Evaluation and Biological Properties of Reactive Ligands for the Mapping of the Glycine Site on the *N*-Methyl-D-aspartate (NMDA) Receptor

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The glycine-binding site of the *N*-methyl-D-aspartate (NMDA) receptor, given its potential as pharmacological target, has been thoroughly studied by structure−activity relationships, which has made possible its description in terms of spatial limits and interactions of various types. A structural model, based on mutational analysis and sequence alignements, has been proposed. Yet, the amino acid residues responsible for the interactions with the ligand have not been unambiguously characterized. To evidence nucleophilic pocket-lining residues, we have designed and synthesized reactive glycine-site ligands derived from 3-substituted 4-hydroxy-quinolin-2(1*H*)-ones by introducing various electrophilic groups at different positions of the molecule. These ligands were found to have high affinity at the glycine site and to be functional antagonists by inhibiting glycine/glutamate-induced currents in transfected oocytes. The correlation between their potency and their substitution pattern was strictly consistent with previously established structure−activity relationships. Most ligands displayed intrinsic reactivity toward cysteine, but none inactivated wild-type receptors. This is consistent with the model since it indicates the absence of exposed cysteine in the glycine-binding site. A strategy of cysteine incorporation by point mutations at selected polypeptide positions will create unambiguously localized targets for our reactive probes.

Introduction

The *N*-methyl-D-aspartate (NMDA) receptor is involved in the majority of neuroexcitatory events in the CNS. Among its specific features, its high Ca^{2+}/Na^{+} permeability ratio, its adaptative properties, and the presence of numerous regulatory sites could be the basis of its role in development, in learning, as well as in various neurodegenerative pathologies (for review, see refs 1 and 2). As channel blockers such as phencyclidine and dizocilpine are endowed with unwanted side effects, a number of antagonists for the glycine co-agonist binding site have been synthesized in various laboratories, as potential neuroprotective agents; the structural requirements for their affinity and for their selectivity toward the NMDA receptor could be described.[3,4] A general pharmacophore could be defined for several families of glycine-site antagonists, including indole-carboxylate, kynurenate, and quinoxaline-dione derivatives.[4] However, the amino acid residues corresponding to these interactions are still unknown. Mutational analysis has pointed out a few residues which are involved in the binding of glycine agonists and antagonists,[5−10] either because they frame the binding site, or because they line it, or because they interfere with its accessibility. Most, if not all, of these residues are located ca. 260 positions upstream from the first transmembrane segment of the receptor polypeptide (on the extracellular side) and on the extracellular chain which links the transmembrane segments 3 and 4,[5−10] consistent with the general topology of the receptor.[6,11,12] In fact, a fusion polypeptide, genetically engineered to contain these two portions of the NR1 subunit assembled by a short linker, can be expressed and isolated as a soluble protein which displays binding properties similar to those of the NMDA receptor in vivo.[13]

Affinity labeling is a powerful method to investigate the presence of nucleophilic amino acid residues on the surface of enzyme active-sites[14] or of receptor binding-sites.[15] The complete sequence of the NMDA receptor NR1 subunit, to which glycine-site agonists and antagonists bind[16−18] is known.[19] Reactive affinity probes for the glycine site would make possible the characterization of nucleophilic residues in this site, and assumptions could then be made as to which part of the sequence is actually in contact with the ligand. A structural model, based on sequence homologies between NR1 and crystallized, soluble amino acid-binding proteins, has been proposed for this site, with the high-affinity antagonist L-701.324[20,21] bound in its cavity.[7] This model can be used for the design of affinity probes, and, in turn, the results from affinity labeling could be entered into the modeling process, as they convey information different in nature from that resulting from mutational analysis.

In this paper, we describe the synthesis and properties of twelve reactive analogues of L-698.532 (**1**), L-701.324 (**2**),[20] and its 4-amino derivative (**3**)[22] (Chart 1). Their binding to rat brain membrane NMDA receptor of the wild-type is reported and discussed in terms of structure−activity relationships; their affinity is correlated to their antagonistic activity against glutamate/glycine-induced currents in oocytes expressing the

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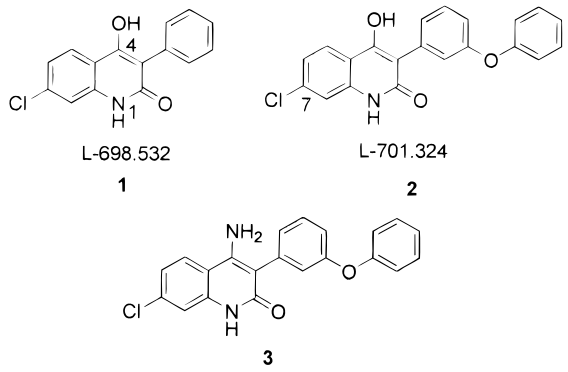
Chart 1



$NR1_A$/NR2B combination of receptor subunits. The stability and reactivity of these ligands, as determined in chemical model reactions, is compared to the absence of irreversible binding in the membrane receptor assay and to the absence of irreversible antagonistic effect in the oocyte activity assay. Some of these results have been published in a preliminary form.[23]

