# *Brief Articles*

## Design, Synthesis, and Pharmacology of a Highly Subtype-Selective GluR1/2 Agonist, (*RS*)-2-Amino-3-(4-chloro-3-hydroxy-5-isoxazolyl)propionic Acid (Cl-HIBO)

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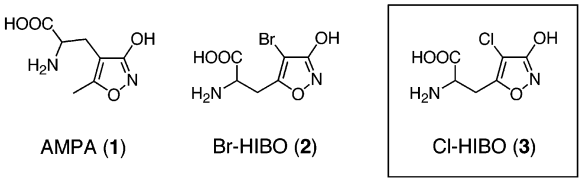
*Received December 27, 2002*

On the basis of structural studies, chloro-homoibotenic acid (Cl-HIBO) was designed and synthesized. Cl-HIBO was characterized in binding and electrophysiology experiments on native and cloned subtypes of GluRs. Electrophysiological selectivities ranged from 275 to 1600 for GluR1/2 over GluR3/4. The potent AMPA receptor activity was strongly desensitizing and the neurotoxicity similar to AMPA. Thus, Cl-HIBO is the most subtype selective agonist reported to date on GluR1/2, and offers a new standard for selectively studying subtypes of AMPA receptors.

**Introduction.** Glutamic acid (Glu) is the major excitatory neurotransmitter in the central nervous system (CNS) and activates two major classes of receptors, the ionotropic and metabotropic Glu receptors (iGluRs and mGluRs). Both receptor classes play important roles in the CNS, and the involvement of Glu receptor systems in memory and learning processes and in a number of neurological disorders make Glu receptors important therapeutic targets.[1−4] The iGluRs mediate fast excitatory responses via the opening of ligand-gated ion channels, whereas the mGluRs exhibit slower modulatory effects via G-protein-coupled receptors. The iGluRs are divided into (*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA, **1**), kainic acid (KA), and *N*-methyl-D-aspartic acid (NMDA) receptors,[1] while the mGluRs are divided into groups I, II, and III.[4]




AMPA receptors assemble as homo- or heteromeric tetramers from various combinations of the four different receptor subunits GluR1−4.[5,6] Very few subtype-selective AMPA receptor agonists are known. We have previously shown that (*RS*)-2-amino-3-(4-bromo-3-hydroxy-5-isoxazolyl)propionic acid (bromo-homoibotenic acid, Br-HIBO, **2**) has selectivity for GluR1 vs GluR3 in receptor binding and functional assays.[7] The structural basis of this selectivity was first modeled and confirmed by mutation studies,[8] and recently characterized by X-ray crystallography,[9] as arising via preferential hydrogen bonding of the distal anion through a water molecule to a nonconserved GluR1/2 binding site tyrosine (Y702 in GluR1/2, F in GluR3/4, see Figure 1). This prompted us to further structurally modify the homoibotenic acid structure in order to develop a more selective compound. This paper describes the design, synthesis, in vitro pharmacology, and excitotoxicity of the new analogue chloro-homoibotenic acid (Cl-HIBO, **3**).

**Design.** We hoped to increase selectivity by increasing the hydrogen bond acceptor strength and electron density around the 3-position oxyanion of the carboxylate bioisostere of homoibotenic acid. A stronger interaction with water molecule W1 (Figure 1A) ought to favor GluR1/2 binding while the cost of burying the negative charge in a hydrophobic region (Figure 1B) should penalize binding to GluR3/4. Automated docking[10] of Cl-HIBO to the complexes of Br-HIBO with GluR2-S1S2J and (Y702F)GluR2-S1S2J[9] gave more favorable energy scores and a greater differentiation between these constructs than seen with Br-HIBO. Density Functional Theory calculations on a model system revealed a stronger complex between Cl-HIBO and W1 than for Br-HIBO ($\Delta\Delta G$ = 0.33 kcal/mol). In addition, receptor binding domain closure, selectivity, and desensitization properties seem to be acutely sensitive to substituent size in this region in parallel series,[9,11] indicating that this alteration might produce a novel pharmacological profile.

