound **2b**.[29)] However, it emerged that moving the 8-heteroaryl substituent to position-9 caused a modest reduction in potency: in fact, compounds **2a** and **2b**, bearing the (1,2,4-triazol-4-yl) moiety at position-9, were about 3-fold less active than the corresponding 8-substituted **TQX-197** and **TQX-173**. In general, this can be observed also when comparison was performed among the other TQX derivatives bearing different heteroaryl substituents (*i.e.* pyrrol-1-yl, 3-formylpyrrol-1-yl) on the fused benzo moiety.[29)]

Affinities of the 9-heteroaryl-substituted derivatives **2a**—**4a**, **2b**—**4b**, **5a**, for both the Gly/NMDA and KA receptors, are generally lower than those for the AMPA receptor. Only compounds **2a**, **2b** and **3b** showed a Gly/NMDA binding affinity in the micromolar range: the same order of potency toward the KA receptor is peculiar for the 9-(pyrrol-1-yl)-substituted compound **3b** showing an IC_{50} value below 10 μM.

As previously observed,[26,27,29,31)] the presence of a free carboxylic group at position-2 does not seem to be an essential feature for the anchoring at the AMPA receptor, but it positively influences the potency of these compounds. In fact, all the 9-heteroaryl-2-carboxylic acids **2b**—**4b** are four to eight fold more active than the corresponding ethyl esters **2a**—**4a** at the AMPA receptor. The differences between the binding affinities of the esters with respect to those of the corresponding acids does not seem to be highly dependent on the nature

Table 1. Binding Affinity at AMPA, Gly/NMDA and KA High-Affinity Receptors of 9-Substituted TQX Derivatives[a)]

Compd.	R	R_9	K_i (μM)[b)] or I%[c)]		IC_{50} (μM)[d)] or I%[c)]
			[^{3}H]AMPA	[^{3}H]Gly	[^{3}H]KA
1a	Et	NH_2	27%	0%	3%
1b	H	NH_2	3.2±0.3	0.61±0.13	34.1±3
2a	Et	N N N—	2.2±0.2	5.9±0.7	30%
2b	H	N N N—	0.4±0.07	2.3±0.4	37±2
3a	Et	N—	6.8±1.0	40%	38%
3b	H	N—	0.80±0.05	1.6±0.2	8.1±0.8
4a	Et	OHC N—	3.4±0.4	91±10	33%
4b	H	OHC N—	1.2±0.08	35±6	21±3
5a	Et	HOOC N—	8.9±0.7	36±4.6	35%
TQX-197[e)]	—	—	0.70±0.13	15%	42%
TQX-173[e)]	—	—	0.14±0.02	33.5±5.3	11.3±1.3
NBQX	—	—	0.07±0.06	3%	7.0±1.1
DCKA	—	—	5%	0.09±0.02	8%

a) The tested compounds were dissolved in DMSO and then diluted with the appropriate buffer. *b*) Inhibition constant (K_i) values were means±S.E.M. of three or four separate determinations in triplicate. *c*) Percentage of inhibition (I%) of specific binding at 100 μM concentration. *d*) Concentration necessary for 50% inhibition (IC_{50}). The IC_{50} values were means±S.E.M. of three or four separate determinations in triplicate. *e*) Refs. 27, 29.

of the heteroaryl substituent as it was in the previously reported 8-substituted TQX series.[29,31)]

Replacement of the heteroaryl substituent with an amino group caused a decrease of the AMPA binding affinity. In fact, compound **1b** was eight fold less potent than the corresponding 9-(1,2,4-triazol-4-yl) substituted derivative **2b**. In contrast, **1b** was the most active compound at the Gly/NMDA receptor among the reported series.

Compound **2b**, which was the most potent compound of this series at the AMPA receptor, together with the well known **NBQX** and **DCKA**, was evaluated for functional antagonist activity by assessing its ability to inhibit depolarization induced by 5 μM AMPA in mouse cortical wedge preparations.[29)] Compound **2b** inhibited AMPA responses in a reversible manner with an $IC_{50}=8.0\pm1$ μM. The electrophysiological potency of compound **2b** is in accordance with the binding data at the AMPA receptor, thus confirming that this compound is an AMPA receptor antagonist.