Chemistry

The affinity probes **13a**−**e** and **15a**−**f**, derived from the lead compounds L-701.324 and L-698.532 (Chart 1), were prepared as shown in Scheme 1. The synthesis of ligands with electrophilic substituents in position 7 (**13a**−**d**) started by the Hofmann rearrangement of 4-nitrophthalimide (**4**) followed by an esterification. Isomers **5a** and **5b** (not shown in Scheme 1) were obtained as a mixture in a 1:3 ratio (NMR), which was resolved by column chromatography. 3-Phenoxyphenylacetic acid was converted into the corresponding acid chloride by treatment with thionyl chloride/DMF at room temperature and was then coupled to isomer **5a**. The obtained compound **10a** was cyclized to the quinolinone **11a** as described for the preparation of L-701.324.[20] Catalytic hydrogenation over palladium on charcoal in methanol/hydrochloric acid gave the amine **12**. The target ligand **13a** was synthesized by treatment of **12** with thiophosgene in a dioxane/aqueous Na_2CO_3 solution mixture. The monoacylated compound **13b** was prepared by a reaction of **12** with chloroacetic anhydride in pyridine at 0 °C, whereas the bis-acylated derivative **13c** was obtained by a reaction with chloroacetic anhydride in CH_2Cl_2 at room temperature in the presence of triethylamine. The diazonium **13d** was obtained according to a protocol recently developed in our laboratory.[24] Acylation of the 4-hydroxy group in L-701.324 with *p*-(fluorosulfonyl)benzoyl chloride in CH_2Cl_2 gave the target compound **13e**. Ligands **15a**−**c** bearing a single phenyl ring in position 3 of the quinolinone were prepared following the described synthesis of L-701.324 except that 3-nitrophenylacetic acid was used instead of 3-phenoxyphenylacetic acid. Hydrogenation of the nitro group with palladium on charcoal (5%) as the catalyst in methanol led to a loss of the chloride at position 7. Since its presence is necessary for a high-affinity binding to the glycine site, the reduction was accomplished with a solution of 20% $TiCl_3$ in hydrochloric acid. Ligands **15a**,**b** were synthesized by the same procedures used to prepare compounds **13a**,**b**. The ethanesulfonyl fluoride group in **15c** was introduced by treating the amine (**14a**) with ethenesulfonyl fluoride in DMF. This reagent was synthesized in two steps from commercially available 2-chloroethanesulfonyl chloride according to a published procedure.[25] The affinity probes **15d**−**f** bearing a reactive group on the second phenyl ring were prepared via a copper-catalyzed (Ullmann) coupling of 3-hydroxyphenylacetic methyl ester with 2- or 3-bromonitrobenzene.[26] Basic hydrolysis of the biaryl ethers **9a**,**b** and subsequent treatment with thionyl chloride/DMF afforded the corresponding acid chlorides, which were reacted with methyl 4-chloroanthranilate. The obtained derivatives **10d**,**e** were cyclized as reported.[20] Reduction of the nitro group was performed with $TiCl_3$ as for the preparation of **14a**. Amine **14b** was converted into two different affinity probes (**15d**,**e**) by procedures already used for the preparation of **15b**,**c**. Acylation of amine **14c** with chloroacetic anhydride in pyridine provided ligand **15f**.

