Pharmacological Characterization of Some Selected 4,5-Dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylates and 3-Hydroxyquinazoline-2,4-diones as (*S*)-2-Amino-3-(3-hydroxy-5-methylisoxazol-4-yl)-propionic Acid Receptor Antagonists

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In the present study, some selected, previously reported 4,5-dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylates (TQXs) and 3-hydroxy-quinazoline-2,4-diones (QZs), were evaluated for their affinity at the (*S*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)-propionic acid (AMPA) receptor in the [^{3}H]-6-cyano-7-nitroquinoxaline-2,3-dione ([^{3}H]-CNQX) binding assay. Electrophysiological experiments were performed in oocytes expressing rat homomeric GluR3 subunits in order to assess the pharmacological profile of the tested compounds. The binding data, together with those regarding the functional activity, confirmed that most of the TQXs and QZs reported herein are potent AMPA receptor antagonists. When tested for their ability to prevent sound-induced seizures in DBA/2 mice, some of these derivatives showed anticonvulsant properties.

Key words competitive antagonist; triazoloquinoxaline; 3-hydroxyquinazoline; (*S*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)-propionic acid; audiogenic seizure; anticonvulsant

Glutamate (Glu), the primary excitatory neurotransmitter in the mammalian central nervous system (CNS), plays pivotal roles in regulating neuronal activity in many physiological processes such as synaptic plasticity, learning and memory.[1,2] Glu is also important for neuronal migration, differentiation, and for the establishment or elimination of synapses in the developing brain.[3—5] The machinery for signal input consists of glutamate receptors including G-protein coupled receptors (metabotropic receptors or mGluRs), and ligand–gated ion channels (ionotropic receptors or iGluRs).[6] The latter are divided into three distinct subtypes, namely (*S*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)-propionic acid (AMPA), kainate (KA), and *N*-methyl-D-aspartic acid (NMDA) receptors, according to the synthetic agonist that activates them preferentially. The NMDA ion–channel complex possesses different binding sites including the glycine coagonist binding site (Gly/NMDA).[7] Based on molecular cloning seven subunits (NR1, NR2A-D, and NR3A-B) for the NMDA receptor, four (GluR1-4) for the AMPA and five (GluR5-7, KA1-2) for the KA receptor have been identified.[8—10]

There is considerable evidence that GluRs are involved in many neurological processes in the diseased CNS. In fact, glutamatergic hyperactivity can induce an increase of intracellular free Ca^{2+} levels and cause collapse of mitochondrial function, potentially leading to secondary neurotoxic events and cell death. These processes are implicated in a large number of acute and chronic neurodegenerative pathologies such as cerebral ischemia,[11] epilepsy,[2] Alzheimer's[12,13] and Parkinson's[14] diseases, and amyotrophic lateral sclerosis (ALS).[15] A large body of data indicate that glutamatergic neurotransmission is involved in nociceptive reflexes at the spinal cord level.[16]

Several studies indicated that many compounds acting as iGluR antagonists have beneficial effects against neurodegenerative disorders.[17—20] These findings gave life to extensive investigations of AMPA receptor antagonists belonging to the classical quinoxalinedione and heterocyclic-fused quinoxalinedione series most of which are endowed with interesting pharmacological profiles.[21]

The success of AMPA receptor antagonists over other well pharmacologically-characterized iGluR antagonists is probably due to their greater clinical potential. In fact, most of the NMDA receptor antagonists, though showing interesting neuroprotective activity in many animal models of cerebral ischemia, also produced important adverse effects including psychotomimetic activity.[22]

In the near future, the emerging clinical application for competitive AMPA receptor antagonists will probably be as neuroprotectants in neurodegenerative diseases, such as epileptic conditions,[23—26] as an alternative to current therapies. In fact, most of the currently used antiepileptic drugs (AEDs) produce adverse effects, and show to be ineffective in refractory epilepsy.[27] As approximately 0.5—2% of the world population is affected by epilepsy, the development of safer and more effective new AEDs is necessary.[28]

Earlier, we have reported extensive studies on a series of 4,5-dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylates (TQXs, Fig. 1), bearing different substituents on the fused benzo moiety, which are endowed with high binding affinity and selectivity for the AMPA receptor.[29—32]