**Chemistry.** Homoibotenic acid has previously been synthesized through intermediate **4**[12] (Scheme 1). Using this intermediate, Cl-HIBO (**3**) was synthesized in a two-step procedure by chlorination with $SO_2Cl_2$ in 75%

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10.1021/jm020588f CCC: $25.00 
Published on Web 04/04/2003

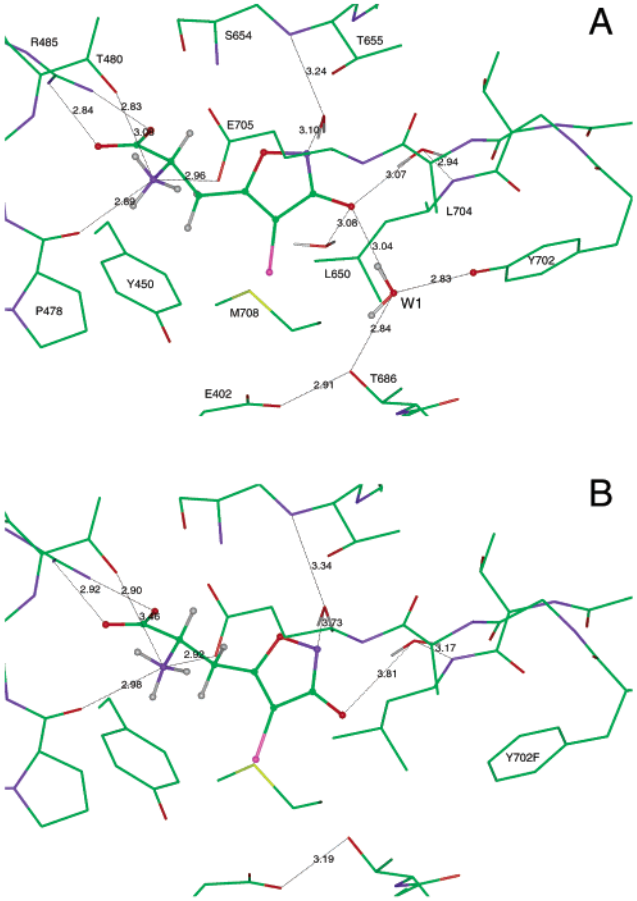
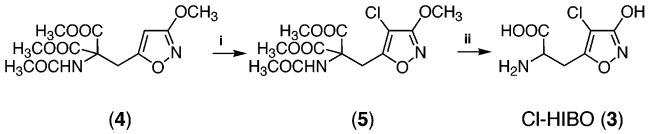



**Figure 1.** Cl-HIBO cominimized (MMFFs)[10] within the AMPA receptor binding site. RMSD under minimization 0.5 Å. (A) Coordinates taken from Br-HIBO:GluR2-S1S2J crystal structure (RCSB Protein Data Bank:1M5C). W1: water molecule observed in all native GluR2-agonist complexes but no (Y702F)-GluR2 structures. Distal anion is tightly bound via W1 to Y702 and the domain-bridging T686. (B) Coordinates taken from GluR3-like Br-HIBO:(Y702F)GluR2-S1S2J complex (RCSB Protein Data Bank:1M5D). In the mutant, the distal anion forms fewer, weaker interactions with domain 2 (right side) due to the loss of the water network accompanied by minor hydrophobic side chain rearrangement.

yield, followed by full deprotection of compound **5** to give Cl-HIBO (**3**) after refluxing in 6 M HCl. Cl-HIBO (**3**) was isolated in 75% yield as a zwitterion after pH adjustment with triethylamine and recrystallization.