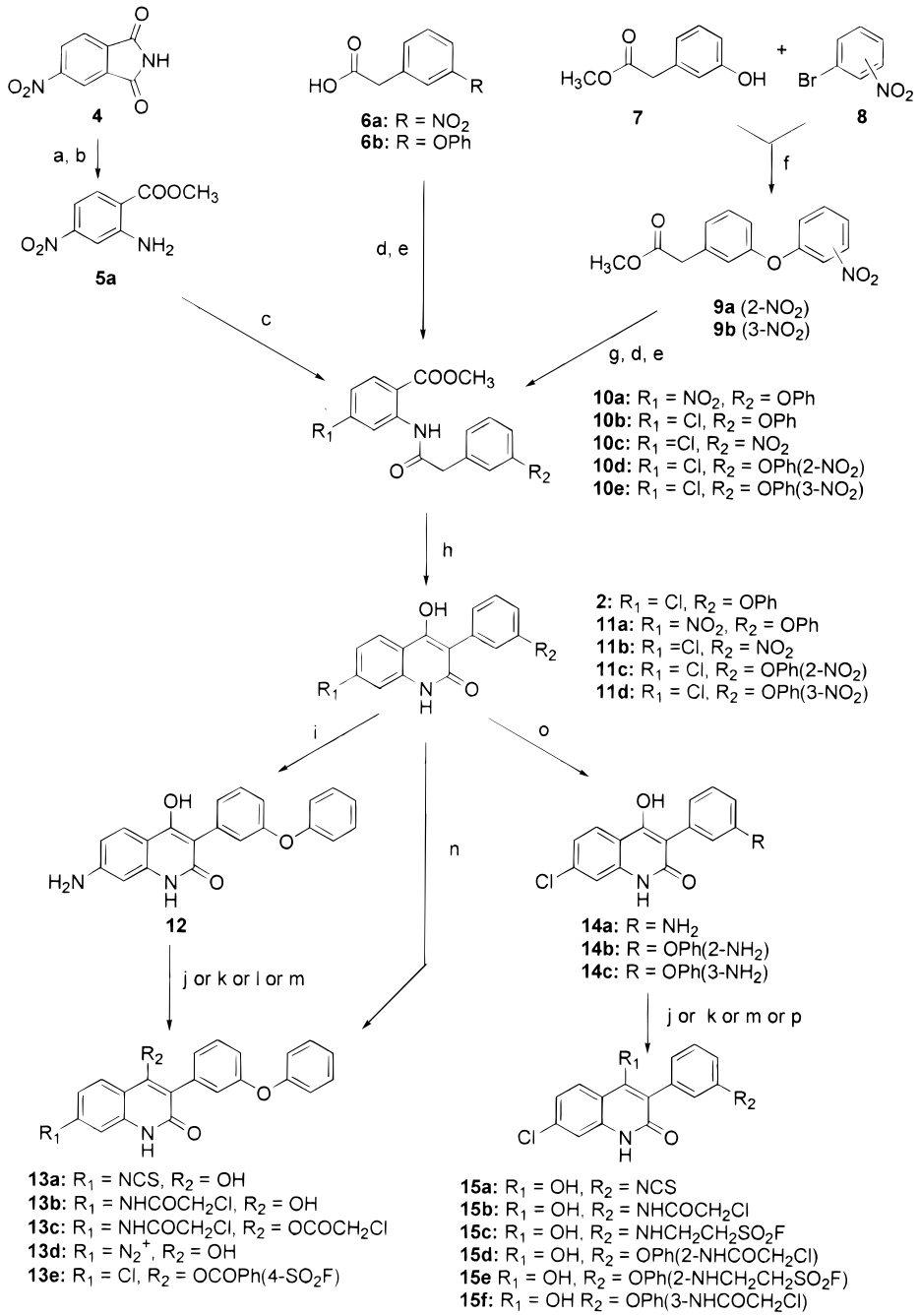
The synthetic route to the affinity probe **20** is illustrated in Scheme 2. Compound **3**, the precursor of ligand **20**, was prepared as already reported.[22] However, the first steps in this synthesis were too slow (3 days), and an alternative procedure was developed. 4-Chloro-2-nitrobenzoic acid (**16**) was treated with thionyl chloride followed by aqueous ammonia (30%) to afford the corresponding amide **17**. Conversion of **17** into the nitrile **18** was carried out by a method described in the literature.[27] Subsequent hydrogenation with a solution of 20% $TiCl_3$ in hydrochloric acid gave the amine **19**, which was acylated with 3-phenoxyphenylacetic chloride in dioxane in the presence of triethylamine. Cyclization of **19** into **3** was performed according to the published procedure.[22] Acylation of the 4-amino group with chloroacetic chloride in dioxane/triethylamine by heating under reflux generated the target compound (**20**). Under these conditions no acylation of the NH group of the quinolinone was observed, at variance from previously reported synthesis performed in different conditions.[22]

Pharmacology

The potency of our reactive ligands for the NMDA receptor glycine-site was measured by competition against [^{3}H]-5,7-dichlorokynurenic acid ([^{3}H]-DCKA) binding in rat brain membrane homogenate, which was prepared according to Danysz et al.[28] For binding, the stock membrane suspension was thawed and further washed.[29] An aliquot of final suspension was incubated with [^{3}H]-DCKA (20 nM), in the absence or in the presence of 1 mM glycine, for total and nonspecific binding, respectively, or in the presence of increasing concentrations of the affinity probes. To detect the occurrence of a ligand−receptor covalent reaction, which would result in a gradual shift of equilibrium binding by decreasing the available amount of free receptor, samples containing the affinity marker at the concentration of its IC_{50} were incubated 2 h longer.

The potency of our ligands as antagonists were determined electrophysiologically in *Xenopus laevis* oocytes expressing the cloned NMDA receptor 1_A/2B subunit combination.[5,30] IC_{50}s were the calculated concentrations of antagonist corresponding to the suppression of one-half of the membrane current response elicited by fixed concentrations of glycine and glutamate. The oocytes were washed with superfusion medium between each stimulation, and, in some cases (the high-

Scheme 1[a]