All the newly synthesized compounds were biologically evaluated for their binding at the AMPA, KA and Gly/NMDA receptors. KA and Gly/NMDA binding assays were performed in order to assess selectivity toward the AMPA receptor. These studies established that the presence of a N^3-nitrogen containing heterocycle at position-8 of the TQX framework is an essential feature for potent and selective AMPA receptor antagonists. Moreover, the presence of potent electron-withdrawing groups, such as chlorine atom, trifluoromethyl or nitro substituents at position-7 on the fused benzo-ring positively influences potency and selectivity to-

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ward the AMPA receptor.[30—32] The obtained results pointed out that the 4,5-dihydro-7-nitro-4-oxo-8-(3-carboxypyrrol-1-yl)-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylic acid and its corresponding ethyl ester (compound **6**, Table 1) are the most potent and selective AMPA receptor antagonists among the TQX series.[32] Some selected compounds were also tested for their functional antagonism at the AMPA receptor, thus confirming the antagonistic activity of these derivatives.[30—32] In contrast, no data have been published on the *in vivo* pharmacological effects of this series.

In the meantime, further investigations in our laboratory disclosed the 3-hydroxy-quinazoline-2,4-dione (QZ, Fig. 1) as a useful scaffold to obtain selective iGluR antagonists.[33,34]

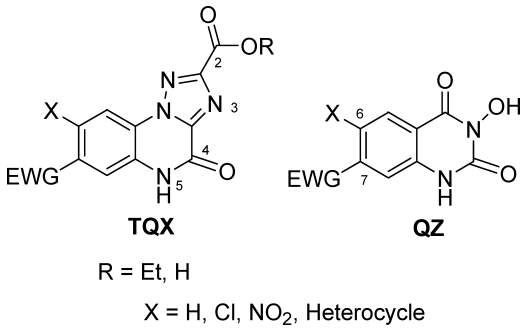


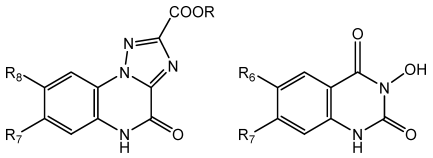
Fig. 1. TQX and QZ Derivatives

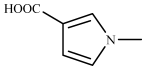
The structure–activity relationships (SARs) of the newly synthesized derivatives at the AMPA receptor were similar to those of the previously reported TQX compounds, thus suggesting a similar binding mode. In fact, in the AMPA receptor binding assays, the 7-chloro-6-(1,2,4-triazol-4-yl)-3-hydroxy-quinazoline-2,4-dione (compound **8**, Table 1) was equipotent to the equally substituted TQX derivative, but more selective.[33]

In the present study, some selected TQX and QZ derivatives were evaluated for their binding affinity at the AMPA receptor in the [^{3}H]-CNQX binding assay[35] in order to confirm previously reported data. The functional activity was measured in *Xenopus* oocytes expressing homomeric rat GluR3 AMPA receptors,[36] using electrophysiological measurements. Moreover, the anticonvulsant properties of these derivatives were estimated against audiogenic seizures (AS) in DBA/2 mice, which is considered an excellent animal model for generalized epilepsy and for screening new anticonvulsant drugs.[37]

In order to be useful therapeutic agents, compounds acting at the CNS level should possess good blood–brain barrier (BBB)-penetration properties. In fact, CNS activity (and membrane permeability, in general) is a complex function of physical/chemical properties of molecules, such as size, lipophilicity and hydrogen-bonding potential. However, for any given molecule, one of these factors may dominate others. Thus, in an effort to explain the newly obtained re-