**Pharmacology. 1. Receptor Binding Assays.** Receptor binding affinities of Cl-HIBO were determined both at native and recombinant receptors. In binding assays using [$^3$H]AMPA, [$^3$H]KA, and [$^3$H]CGP39653 to represent native AMPA, KA, and NMDA receptors, respectively, high affinity for AMPA receptors was observed ($IC_{50}$ = 0.22 $\mu$M). Affinity for NMDA receptors was approximately 2 orders of magnitude lower, and no significant affinity for KA receptors was observed (Table 1). The affinity at AMPA receptors was studied further on homomeric GluR1−4 expressed in Sf9 insect cells, where Cl-HIBO exhibited very high selectivity for GluR1 and 2 with respect to GluR3 and 4 (Table 2). The selectivity ratio was highest for GluR1 vs GluR3 (greater than 200 times). Affinity was also determined at the KA receptor subtypes GluR5 and GluR6, and Cl-HIBO was found to have only weak affinity for GluR5 and no affinity for GluR6 ($K_i$ > 100 $\mu$M) (Table 2).

**Scheme 1**[a]




[a] (i) $SO_2Cl_2$; (ii) 6 M HCl.

**Table 1.** Receptor Binding and Electrophysiological Data at Native IGluRs

| | [$^3$H]AMPA $IC_{50}$ ($\mu$M) | [$^3$H]KA $IC_{50}$ ($\mu$M) | [$^3$H]CGP39653 $K_i$ ($\mu$M) | electrophysiology $EC_{50}$ ($\mu$M) |
|---|---|---|---|---|
| AMPA[a] | 0.040 ± 0.005 | >100 | >100[b] | 3.5 ± 0.2 |
| Br-HIBO | 0.65 ± 0.12[c] | >100 | 34 ± 1 | 22 ± 0.6[c] |
| Cl-HIBO | 0.22 ± 0.01 | >100 | 18 ± 1 | 39 ± 3.4 |

Mean ± SEM from at least three experiments. [a]Reference 13. [b] [$^3$H]CPP. [c] Reference 14.

**2. Electrophysiology Assays.** The electrophysiological properties were studied on native iGluRs using the rat cortical slice model and at recombinant homomeric rat GluR1−4 expressed in *Xenopus laevis* oocytes. At native receptors, Cl-HIBO was almost equipotent with Br-HIBO as an AMPA receptor agonist (Table 1). The activity of Cl-HIBO could be fully antagonized by the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline (NBQX) as with Br-HIBO and AMPA itself. At recombinant GluR1−4, a similar selectivity profile for Cl-HIBO was observed as was found in the binding studies. However, the selectivity for GluR1 over GluR3 was even more pronounced (Table 3). The observed selectivity ratio of Cl-HIBO between GluR1 and GluR3 was almost 600, whereas Br-HIBO showed a selectivity ratio of approximately 14. High activity was observed at both GluR1 and GluR2 and very low activity at GluR3 and GluR4. Concentration−response curves for Cl-HIBO at GluR1−4 are shown in Figure 2.

The receptor desensitization properties of Cl-HIBO were studied in experiments with or without cyclothiazide, which blocks receptor desensitization. Like Br-HIBO and AMPA, on oocytes expressing GluR1, Cl-HIBO proved to be a strongly desensitizing agonist. Addition of cyclothiazide resulted in a more than 60-fold potentiation of the steady-state response for Cl-HIBO compared to an approximately 25-fold increase for Glu (results not shown). Currents evoked under blockade of receptor desensitization with cyclothiazide were of the same magnitude as those evoked by Glu (Figure 3A), whereas the currents for Cl-HIBO and Br-HIBO without cyclothiazide present were lower than for Glu (Figure 3B). Thus, Cl-HIBO is a strongly desensitizing full agonist at GluR1.

**3. Metabotropic Receptor Assays.** Activity at mGluRs was studied in second messenger assays on mGluR1α, mGluR2 and mGluR4a, representing groups I, II and III. As with Br-HIBO, Cl-HIBO proved inactive, apart from weak activity at mGluR1α. The antagonist activity observed at mGluR1α ($K_i$ = 860 ± 160 $\mu$M) is 2−3 times weaker than that of Br-HIBO ($K_i$ = 329 ± 6 $\mu$M). Neither compound showed agonist or antagonist activity at 1 mM at mGluR2 or mGluR4a.