To rationalize the trend of AMPA binding affinities of the most active derivatives **2a** and **2b**, a molecular modeling study was performed by docking these new compounds and the leads **TQX-197** and **TQX-173** in the domain-open state of the GluR2 ligand-binding core (GluR2-S1S2J/(*S*)-ATPO crystal structure, pdb entry 1n0t),[37)] ((*S*)-ATPO, namely (*S*)-2-amino-3-[5-*tert*-butyl-3-(phosphonomethoxy)-4-isoxazolyl]propionic acid).

The antagonist binding core of ionotropic glutamate receptor, iGluRs, is formed by two domains, D1 and D2, composed of residues from two discontinuous polypetide segments S1 and S2. They fold into a clamshell-like structure that undergoes conformational change upon ligand binding, switching from an open cleft apo conformation to a domain closed state when glutamate or agonists bind. Antagonists prevent the domain closure *via* a foot-in-the-door mechanism that involves residues from both domains, namely E402, T686 and residues from the base of the helix F (S654, T655), depending on the different volume of the binding cavity occupied by the ligands.

The results from QM-polarized docking simulations[38—40)] pointed out the similar binding mode of the leads **TQX-197** and **TQX-173**, and the newly synthesized compounds **2a** and **2b** (Fig. 2). The interactions between the ligands and the residues in D1 and D2 involve the highly conserved residue triad among iGluRs: R485, T480 and P478. In particular, the 4-carbonyl group interacts with R485 and T480, and the NH at position-5 engages a hydrogen bond interaction with the D1 residue P478. Moreover, the triazoloquinoxaline scaffold is stabilized by a π–π stacking interaction with the aromatic Y450 residue (distance=about 4.2 Å), located at the top of the binding pocket. The OH group of the S654 in D2 is hydrogen bonded with the oxygen of the 2-carboxylate group. The distances of all potential hydrogen bonds (<3.3 Å) between GluR2-S1S2J residues and the ligands are reported in Table 3. The (1,2,4-triazol-4-yl) moiety of the lead compounds and derivatives **2a**, **2b** occupies, in the receptor, the binding cleft formed by E705, L704, T655, T684 and L650; in this way, the (1,2,4-triazol-4-yl) group, either at position-8 or -9 of the TQX framework, blocks the interaction of T686 on D2 with E402 on D1. This interaction is known to form the interdomain lock that stabilizes the closed agonist in-

Table 2. Physical Data of the Newly Synthesized Compounds

Compd.	mp [°C]	Solvent[a)]	Yield [%]
1a	>300	D	53
1b	>300	E	93
2a	>300	A	20
2b	234—236	B	97
3a	260—262	A	58
3b	221—223	A	55
4a	279—281	A	17
4b	189—191	B	43
5a	289—291	C	6
6	124—126	A	74
7	172—174	F	85
8	170—172	F	80
9	176—177	F	74

a) Recrystallization solvents: A=ethanol; B=the title compound was dissolved in the minimal amount of NaOH 1 N, the insoluble material was filtered off and the resulting clear solution acidified with HCl 1 N. The resulting solid was collected, treated with boiling ethanol, collected again and washed with fresh ethanol; C=purification of the title compound was performed by silica gel column chromatography as reported in the Experimental; D=glacial acetic acid; E=ethanol/water; F=cyclohexane/ethyl acetate.

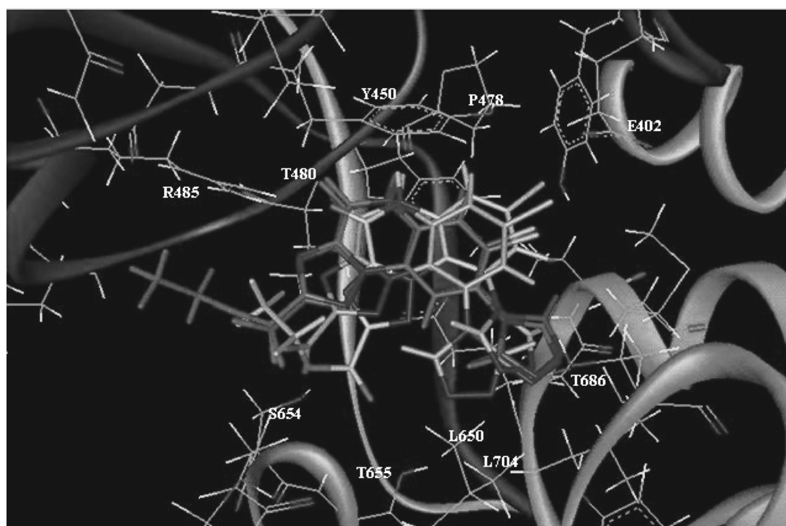


Fig. 2. The Leads **TQX-197** and **TQX-173** , and Compounds **2a** and **2b** Docked into the X-Ray GluR2-S1S2J:(*S*)-ATPO Crystal Structure (1n0t)

Side chains of the domain residues directed towards the binding cavity are shown.