Table 1. Previously[a] and Currently Reported Binding Affinity at AMPA Receptor, Functional Activity in *Xenopus* Oocytes and Anticonvulsant Effect against AS in DBA/2 Mice of TQX (**1**—**6**) and QZ (**7**, **8**) Derivatives



n	R	R_7	R_8/R_6	pIC_{50}[b] [^{3}H]CNQX	IC_{50} (μM)[c] Oocytes GluR3 (95% confidence limits)	% protection against tonic seizures @ 30 mg/kg Administration *p.o.*	i.p.	MW/c Log P/PSA
1	Et	CF_3		6.23±0.005 (*n*=2)	2.62 (2.09—3.28)	6%	30%	393.29/−0.35/121
2	H	CF_3		7.47±0.02 (*n*=3)	0.4 (0.28—0.4)	N.T.[d]	70%	365.23/−0.11/131.58
3	H	CF_3		7.67±0.07 (*n*=2)	0.38 (0.35—0.41)	0%	100% (*)[e]	364.25/1.02/119
4	H	CF_3		7.58±0.08 (*n*=2)	0.34 (0.3—0.38)	0%	29%	363.26/2.33/106
5	H	NO_2		7.58±0.01 (*n*=2)	N.T.	N.T.	0%	340.26/1.45/152
6	Et	NO_2		7.00±0.03 (*n*=3)	(**)[f]	N.T.	83%	412.32/0.99/178
7	—	Cl	NO_2	6.21±0.09 (*n*=2)	5.26 (4.0—6.9)	6%	10%	257.59/−0.59/121
8	—	Cl		6.47±0.04 (*n*=2)	1.35 (1.17—1.56)	6%	80%	279.64/−1.31/106

a) Refs. 32, 33. *b*) IC_{50}=concentration necessary for 50% inhibition. The IC_{50} values are means±S.E.M. of (*n*) separate experiments, obtained from 9 concentrations of each compound, run in triplicate. *c*) Concentration necessary for 50% inhibition (IC_{50}). The IC_{50} values are obtained from 9 concentrations of each compound, run in triplicate. *d*) N.T.=not tested. *e*) 38% of protection @ 10 mg/kg. *f*) Compound precipitated.

sults, we evaluated the hydrophobic pattern of the tested compounds by calculating molecular weight (MW), the calculated octanol/water partition coefficient (c Log P), polar surface area (PSA) (Table 1), and number of H-bond acceptors and donors (not shown).The rule-of-five is now well accepted as a qualitative absorption/permeability predictor.[38] It can be hypothesized that compounds are most likely to have poor absorption when their MW is $\geq$500, c Log P is $>$5, number of H-bond donors is $>$5, number of H-bond acceptors is $>$10, and PSA is $\geq$140 Å^2. The PSA, in addition to being one of the predictive descriptors for BBB crossing together with MW and c Log P parameters, is also well correlated with oral absorption.[39—41]

Results and Discussion

In this study, the triazoloquinoxalines **1**—**6**[32] and the quinazolines **7**, **8**[33] were tested in the [^{3}H]-CNQX binding assay[35] in order to confirm their affinity for the AMPA receptor. Compounds **2**—**6** are the most active, showing IC_{50} values below 0.1 μM. By comparing previously reported binding data with currently obtained results (Table 1), it emerged that all the tested derivatives **1**—**8** broadly showed the expected potencies in the herein used binding assay.

To determine functional antagonism at the AMPA receptor, experiments were performed on Xenopus oocytes expressing rat GluR3 AMPA receptor subunits.[36] GluR3 plays a specific functional role in the CNS and it is also involved in neurological diseases.[42] In oocytes expressing GluR3 subunit, the TQX derivatives **2**—**4** antagonized L-Glu-induced currents with IC_{50} values in the low micro-molar range, showing to be the most potent antagonists among the tested compounds. These functional data are in accordance with AMPA receptor binding results.

Derivatives **1**—**8** were also evaluated for their *in vivo* anticonvulsant activity against audiogenic seizures in the DBA/2 mice (Table 1).[37] Compounds were intra-peritoneally (*i.p.*) or *per so* (*p.o.*) administered with a pretreatment time of 1 h at a dose of 30 mg/kg. While compounds **2**, **3**, **6** an **8** gave protection against tonic seizures when administered *i.p.*, none of the tested compounds was orally active in this test. This inactivity could be explained on the basis of their PSA values. In fact, even though 140 Å^2 is the highest PSA value permitted for BBB penetration according to the now accepted criteria of druglikeness,[40,41] orally administered drugs with PSA $>$120 Å^2 are in general hardly absorbed by the passive transcellular route.[39—41] In contrast, drugs with a low PSA value ($<$60 Å^2) could be almost completely absorbed. Most of the compounds reported herein have PSA values $\geq$120 Å^2 with the only exceptions being derivatives **4** and **8** whose PSA values are however $>$60 Å^2. Nevertheless, the calculated log P (c Log P) reported here, indicated most of the TQX and QZ compounds as sufficiently lipophilic for penetrating biological membranes.