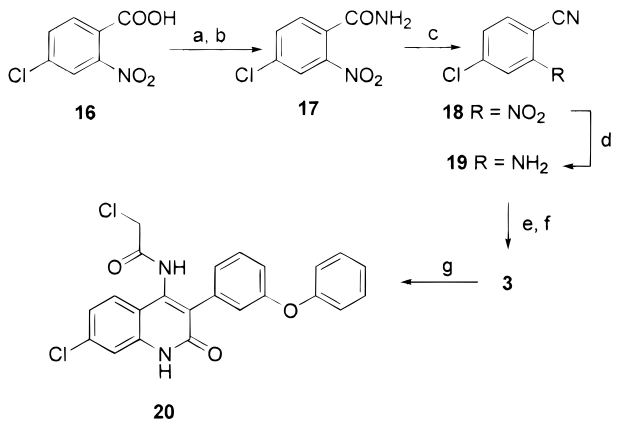
[a] Reagents: (a) aqueous NaOH/dioxane, $Ca(OCl)_2$, 5 °C then 100 °C, concentrated HCl 5 °C; (b) methanol, H_2SO_4; (c) 3-phenoxyphenylacetyl chloride, NEt_3, THF, reflux; (d) $SOCl_2$, DMF, CH_2Cl_2, rt; (e) methyl 4-chloroanthranilate, NEt_3, tetrahydrofuran, reflux; (f) CuO, K_2CO_3, pyridine, reflux; (g) EtOH/aqueous NaOH, reflux; (h) NaHMDS, THF, rt, then TFA; (i) H_2, Pd/C (10%), methanol, HCl, rt; (j) thiophosgene, Na_2CO_3, THF/H_2O rt; (k) chloroacetic anhydride, pyridine, 5 °C; (l) chloroacetic anhydride, NEt_3, CH_2Cl_2, rt; (m) TFA, isoamyl nitrite, CH_2Cl_2/CH_3CN, −15 °C, then −78 °C, ether; (n) *p*-(fluorosulfonyl)benzoyl chloride; (o) $TiCl_3$, THF/MeOH, rt; (p) ethenesulfonyl fluoride, TFA, DMF, rt.

affinity antagonists, including L-701.324), pulses of 1 mM glycine were incorporated in the washing procedure to assess the reversibility of the antagonistic effect.

Results and Discussion

Binding and Activity Data. The structure−activity relationships of our ligands are based on the analysis of the binding values in Table 1. The substitution of the chlorine in position 7 of L-701.324 resulted in a sharp decrease in affinity, with the isothiocyanate group in compound **13a** being less detrimental to the affinity than the chloroacetamide group in compounds **13b** and **13c**. Their synthetic precursor **12** bound with an affinity close to that of the isothiocyanate (data not shown), while the diazonium derivative **13d** had lost almost all affinity. It is therefore concluded that the lipophilicity is the first key factor in this position, with the size of the substituent in second. These conclusions are consistent with previous reports.[3,31,32] Substituting position 4 has little effect on the binding: L-701.324 and **13e** have similar K_I values, as well as **13b** and **13c**. These features have already been reported by others.[22] The

Scheme 2[a]



[a] Reagents: (a) thionyl chloride, DMF, toluene, reflux; (b) 30% NH_4OH, dioxane, rt; (c) TFA, pyridine/dioxane, 5 °C, then rt; (d) 20% $TiCl_3$ in hydrochloric acid, acetone, then aqueous 15% NaOH; (e) 3-phenoxyphenylacetic chloride, NEt_3, dioxane, reflux; (f) NaH, DMF, 100 °C; (g) chloroacetic chloride, NEt_3, THF, reflux.

difference in K_I between **13e** and **20** may result from the replacement of the hydrogen bond acceptor oxygen by a nitrogen atom, H-bonding in this position being one of the structural requirements in this series.[22] With regard to position 3, several authors have reported the beneficial effect of a phenyl substituent,[29,33,34] as an aromatic ring in this position could be involved in the formation of a π-cation interaction.[35,36] Substitutions on this ring (in particular at the meta position) have evidenced that electroattracting and aromatic substituents further improve the affinity.[20,22,29,33,36] Replacement or substitution of the phenoxy group of L-701.324 does not alter significantly the affinity (Table 1). This is consistent with observations made by others[22,29] with similar ligands. No substitution was attempted in positions 6 and 8 of the quinolinone ring, since it is well documented that they result in a dramatic loss of NMDA receptor selectivity and of affinity, respectively.[37−39] Position 5 was also left unsubstituted; it is known that, as in position 7, only small hydrophobic substituents can improve the affinity.[3,40] It was therefore anticipated that substitution of this position by our comparatively large and hydrophilic reactive groups would result in a drop in affinity, as previously observed in position 7. In ligands **13c**,**e** and **20**, the reactive side chains of position 4 are long and flexible enough to reach the amino acid residues which face the 5-vicinal position. Altogether, the binding data for the new ligands presented here evidence structural features which are common to the best antagonists known so far for the NMDA receptor glycine-binding site, to which they bind with nanomolar to sub-micromolar affinities. This qualifies them as good ligands for this site. The level of [^{3}H]-DCKA binding at equilibrium, in experiments where our reactive probes (at the concentration of their IC_{50}) compete with [^{3}H]-DCKA (20 nM), does not significantly change with time. This indicates that no receptor−ligand reaction is taking place.