Table 3. Distances of Potential Hydrogen Bonds (<3.3 Å) between GluR2-S1S2J Residues and the Ligands

Compd.	H-bond	Distances (Å)
2a	4C=O···HN(R485)	2.0
	4C=O···HN(T480)	2.0
	5NH···CO(P478)	1.83
	2COO···HN(S654)	1.73
	2COO···HO(S654)	1.73
2b	4C=O···HN(R485)	2.4
	5NH···CO(P478)	2.0
	2COO···HN(S654)	1.9
TQX-197	4C=O···HN(R485)	1.9
	4C=O···HN(T480)	2.0
	5NH···CO(P478)	1.77
	2COO···HN(S654)	1.7
	2COO···HO(S654)	2.3
TQX-173	4C=O···HN(R485)	1.7
	4C=O···HN(T480)	2.0
	5NH···CO(P478)	1.78
	2COO···HN(S654)	1.58
	2COO···HO(S654)	2.2

duced conformation of the iGluR2. In addition, the open state of the cleft induced by the herein docked compounds ensued by the direct interactions of the 2-carboxylate group with the hydroxylic oxygen of S654.

Slight differences in compound location interest the 7-Cl substituent. In fact, while the position and distance of the 7-chloro atom of the lead compounds (**TQX-173** and **TQX-197**) are optimal for a Van der Waals contact with M708, compounds **2a** and **2b** are not stabilized by this interaction with the same residue. This, at least partly, might account for the slightly lower AMPA binding affinities of compounds **2a** and **2b** with respect to the leads **TQX-197** and **TQX-173**.

In conclusion, the study on the herein reported compounds has allowed us to further explore the SAR of the TQX derivatives, affording some new AMPA receptor antagonists. Moving the crucial heteroaryl substituent from position-8 of **TQX-173** and **TQX-197** to position-9 gave rise, respectively, to **2a** and **2b** which are slightly less potent than the lead compounds. The binding mode of the 9-substituted heterocyclic compounds, as it results from docking experiments, does not substantially differ from that of the 8-substituted analogs belonging to the TQX series. Thus, the lower AMPA binding affinity of the 9-heteroaryl-TQX derivatives with respect to the 8-substituted ones, does not seem to be correlated with the movement of the substituent to position-9. In fact, the task which accommodates the 9-substituent seems to be large enough to tolerate the herein studied 9-heteroaryl moieties. In contrast, the weak interaction between the 7-chloro group and the receptor protein might explain the small difference in binding affinities of the herein reported compounds and leads. Nevertheless, the modification performed on TQX derivatives was not so lucky and does not warrant the synthesis of any other compound belonging to this series with a flat heteroaryl substituent at position-9.

Experimental

Chemistry Silica gel plates (Merck F_{254}) and silica gel 60 (Merck; 70—230 mesh) were used for analytical and column chromatography, respectively. All melting points were determined on a Gallenkamp melting point apparatus. Microanalyses were performed with a Perkin-Elmer 260 elemental analyzer for C, H, N, and the results were within ±0.4% of the theoretical values except where otherwise stated (Table 2). The IR spectra were recorded with a Perkin-Elmer 1420 spectrometer in Nujol mulls and are expressed in cm^{-1}. The ^{1}H-NMR spectra were obtained with a Varian Gemini 200 instrument at 200 MHz. The chemical shifts are reported in δ (ppm) and are relative to the central peak of the solvent. The following abbreviations are used: s=singlet, d=doublet, dd=double doublet, t=triplet, q=quartet, m=multiplet, br=broad, and ar=aromatic protons. The physical data of the newly synthesized compounds are shown in Table 2.