The 7-trifluoromethyl-8-(imidazol-1-yl)-2-carboxylic acid derivative **3** was the most potent compound, among the herein reported, in the *in vivo* anticonvulsant test when administered *i.p.* It turned out to be active at 30 mg/kg after a pretreatment time of 1 h, leading to a 100% protection against tonic seizures. This result could be indicative of good BBB penetration and long duration of action. However, at the dose of 30 mg/kg, sedation was observed as the side effect in all the animals, and two animals, laying on their sides, showed severe ataxia. Thus, compound **3** was retested in the anticonvulsant test at 10 mg/kg, which led to a 38% protection against tonic seizures. No side effect was observed at this lower dose.

The ethyl 7-nitro-8-(3-carboxypyrrol-1-yl)-2-carboxylate derivative **6** showed a protection in 80% of the animals (83%) against tonic seizures in the AS model for epilepsy in DBA/2 mice, despite its high PSA value of 178. The side effects observed with this compound were important at the dose of 30 mg/kg. The most frequently observed (8/10 animals) ones were: stereotype behaviour, scratching movements, ptosis and hypotonia. Two animals also exhibited dyspnoea. In contrast, it was surprising that 2/10 animals did not have any side effects. This result suggests that, in general, the 7-trifluoromethyl-substituted derivatives could be preferentially chosen as drug candidates over the nitro-analogues. Such prudence is also supported by the fact that the nitro-group is often reported to have a negative impact on the pharmacokinetic profile of compounds and may also cause toxicity problem.[43]

Among the QZ derivatives, compound **8** showed 80% protection in the AS anticonvulsant test despite its low c log P value of $-$1.31 which is lower than those usually expected to allow brain penetration.

The 7-trifluoromethyl-8-(1,2,4-triazol-4-yl)-2-carboxylic acid derivative **2** was one of the most potent TQX compounds in both the [^{3}H]-CNQX binding assay and the functional test. It also showed anticonvulsant activity, giving 70% protection against tonic seizures. In contrast, its corresponding ethyl ester (compound **1**) was markedly less active than **2** as AMPA receptor antagonist both in the binding assay and in the functional test. The low anticonvulsant activity of **1** could be partly explained on the basis of its modest AMPA binding affinity as shown in the [^{3}H]-CNQX assay. The same can be hypothesized for the QZ compound **7**. In contrast, the remaining 7-trifluoromethyl- and 7-nitro-8-(pyrrol-1-yl)-2-carboxylic acid derivatives **4** and **5**, though being two of the most potent compounds in the binding test, gave, respectively, low and null protection against seizures in the convulsion model employed here after *i.p.* administration. Thus, this inactivity might be due to a very poor permeability across the BBB or to a low water solubility.

In conclusion, this work pointed out that some of the TQX and QZ derivatives, evaluated in this study, showed anticonvulsant properties in the AS model in DBA/2 mice when administered *i.p.* However, they were not active when tested in the same model of convulsion after oral administration. These results prompt us to develop new compounds belonging to these series in order to improve their physicochemical properties. Further modifications of TQX compounds are in progress.

Experimental

Pharmacology Whole rat brain membranes, prepared according to standard procedures, were obtained from Analytical Biological Services Inc. (ABS, Wilmington, DE, U.S.A.).