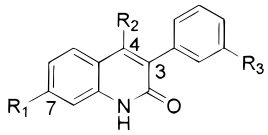
Except for compounds **13d** and **20** all ligands display antagonistic properties at this site in electrophysiological measurements by suppressing the current elicited by glycine (in the presence of glutamate) in a dose-dependent manner (Table 1). This inhibition requires higher doses of antagonists than the inhibition of [^{3}H]-DCKA binding in rat brain membranes: the IC_{50}s for current suppression spanned 3 orders of magnitude from 20 nM to 50 μM. This difference can be accounted for by the difference in the experimental conditions in each system: in the binding experiments, the temperature, the buffer ionic strength, and the ratio of the competed ligand concentration versus its K_D were lower than in the activity recordings. In addition, the NMDA receptor in rat brain membranes is heterogeneous, while only the $NR1_A$/NR2B combination of subunits was expressed by the transfected oocytes; glycine-site ligands bind to the different receptor subpopulations with different affinities, depending on the nature of the NR2 subunit[16] rather than on the NR1 splice variant[41] of the combination. Finally, the effects observed in activity or in binding assays may relate to different states of the receptor (active or desensitized), which were shown to correspond to different affinities of the glycine site toward a ligand (although this ligand was an antagonist).[42] The order of binding affinities and of antagonist potencies are roughly similar: high-affinity compounds are the most potent, and vice-versa, even though the IC_{50}/K_I ratio tends to increase as the K_I increases. The poor solubility of some compounds in frog's Ringer might have biased the actual ligand concentration.[43] More importantly, these compounds were all found to be reversible antagonists: after appropriate washing, the oocytes could recover most, if not all, of their control activity upon subsequent applications of 1 μM glycine. This indicates that no irreversible reaction has taken place between our reactive ligands and binding-site amino acid residues.

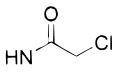
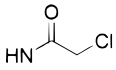
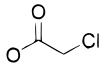
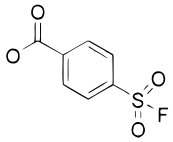
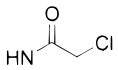
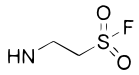
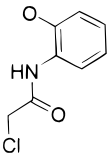
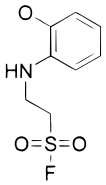
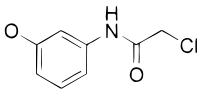
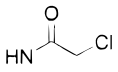
Stability and Reactivity of the Markers. Representatives of each reactive group were assayed in buffers for stability and in the presence of amino acid model compounds to test their potential as affinity markers. In a classical experiment the ligand was dissolved in the corresponding buffer in the absence or in the presence of excess model amino acid. Aliquots were taken up at different times and analyzed by HPLC. The decrease of the peak area corresponding to the marker and the appearance of new peaks were followed up by UV detection at 320 nm. Both the degradation and reaction products were not identified, but they were unambiguously discriminated via their retention times.

All ligands were shown to be stable in the conditions of the binding assay. However, several compounds (**15c**, **15e**) decomposed in the conditions used for electrophysiological measurements with half-lives of 5−12 h. It is well known that sulfonyl fluorides can undergo spontaneous hydrolysis;[44,45] ligands **15c** and **15e** bear an aliphatic fluorosulfonyl substituent. In their case, fresh solutions were always used for the determination of their constants. By contrast, **13e**, which bears the aromatic *p*-(fluorosulfonyl)benzoyl group, was shown to be more stable ($t_{1/2}$ > 24 h).

Amino- and carboxy-protected cysteine was used as a model in reactivity measurements, as other amino acids with nucleophilic residues (tyrosine, histidine) did not react significantly with our reactive electrophilic substituents (data not shown). For the isothiocyanate substituted compound (**15a**) the reaction with cysteine was finished within 3 min, whereas the chloroacetamide derivatives (**13b**, **15f**) showed longer reaction times ($t_{1/2}$

Table 1. Binding and Activity Data for Compounds **13a**−**e**, **15a**−**f**, **20**, and L-701.324[a]



compound	R_1	R_2	R_3	K_i (nM) ([^{3}H]-DCKA)	IC_{50} (μM) (glycine)
L-701.324	Cl	OH		14±4.2	0.018±0.0015
13a	NCS	OH		64.3±8.5	24.0±2.9
13b		OH		278±51	38.0±4.5
13c				427±91	63±7.8
13d	N_2^+	OH		> 1000[b]	n.d.
13e	Cl			24.2±8.2	2.41±0.21
15a	Cl	OH	NCS	47.6±11.7	0.20±0.014
15b	Cl	OH		42±3.7	0.155±0.053
15c	Cl	OH		24±7.8	0.335±0.07
15d	Cl	OH		7.1±1.5	0.024±0.011
15e	Cl	OH		26.8±7.0	0.032±0.014
15f	Cl	OH		13.8±1.3	0.039±0.022
20	Cl			216±13.9	> 100

[a] Dissociation constants (K_i) were obtained by competition against [^{3}H]-DCKA binding in rat brain membrane homogenate; antagonistic potencies were calculated from dose-dependent inhibitions of the currents induced by glycine plus glutamate in oocytes expressing active NMDA receptors (see Experimental Section for details). [b] The binding assay was performed in phosphate buffer (50 mM, pH 7.2) because compound **13d** underwent rapid decomposition in HEPES/KOH.