Ethyl *N*^{1}-(4-Chloro-2,6-dinitrophenyl)hydrazono-*N*^{2}-chloroacetate 6 Commercially available 2,6-dinitrophenylhydrazine (5.06 mmol) was portionwise added, in an overall time of 40 min, to a cooled (−10 °C) solution of $NaNO_2$ (5.06 mmol) in concentrated H_2SO_4 (15 ml). Ice (40 g), and then a cold solution of $NaBF_4$ (3.0 g) in water (7.5 ml), were carefully added to the reaction mixture rigorously kept below 5 °C. After elimination of the solid by filtration under reduced pressure, cold (0—5 °C) methanol (20 ml) and ethyl 2-chloro-3-oxobutanoate (1.6 ml) were added to the clear solution. The reaction mixture was kept at room temperature for 20 h. The yellow solid which precipitated was collected by filtration and washed with water. ^{1}H-NMR (DMSO-d_6) δ: 1.27 (t, 3H, CH_3, J=6.96 Hz), 4.26 (q, 2H, CH_2, J=6.96 Hz), 8.52 (s, 2H, ar), 10.92 (s, 1H, NH); IR. 3290, 3120, 3100, 1750. *Anal.* Calcd for $C_{10}H_8Cl_2N_4O_6$: C, 34.21; H, 2.30; N, 15.96. Found: C, 34.00; H, 2.07; N, 16.21.

Ethyl *N*^{1}-(4-Chloro-2,6-dinitrophenyl)-*N*^{2}-oxamidrazonate 7 Ammonia was bubbled until saturation into a stirred solution of **6** (3.42 mmol) in anhydrous dioxane (13 ml). The mixture was stirred at room temperature for 30 min and then was diluted with water (60 ml) to yield a red solid which was collected and washed with water. ^{1}H-NMR (DMSO-d_6) δ: 1.23 (t, 3H, CH_3, J=6.95 Hz), 4.17 (q, 2H, CH_2, J=6.95 Hz), 7.00 (s, 2H, NH_2), 8.32 (s, 2H, ar), 9.84 (s, 1H, NH); IR 3490, 3380, 3300, 1730, 1670. *Anal.* Calcd for $C_{10}H_{10}ClN_5O_6$: C, 36.21; H, 3.04; N, 21.12. Found: C, 35.99; H, 3.29; N, 21.35.

Ethyl *N*^{1}-(4-Chloro-2,6-dinitrophenyl)-*N*^{3}-ethoxalyl-*N*^{2}-oxamidrazonate 8 A solution of ethyloxalyl chloride (5.72 mmol) in anhydrous diethyl ether (14 ml) was dropwise added to a suspension of compound **7** (3.02 mmol) in anhydrous toluene (11 ml). Then, the reaction mixture was heated at reflux for 1.5 h. After evaporation of the solvent under reduced pressure, the resulting solid was worked up with diethyl ether (7 ml) and collected by filtration. ^{1}H-NMR (DMSO-d_6) δ: 1.20—1.35 (m, 6H, $2CH_3$), 4.18 (q, 2H, CH_2), 4.33 (q, 2H, CH_2), 8.44 (s, 2H, ar), 10.75 (s, 1H, N^1H), 11.25 (s, 1H, N^3H); IR 3340, 3200, 3110, 1745, 1730, 1720. *Anal.* Calcd for $C_{14}H_{14}ClN_5O_9$: C, 38.95; H, 3.27; N, 16.22. Found: C, 39.11; H, 3.47; N, 16.47.

Diethyl 1-(4-Chloro-2,6-dinitrophenyl)-1,2,4-triazolo-3,5-dicarboxylate 9 A suspension of compound **8** (2.1 mmol) in concentrated H_2SO_4 (8 ml) was kept at room temperature for 4.5 h. Then, the resulting solution was diluted with ice/water (180 ml) and extracted with ethyl acetate (120 ml×2). The two portions of ethyl acetate, put together, were washed with an aqueous solution of NaOH (0.5%, 120 ml×2) and then with water (100 ml×3). Evaporation of the dried (Na_2SO_4) organic layers to small volume (2.5 ml) gave a solid which was collected by filtration and washed with a little petroleum ether. ^{1}H-NMR (DMSO-d_6) δ: 1.19 (t, 3H, CH_3, J=7.0 Hz), 1.33 (t, 3H, CH_3, J=7.0 Hz), 4.26—4.45 (m, 4H, $2CH_2$), 8.96 (s, 2H, ar). IR 3100, 1735. *Anal.* Calcd for $C_{14}H_{12}ClN_5O_8$: C, 40.64; H, 2.92; N, 16.93. Found: C, 40.40; H, 3.09; N, 17.11.