**4. Excitotoxicity Assay.** Neurotoxicity was determined in cultured mouse cortical neurons. Cl-HIBO induced cytotoxicity to the same extent as AMPA and

**Table 2.** Receptor Affinity at Cloned Subtypes Expressed in Sf9 Cells[a]

| | $GluR1_o$ $K_i$ (nM) | $GluR2(R)_o$ $K_i$ (nM) | $GluR3_o$ $K_i$ (nM) | $GluR4_o$ $K_i$ (nM) | GluR5(*Q*) $K_i$ (nM) | GluR6(*V,C,R*) $K_i$ (nM) |
|---|---|---|---|---|---|---|
| AMPA | 22 ± 4 | 17 ± 3 | 21 ± 3 | 40 ± 20 | 740 ± 260 | >100000 |
| Br-HIBO | 170 ± 64 | 250 ± 67 | 12000 ± 4100 | 11700 ± 1500 | 4800 ± 600 | >100000 |
| Cl-HIBO | 130 ± 26 | 370 ± 24 | 27500 ± 6400 | 11900 ± 2700 | 6800 ± 2200 | >100000 |

[a] Mean ± SD from at least three experiments.

**Table 3.** Electrophysiology on Cloned Receptors Expressed in *Xenopus* Oocytes and Toxicity on Mouse Cortical Neurons[a]

| | $GluR1_i$ $EC_{50}$ ($\mu$M) | $GluR2(Q)_o$ $EC_{50}$ ($\mu$M) | $GluR3_i$ $EC_{50}$ ($\mu$M) | $GluR4_i$ $EC_{50}$ ($\mu$M) | toxicity $EC_{50}$ ($\mu$M) |
|---|---|---|---|---|---|
| AMPA | 8.7 ± 1.3[b] | - | 1.4 ± 0.2[b] | - | 13 ± 3 |
| Br-HIBO | 14 ± 2 | 5.4 ± 1.3 | 200 ± 68 | 38 ± 4 | 16 ± 4 |
| Cl-HIBO | 4.7 ± 1.1 | 1.7 ± 0.6 | 2700 ± 350 | 1300 ± 130 | 27 ± 8 |

[a] Mean ± SEM from at least three experiments. [b] Reference 15 (at *flop* receptors).

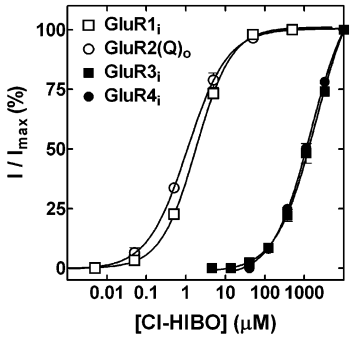



**Figure 2.** Concentration−response curves for Cl-HIBO-induced currents from *Xenopus* oocytes expressing $GluR1_i$, $GluR2(Q)_o$, $GluR3_i$, or $GluR4_i$.

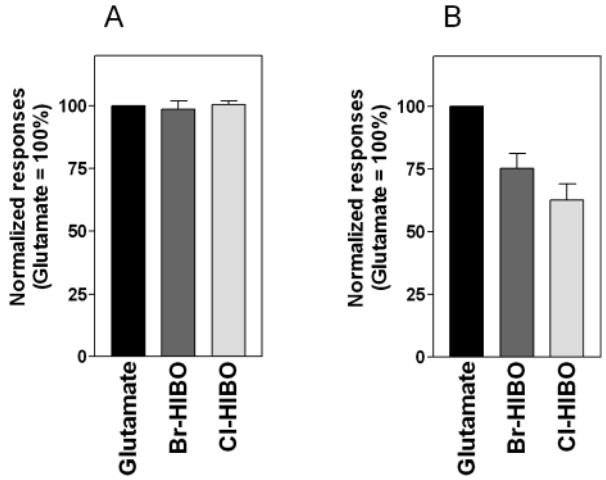



**Figure 3.** Levels of agonist-induced (Glu, Br-HIBO, or Cl-HIBO at saturating concentrations) currents from *Xenopus* oocytes expressing $GluR1_i$ in the presence (A) or absence (B) of 100 $\mu$M cyclothiazide, normalized to the mean maximal Glu response of each oocyte ($n$ = 4 oocytes, with three or more replicates per drug per oocyte).