= 0.5−0.9 h at pH 7.5). These reaction half-times are comparatively long, but still compatible with our experimental conditions. In addition, in affinity labeling the reaction of amino acids with ligands is preceded by the formation of a reversible complex, and the intrinsic rate of the reaction is strongly influenced by the positioning of the reacting groups; it is therefore anticipated that the reaction rate will be increased under a binary complex form. The incubation of compounds **13b** and **13c** with cysteine yielded similar HPLC profiles (in fact, co-injection of **13b** and **13c** incubated with cysteine resulted in the pattern obtained with cysteine-reacted **13b** plus an extra peak corresponding to **13c**). Thus, it can be suggested that in the presence of cysteine **13c**

was decomposed into **13b**, which subsequently reacted with cysteine. In the same manner, the incubation of **13e** with cysteine led to the liberation of the *p*-(fluorosulfonyl)benzoyl substituent in the 4 position of the ligand. None of the fluorosulfonyl substituted ligands (**13e**, **15c**, **15e**) showed a reaction with cysteine (as well as with tyrosine or histidine). This was unexpected, since less nucleophilic amino acids (such as tyrosine, lysine, histidine, and serine) have been reported to be labeled by these groups in various proteins.[44,46−49] This discrepancy could result from an enhanced reactivity or nucleophilicity of amino acid residues in the active/catalytic site of the proteins, through interactions with adjacent amino acid residues.

Reversibility of the Ligands as Competitors and as Antagonists. Both binding and activity data indicate that no ligand−receptor reaction has taken place in the glycine-binding site, even with the most reactive isothiocyanate compounds. In the portion of the NR1 polypeptide sequence which is relevant to glycine binding,[13] a maximum of 66 residues are nucleophilic (including 7 cysteines), and, among them, 26 belong to sequence fragments whose homologue in other glutamate receptors is highly conserved (including 4 cysteines). Only 12 of them are highly (7) or absolutely (5) conserved residues, including 2 cysteines which are thought to be involved in a disulfide bridge (cysteine 734 and cysteine 781, numbering according to Ishii et al.[19]). The fact that our ligands do not react in the site has two possible explanations. One is that these nucleophilic residues do not interact with the ligand; they may be located in the protein core or they may line the binding site, but in both cases they are involved in side chain to side chain interactions and therefore participate in the framing of the site. The other possibility is that they do interact with the ligand, but that their reciprocal positioning is such that they cannot react with its electrophilic substituents. A structural model of the part of the NR1 subunit which binds glycine-site ligands is currently available,[7] and our ligands could be docked in its structure; their quinolinone ring was, in most cases, superimposed with that of L-701.324, and substituents in the 4 position and on the phenyl rings could rotate, with their electrophilic center thus covering an umbrella-like area. In fact, in this model, most of the 26 critical nucleophilic residues defined above do not belong to the binding crevice of the model. Among those which do belong to the binding site, serine 530 has its side chain pointing opposite to the ligand and threonine 539 is a little too distant. By contrast, serine 688 and tyrosine 692 hydroxyl groups appear to be adequately oriented to react with 4 position- and phenyl ring-substituted compounds. Presumably, the reactivity of these amino acid residues is, as in model-reaction studies, too low to be observed. The corresponding region of GluR2, the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor, has been crystallized, and atom coordinates are available for modeling studies.[50] Tentative docking of our compounds in its ligand-binding site by computer analysis showed that they did not fit in this structure; in particular, the regions which are conserved between the two glutamate receptors (as well as in glutamate, glutamine, and other amino acid-binding proteins) in the NR1 model do not exactly superimpose with their counterpart in the GluR2 structure.

Conclusion

We have synthesized structural probes with reactive groups at different positions of the ligand-pharmacophore to investigate the presence of nucleophilic amino acid residues in the glycine-binding site of the NMDA receptor. Their binding properties have been determined by competition against a prototype NMDA receptor glycine-site ligand, and they are consistent with the description of the structural requirements for antagonists at this site, as established by a number of structure−activity relationships. The interactions of our probes with their binding site therefore appear to be those we intend to characterize. This and the high affinity of these ligands suffice to make them valuable tools for structural studies. These probes are antagonists in electrophysiological activity measurements. There are variations, which can be explained, between binding and activity data; these latter data are nonetheless useful to set up protocols of receptor inactivation. Several of our probes were shown to be reactive with cysteine in solution; however, they do not inactivate the receptor, either in binding or in current response, which means they do not react in the receptor glycine-site. This is consistent with the structural model of this site in its present form: there is no cysteine in the site. In addition, most identified nucleophilic residues (other than cysteines) are core residues, unlikely to interact with the ligand. Introducing cysteines in the receptor polypeptide by point mutation will create targets for our reactive ligands. When observed, the inactivation of these mutant receptors would mean unambiguously that a covalent bond has been formed between the ligand reactive substituent and the amino acid residue at the mutated position. Clear assumptions can then be made as to its location in the protein structure and the orientation of the secondary structure element thereof. This strategy has the potential to bridge the gap between pharmacophore definition and receptor modeling.

Taken together, the results reported here are consistent with the predictions of the model. Point mutations in the protein, designed at creating structurally informative reaction centers in the binding site, should complement our collection of topological probes.