Ethyl 9-Amino-7-chloro-4,5-dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]-quinoxaline-2-carboxylate 1a Iron powder (1.52 g) was added to a solution of **9** (1.52 mmol) in glacial acetic acid (1 ml). The mixture was heated at reflux for 5 h. Evaporation of the solvent at reduced pressure yielded a residue which was dried under vacuum and extracted in soxhlet with acetone (250 ml). Most of the solvent was evaporated to yield a solid which was collected by filtration. ^{1}H-NMR (DMSO-d_6) δ: 1.36 (t, 3H, CH_3, J=6.96 Hz), 4.43 (q, 2H, CH_2, J=6.96 Hz), 6.61 (s, 1H, ar), 6.71 (s, 1H, ar), 7.00 (s, 2H, NH_2), 12.34 (s 1H, NH); IR 3460, 3340, 1750, 1740, 1710, 1650, 1625. *Anal.* Calcd for $C_{12}H_{10}ClN_5O_3$: C, 46.84; H, 3.28; N, 22.76. Found: C, 46.99; H, 3.45; N, 22.61.

Ethyl 7-Chloro-4,5-dihydro-4-oxo-9-(1,2,4-triazol-4-yl)-1,2,4-triazolo-[1,5-*a*]quinoxaline-2-carboxylate 2a Diformylhydrazine (4.86 mmol) and then, drop by drop, trimethylsilyl chloride (24.3 mmol) and triethylamine (11.3 mmol), were added to a suspension of **1a** (1.62 mmol) in anhydrous pyridine (8.5 ml). The mixture was heated at 100 °C for 11 h. Evaporation of the solvent at reduced pressure yielded a solid which was treated with water (10 ml), collected by filtration and purified by silica gel column chromatography, eluting system $CHCl_3$/MeOH (9 : 1). Further purification was per-

formed by crystallization. ^{1}H-NMR (DMSO-d_6) δ: 1.30 (t, 3H, CH_3, J=6.96 Hz), 4.32 (q, 2H, CH_2, J=6.96 Hz), 7,65 (d, 1H, ar, J=2.20 Hz), 7.71 (d, 1H, ar, J=2.20 Hz), 8.70 (s, 2H, triazole H-2, H-5), 12.9 (br s, 1H, NH); IR 1750, 1710, 1610. *Anal.* Calcd for $C_{14}H_{10}ClN_7O_3$: C, 46.74; H, 2.80; N, 27.26. Found: C, 46.58; H, 2.56; N, 27.41.

Ethyl 7-Chloro-4,5-dihydro-4-oxo-9-(pyrrol-1-yl)-1,2,4-triazolo[1,5-*a*]-quinoxaline-2-carboxylate 3a A solution of 2,5-diethoxytetrahydrofuran (2.4 mmol) in glacial acetic acid (4.5 ml) was dropwise added to a suspension of **1a** (8.13 mmol) in glacial acetic acid (9 ml). The reaction mixture was heated at 90 °C for 20 min. Dilution with water (50 ml) afforded a solid which was collected by filtration and washed with water. ^{1}H-NMR (DMSO-d_6) δ: 1.27 (t, 3H, CH_3, J=6.96 Hz), 4.28 (q, 2H, CH_2, J=6.96 Hz), 6.23 (t, 2H, pyrrole H-3, H-4, J=2.2 Hz), 6.89 (t, 2H, pyrrole H-2, H-5, J=2.2 Hz), 7.39 (d, 1H, ar, J=2.2 Hz), 7.51 (d, 1H, ar, J=2.2 Hz), 12.7 (br s, 1H, NH); IR 1720, 1700. *Anal.* Calcd for $C_{16}H_{12}ClN_5O_3$: C, 53.72; H, 3.38; N, 19.58. Found: C, 53.91; H, 3.10; N, 19.71.