[^{3}H]CNQX Binding Assay (AMPA Receptor) The assay was performed in 96-well microtiterplates in a final volume of 0.25 ml. The assay mixture contained 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES)/KOH, pH 7.2, membranes (*ca.* 70 μg protein), 10 nM [^{3}H]-CNQX (*ca.* 20 Ci/mmol, NEN Perkin Elmer, Italy) and the compound to be tested at the appropriate concentrations. To the samples for measuring non-

specific binding 10 μM unlabeled CNQX was added. The plates were incubated at 4 °C for 60 min before bound and free radioligand were separated by centrifugation at 3700×***g*** for 30 min. The pellet was washed once with cold buffer and then dissolved in 0.02 ml biolute-A/propanol (1 : 1) for 20 min. Two hundred microliters Microscint 20 (Packard) was added and the radioactivity was counted in a Packard Topcount scintillation counter.

Electrophysiological Assay. Oocyte Electrophysiology Oocyte experiments were performed as described previously.[44] Briefly, plasmids coding for the rat GluR3-(flop) receptor[36] were linearized and transcribed into capped cRNA using an *in vitro* RNA synthesis kit (mCAP mRNA capping kit, Stratagene, Basle, Switzerland) with T7 Polymerase. Rat GluR3-(flop) receptors were expressed as homomers. Oocytes were maintained at 16 °C in a solution containing 88 mM NaCl, 10 mM HEPES, pH 7.5, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.82 mM $MgSO_4$, 0.34 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 100 units/ml penicillin and 100 units/ml streptomycin. The medium was changed every second day. Oocytes were incubated for at least 1 d prior to recording, to allow receptor expression. For recording oocytes were placed in a perfusion chamber and continuously perfused with Mg^{2+} containing Ringer (10 mM HEPES; pH 7.2; 115 mM NaCl; 5 mM KCl; 1.8 mM $CaCl_2$).

All compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted in Ringer solution and applied to the recording chamber by gravity. The compounds were given in a cumulative manner together with the agonist L-Glu.

Two-electrode voltage-clamp recordings were performed with the membrane potential clamped to −70 mV using a digital data acquisition system (New Visions Engineering, Winterthur, Switzerland). Data were analyzed using Prism 3.0 software (GraphPad, San Diego, U.S.A.). Baseline current drifts were corrected using linear interpolations. For concentration–response curves, the induced inward current was measured 2 s prior to the application of the next higher concentration. Data from different oocytes were normalized and fitted by the following equation using a non-linear regression (Eq. 1):

$$\text{calculation of half maximal effects} \quad I(c) = I_{\min} + \frac{I_{\max} - I_{\min}}{1 + 10^{n(\log(IC_{50}) - \log(c))}} \tag{1}$$

with $I(c)$ as the current amplitude evoked by the agonist concentration c, I_{min} as the asymptotic minimal (fixed to 0%) and I_{max} as the asymptotic maximal current (fixed to 100%), IC_{50} as the half-maximal inhibiting concentration and n as the Hill coefficient. Errors of IC_{50} values and Hill coefficients were calculated from the covariance matrix by the fitting routine (Prism 3.0). IC_{50} values are given as mean (lower to upper boundaries of the 95% confidence interval) if not stated otherwise.

***In Vivo* Experiments. Audiogenic Seizures** AS were elicited in DBA/2 mice. Briefly, DBA/2 mice were purchased one week ahead of testing. For testing, 20-day-old animals were placed in a sound attenuated chamber. Following a 60 s habituation period the animals were stimulated using band limited noise (14—20 kHz, 118 dB SPL) lasting for maximally 60 s. Animals that did not show respiratory arrest during acoustic stimulation were immediately euthanised in CO_2.

The compounds were dissolved in 0.2 N NaOH, cremophor EL 10% was added and the pH was adjusted to 7 by adding 0.1 N HCl. A volume of 10 ml/kg was used for oral and intraperitoneal application. Doses of 10 or 30 mg/kg were applied. Seizure tests were performed 1 h subsequent to the compound application. Ten animals were used for a dose group. For every batch of animals a control group (n=5) was tested. The control groups received the NaOH/cremophor/HCl solution *p.o.* or i.p. at a volume of 10 ml/kg.

For data analysis the occurrence of the different behavioural phases was measured. The following phases formed the basis for classification: wild running, clonic seizure, tonic seizure, and respiratory arrest. In the present report only the percentage protection from tonic seizures is given.

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