Experimental Section

Chemistry. Solvents were dried and distilled prior to use: dioxane and THF from sodium/benzophenone; CH_2Cl_2 and acetonitrile from calcium hydride. Silica gel 7734 (70−230 mesh) from Merck was used for column chromatography. Melting points were determined on a Bausch & Lomb apparatus and are uncorrected. NMR spectra were recorded on Bruker AC200 or DPX300 spectrometers. The δ (ppm) scale was in reference with the deuterated solvent. The signals are described as s (singulet), d (doublet), t (triplet), pt (pseudotriplet), m (multiplet), and br (broad). Coupling constants (J) are reported in hertz. UV spectra were recorded on a double beam Uvikon 860 spectrophotometer. IR spectra were performed on a Perkin Elmer 1600 FT-IR spectrometer; absorbances are reported in ν (cm^{-1}). Mass spectra and elemental analyses were obtained at the Faculté de Chimie, Université Louis Pasteur, Strasbourg.

Methyl 2-Amino-4-nitrobenzoate (5a). To an ice-cold solution of 4-nitrophthalimide (13.5 g, 70 mmol) in 70 mL of

aqueous NaOH (10%) and 24 mL of dioxane was added a slurry of $Ca(OCl)_2$ (14.0 g, 98 mmol) in aqueous NaOH (10%, 28 mL). The mixture was stirred in an ice bath for 2 h. Next, the temperature was raised to 30 °C for 10 min and then to 100 °C for 20 min. The resulting suspension was filtered hot, and the residue was washed with hot water (3 × 100 mL). The filtrate was cooled to room temperature, and HCl (36%) was added dropwise until pH 4 was reached. The red precipitate was filtered and dried by coevaporation with toluene to yield a mixture of 5-nitroanthranilic acid and 4-nitroanthanilic acid (1:3, NMR). These acids could not be separated by silica gel column chromatography. They were dissolved in methanol (120 mL) with 11.2 mL of H_2SO_4 (96%), and the solution was heated to reflux overnight. After evaporation of the solvent the crude material was dissolved in CH_2Cl_2 and the organic phase was washed with a 5% $NaHCO_3$ solution (3×) and with water (2×) and then dried (Na_2SO_4). The solvent was evaporated, and the residue was purified by silica gel chromatography (hexane−Et_2O, 6:4, as eluent). Fractions containing **5a** were collected and submitted to a further chromatography (silica gel, CH_2Cl_2−hexane, 6:4, as eluent) for the elimination of formed methyl *N*-methyl-4-nitroanthranilate: yield 7.83 g (57%), orange crystals; mp 156 °C (lit. 156−157 °C[51]);^{1}H NMR (200 MHz, $CDCl_3$) δ 8.01 (d, 1H, J = 8.8, H-6), 7.51 (d, 1H, J = 2.2, H-3), 7.41 (dd, 1H, J = 8.8, 2.2, H-5), 6.07 (br, s, 2H, NH_2), 3.93 (s, 3H, CH_3); MS (EI) m/z 196 [M^+]. Anal. ($C_8H_8N_2O_4$) C, H, N.

Methyl 2-(3-Hydroxyphenyl)acetate (7). 3-Hydroxyphenylacetic acid (7.6 g, 50 mmol) was heated to reflux for 12 h in methanol (150 mL) with *p*-toluenesulfonic acid (300 mg). After standard workup the crude product was purified by silica gel chromatography (CH_2Cl_2−ethyl acetate, 9:1, as eluent) giving 8.12 g (94%) of **7** as a colorless oil. The analytical data are consistent with the literature.[52] Anal. ($C_9H_{10}O_3$) C, H, N.

Methyl 2-[3-(2-Nitrophenoxy)phenyl]acetate (9a). A solution of 2-bromonitrobenzene (9.7 g, 48 mmol), **7** (6.65 g, 40 mmol), and K_2CO_3 (16.6 g, 120 mmol) in dry pyridine (40 mL) under argon was warmed to 100 °C for 2 h. CuO (12.7 g, 160 mmol) was added, and the reaction mixture was treated and worked up as described in the literature for the synthesis of 4-(2-methoxyphenoxy)benzonitrile[26] except that the reaction time was of 6 h instead of 21 h. The crude product was purified by silica gel chromatography (hexane−ethyl acetate, 6:4, as the eluent) to give 7.8 g (68%) of **9a** as a yellow oil: ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.95 (dd, 1H, J = 8.2, 1.5, H-3′), 7.51 (m, 1H, H-5′), 7.33 (t, 1H, J = 7.7, H-5), 7.20 (m, 1H, H-4′), 7.13−6.92 (m, 4H), 3.70 (s, 3H, OCH_3), 3.63 (s, 2H, CH_2); MS (EI) m/z 287 [M^+]. Anal. ($C_{15}H_{13}NO_4$) C, H, N.