Ethyl 7-Chloro-4,5-dihydro-9-(3-formylpyrrol-1-yl)-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylate 4a A solution of 2,5-dimetoxytetrahydrofuran-3-carboxaldehyde (2.4 mmol) in glacial acetic acid (10 ml) was dropwise added to a suspension of **1a** (1.63 mmol) in glacial acetic acid (10 ml). The reaction mixture was heated at 90 °C for 15 min. After distillation of the solvent under reduced pressure, the resulting solid was worked up with a little ethanol, collected by filtration and purified by silica gel column chromatography, eluting system acetone. Evaporation at small volume of the first eluates gave a white solid which was collected by filtration, washed with petroleum ether and recrystallized. ^{1}H-NMR (DMSO-d_6) δ: 1.21 (t, 3H, CH_3, J=6.95 Hz), 4.24 (q, 2H, CH_2, J=6.95 Hz), 6.64 (s, 1H, pyrrole proton), 7.00 (s, 1H, pyrrole proton), 7.61 (s, 2H, ar), 7.78 (s, 1H, pyrrole proton), 9.77 (s, 1H, CHO), 11.9 (br s, 1H, NH); IR 1740, 1715, 1700, 1675. *Anal.* Calcd for $C_{17}H_{12}ClN_5O_4$: C, 52.93; H, 3.14; N, 18.15. Found: C, 53.09; H, 3.38; N, 17.95.

Ethyl 7-Chloro-4,5-dihydro-8-(3-carboxypyrrol-1-yl)-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylate 5a Solid potassium permanganate (0.85 mmol) was portionwise added to a cooled (0 °C) suspension of **4a** (0.83 mmol) in a 1 : 1 acetone/water mixture (10 ml). The reaction mixture was stirred at 0—3 °C for 48 h and then diluted with ice/water (30 g); the small excess of potassium permanganate was quenched with a 40% solution of sodium bisulfite and the resulting suspension was extracted with ethyl acetate (15 ml×4). Evaporation of the dried (Na_2SO_4) organic layers at small volume afforded a solid which was collected by filtration and purified by silica gel column chromatography (eluting system $CHCl_3$/MeOH 9 : 1). ^{1}H-NMR (DMSO-d_6) δ: 1.23 (t, 3H, CH_3, J=6.96 Hz), 4.24 (q, 2H, CH_2, J=6.96 Hz), 6.53 (s, 1H, pyrrole proton), 6.90 (s, 1H, pyrrole proton), 7.45 (s, 1H, pyrrole proton), 7.51 (d, 1H, ar, J=2.2 Hz), 7.55 (d, 1H, ar, J=2.2 Hz), 11.8 (br s, 1H, NH or OH); IR 1735, 1690. *Anal.* Calcd for $C_{17}H_{12}ClN_5O_5$: C, 50.82; H, 3.01; N, 17.43. Found: C, 51.03; H, 2.89; N, 17.27.

General Procedure for the Hydrolysis of the 2-Carboxylate Ethyl Esters 1a—3a An aqueous solution of NaOH (3%, 7.0 ml) was added to a suspension of the ethyl 2-carboxylate esters **1a**—**3a** (0.63 mmol) in EtOH (7.0 ml). The mixture was stirred at room temperature until the disappearance of the starting material (TLC monitoring, eluting system $CHCl_3$/MeOH 9 : 1). The solid was collected by filtration and dissolved in the minimal amount of water; the clear cold (5 °C) solution was acidified with HCl 6 N, and then kept at room temperature for 30 min. The solid which precipitated was collected by filtration and washed with water. Compounds **1b**—**3b** displayed the following spectral data:

9-Amino-7-chloro-4,5-dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylic Acid **1b**: ^{1}H-NMR (DMSO-d_6) δ: 6.56 (d, 1H, ar, J=2.21 Hz), 6.20 (d, 1H, ar, J=2.21 Hz), 7.17 (s, 2H, NH_2). IR 3500, 3365, 3095, 2800—2100, 200—1900, 1720. *Anal.* Calcd for $C_{10}H_6ClN_5O_3$: C, 42.95; H, 2.16; N, 25.04. Found: C, 43.16; H, 2.00; N, 25.16.

7-Chloro-4,5-dihydro-4-oxo-9-(1,2,4-triazol-4-yl)-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylic Acid **2b**: ^{1}H-NMR (DMSO-d_6) δ: 7.66 (d, 1H, ar, J=2.2 Hz), 7.72 (d, 1H, ar, J=2.2 Hz), 8.71 (s, 2H, triazole H-2, H-5), 12.86 (s, 1H, NH), 12.8 (br s, 1H, COOH); IR 3100—2000, 2000—1800, 1710. *Anal.* Calcd for $C_{12}H_6ClN_7O_3$: C, 43.45; H, 1.82; N, 29.56. Found: C, 43.68; H, 2.09; N, 29.39.