Br-HIBO and with similar potency (Figure 4A and Table 3). The toxicities of the three agonists were dependent upon the presence of cyclothiazide, characteristic for strongly desensitizing agonists. Furthermore, the toxicity of the three agonists could be antagonized by the presence of the AMPA receptor antagonist NBQX (Figure 4B).

**Conclusions.** Subtype selective agonist activity has previously been reported for Br-HIBO (**2**), showing 14-fold selectivity for GluR1 compared to GluR3 in electrophysiology experiments on *Xenopus* oocytes. Other compounds have been reported with similar subtype selectivity. Some uracil derivatives such as (*S*)-willardiine[11] and (*S*)-1-(2-amino-2-carboxyethyl)-6,7-dihydro-1*H*-cyclopentapyrimidine-2,4(1*H*,3*H*)-dione [(*S*)-CPW-399][16] have shown preference for GluR1/2. The latter compound is distinguished by being a partially desensitizing agonist and thus a potent neurotoxin. The highest selectivity of (*S*)-CPW399 in the electrophysiology experiments was 16-fold for GluR2 over GluR3.

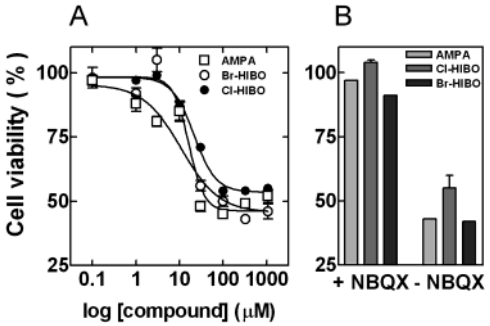



**Figure 4.** Agonist-induced neurotoxicity in mouse cortical neurons in the presence of 50 $\mu$M cyclothiazide. (A) Cell viability after 6 h incubation with AMPA, Cl-HIBO, or Br-HIBO. (B) Cell viability after 6 h incubation with 100 $\mu$M agonist in the presence or absence of 50 $\mu$M of NBQX.

Molecular modeling studies based on the binding mode of Br-HIBO at GluR2 and the GluR3-like (Y702F)-GluR2 suggested that substitution of the 4-bromo atom for a 4-chloro atom could lead to enhanced subtype selectivity. Synthesis and pharmacological characterization of Cl-HIBO (**3**) did indeed show a substantial enhancement of the GluR1/2 selectivity. The approximate increase in binding selectivity is consistent with the increase in hydrogen bond acceptor strength of the 3-position oxyanion, in support of this model for fine-tuning GluR1/2 vs GluR3/4 selectivity. Cl-HIBO shows an even more pronounced selectivity in the electrophysiological studies performed on *Xenopus* oocytes expressing homomeric GluR1−4. The observed selectivities of Cl-HIBO range from a ratio of approximately 275 for GluR1 over GluR4 to a ratio of almost 1600 for GluR2 over GluR3. The greater selectivity observed in the electrophysiology experiments compared to the binding studies (Tables 3 and 2, respectively) may be explained by the desensitizing properties of Cl-HIBO. The neurotoxicity profile observed for Cl-HIBO is similar to those of AMPA and Br-HIBO and depends on the presence of cyclothiazide, showing that Cl-HIBO is a full and strongly desensitizing agonist. In light of its unprecedently high selectivity for GluR1/2, we expect that Cl-HIBO will be a valuable tool for studying AMPA receptors, and in particular for investigating the pharmacological behavior and distribution of AMPA receptor subpopulations.

**Acknowledgment.** The financial support from the Lundbeck Foundation, the Novo Nordisk Foundation, the Novo Scholarship Program, the Danish Medical Research Counsil, the Augustinus Foundation, the Ib Henriksen Foundation and the donation of computing resources by the Australian Centre for Advanced Computing and Communications are gratefully acknowledged.

**Supporting Information Available:** Experimental details (chemistry, pharmacology, docking and quantum mechanical calculations with references). This material is available free of charge via the Internet at http://pubs.acs.org.

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