7-Chloro-4,5-dihydro-4-oxo-9-(pyrrol-1-yl)-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylic Acid **3b**: ^{1}H-NMR (DMSO-d_6) δ: 6.23 (t, 2H, pyrrole H-3, H-4, J=2.2 Hz), 6.92 (t, 2H, pyrrole H-2, H-5, J=2.2 Hz), 7.37 (s, 1H, ar), 7.50 (s, 1H, ar), 12.70 (s, 1H, NH); IR 3500—2600, 1720, 1465, 1375. *Anal.* Calcd for $C_{14}H_8ClN_5O_3$: C, 51.00; H, 2.45; N, 21.24. Found: C, 51.31; H, 2.29; N, 21.00.

7-Chloro-4,5-dihydro-8-(3-carboxypyrrol-1-yl)-4-oxo-1,2,4-triazolo-[1,5-*a*]quinoxaline-2-carboxylic Acid 4b An aqueous solution of NaOH (3%, 7.0 ml) was added to a suspension of **4a** (0.28 mmol) in EtOH (7.0 ml). The mixture was stirred at room temperature for 1 h, and then diluted with water (40 ml). The small amount of insoluble solid was filtered off and the resulting solution was further fined with charcoal and filtered again. The cold (5 °C) alkaline phase was acidified with HCl 6 N and then extracted with ethyl acetate (30 ml×4). Evaporation of the dried (Na_2SO_4) organic layers yielded a solid which was collected by filtration and washed with water. ^{1}H-NMR (DMSO-d_6) δ: 6.68 (s, 1H, pyrrole proton), 7.10 (s, 1H, pyrrole proton), 7.63 (s, 2H, ar), 7.86 (s, 1H, pyrrole proton), 9.80 (s, 1H, CHO); IR 3700—3300, 1700. *Anal.* Calcd for $C_{15}H_8ClN_5O_4$: C, 50.37; H, 2.25; N, 19.58. Found: C, 50.58; H, 2.41; N, 19.35.

Pharmacology. Binding Assay Rat cortical synaptic membrane preparation, [^{3}H]glycine and [^{3}H]AMPA binding experiments were performed following the procedures described in refs. 23 and 41, respectively. High-affinity [^{3}H]kainate binding assays were performed on rat cortical membranes according to previously reported methods.[31]

Electrophysiological Assay The mouse cortical wedge preparation described by Mannaioni *et al.*[42] was used, while the electrophysiological assays were performed following the procedures described in ref. 29.

Sample Preparation and Result Calculation A stock 1 mM solution of the test compound was prepared in 50% DMSO. Subsequent dilutions were accomplished in buffer. The IC_{50} values were calculated from three to four displacement curves based on four to six scalar concentrations of the test compound in triplicate using the ALLFIT computer program[43] and, in the case of tritiated glycine and AMPA binding, converted to K_i values by application of the Cheng–Prusoff equation.[44] In our experimental conditions the dissociation constants (K_D) for [^{3}H]glycine (10 nM) and [^{3}H]-DL-AMPA (8 nM) were 75±6 and 28±3 nM, respectively.

Molecular Modeling Compounds were modeled using the LigPrep Schrodinger ligands preparation procedure (pH 7.4) and minimized with Macromodel8.5.[45]

The experimental crystal structure of GluR2S1S2J/ATPO complex (PDB entry: 1n0t) was used in docking computation. Docking calculations were performed using the Schrodinger QM-Polarized Ligand docking protocol[38] that overcomes the assumption of standard molecular mechanics force field by assuming the charge distribution to be invariant to electrostatic fields of the surrounding environment. In the QM-polarized docking protocol,[38] the ligands are docked with Glide v4.0,[46] then charges are derived from quantum mechanical calculation on the ligand in the field of the receptor using QSitev4.0.[47] This procedure allows the polarization of the charges on the ligand by the receptor to be accounted for.[39,40] Redocking the ligands with these new charges can result in improved docking accuracy.

The crystal structure was prepared according to the protein preparation procedure recommended. Default input parameters were used in all computations. Upon completion of the docking calculation, three poses per ligand were saved. The best-docked structure was chosen using a model energy score (Emodel) derived from a combination of the Glide Score (Gscore, a modified and extended version of the empirically based ChemScore function[48]), Coulombic and the van der Waals energies and the internal strain energy of the ligands. All computations were performed on an Intel®Pentium®4 3 GHz processor running Linux.

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