Methyl 2-[3-(3-Nitrophenoxy)phenyl]acetate (9b). Compound **9b** was prepared according to the synthesis of **9a** starting from 3-bromonitrobenzene (4.85 g, 24 mmol). Purification by silica gel chromatography (hexane−ethyl acetate, 8:2, as the eluent) gave **9b** as a pale yellow oil (2.2 g, 45%): ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.95 (ddd, 1H, J = 8.3, 2.0, 1.0, H-4′), 7.80 (pt, 1H, J = 2.4, 2.0, H-2′), 7.50 (t, 1H, J = 8.3, H-5′), 7.37 (t, 1H, J = 7.8, H-5), 7.34 (ddd, 1H, J = 8.3, 2.4, 1.0, H-6′), 7.14 (br, d, 1H, J = 7.8, H-6), 7.02 (t, 1H, J = 2.0, H-2), 7.00 (dd, 1H, J = 8.0, 2.0, H-4), 3.71 (s, 3H, OCH_3), 3.65 (s, 2H, CH_2); MS (EI) m/z 287 [M^+]. Anal. ($C_{15}H_{13}NO_4$) C, H, N.

Methyl 4-Nitro-2-[[2-(3-phenoxyphenyl)acetyl]amino]benzoate (10a). A mixture of 3-phenoxyphenylacetic acid (2 g, 8.76 mmol) and thionyl chloride (2.54 mL, 35 mmol) in CH_2Cl_2 (20 mL) with DMF (0.2 mL) was stirred overnight under argon. Evaporation of the solvent gave crude 3-phenoxyphenylacetyl chloride, which was without further purification dissolved in THF (10 mL) and added to a solution of **4** (0.86 g 4.38 mmol) in tetrahydrofuran (20 mL) preheated at 80 °C. Triethylamine (1.34 mL, 9.6 mmol) was added, and the reaction mixture was heated under reflux for 5 h. After cooling, the solvent was removed under vacuum and the residue was partitioned between CH_2Cl_2 (1 × 120 mL) and water (3 × 50 mL). The organic layer was dried (Na_2SO_4), filtrated, and evaporated to dryness. Silica gel chromatography of the crude product using 20% ethyl acetate in hexane as eluent gave **10a** as a pale yellow solid (1.19 g, 69%): mp 126 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 11.1 (s, 1H, NH), 9.62 (d, 1H, J = 2.2, H-3), 8.17 (d, 1H, J = 8.8, H-6), 7.88 (dd, 1H, J = 8.8, 2.2, H-5), 7.29−7.4 (m, 3H), 6.95−7.16 (m, 6H), 3.94 (s, 3H, OCH_3), 3.79 (s, 2H, CH_2); MS (FAB) m/z 407 [MH^+]. Anal. ($C_{22}H_{18}N_2O_6$) C, H, N.

The syntheses of compounds **10b**−**e** were performed in the same way as described for **10a** starting with the appropriate amines and acid chlorides.

Methyl 4-Chloro-2-[[2-(3-phenoxyphenyl)acetyl]amino]benzoate (10b). Purification by silica gel chromatography (hexane−ethyl acetate, 9:1, as eluent) gave 0.92 g (93%) of **10b** as a white solid: mp 58−60 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.11 (s, 1H, NH), 8.83 (d, 1H, J = 2.2, H-3), 7.93 (d, 1H, J = 8.5, H-6), 7.37−7.3 (m, 3H), 7.17−7.03 (m, 6H), 6.96 (ddd, J = 8.2, 2.5, 0.2, H-5″), 3.93 (s, 3H, CH_3), 3.75 (s, 2H, CH_2); MS (EI) m/z 395 [M^+]. Anal. ($C_{22}H_{18}ClNO_4$) C, H, N.

Methyl 4-Chloro-2-[[2-(3-nitrophenyl)acetyl]amino]benzoate (10c). Crude material of **10c** was purified by silica gel chromatography (hexane−ethyl acetate, 7:3, as the eluent) followed by heating the product in Et_2O and hot filtration of the suspension. The residue afforded 3.48 g (80%) of **10c** as a pale yellow solid: mp 137−139 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 11.32 (s, 1H, NH), 8.79 (d, 1H, J = 2.2, H-3), 8.27 (t, 1H, J = 1.7, H-2′), 8.21 (m, 1H, H-4′), 7.95 (d, 1H, J = 8.4 Hz, H-6), 7.73 (m, 1H, H-6′), 7.57 (t, 1H, J = 7.7, H-5′), 7.08 (dd, 1H, J = 8.4, 2.2, H-5), 3.90 (s, 3H, OCH_3), 3.89 (s, 2H, CH_2); MS (FAB) m/z 349 [MH^+]. Anal. ($C_{16}H_{13}ClN_2O_5$) C, H, N.

Methyl 4-Chloro-2-[[2-[3-(2-nitrophenoxy)phenyl]acetyl]amino]benzoate (10d). After saponification of **9a** with NaOH (3 equiv) in 50% ethanol under reflux for 30 min the resulting acid was treated with thionyl chloride and coupled with methyl 4-chloroanthranilate as described above. Purification by silica gel chromatography (hexane−ethyl acetate, 7:3, as eluent) gave 5.69 g (73%) **10d** as a pale yellow solid: mp 73−75 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 10.71 (s, 1H, NH), 8.39 (d, 1H, J = 2.0, H-3), 8.05 (dd, 1H, J = 8.3, 1.5, H-3″), 7.90 (d, 1H, J = 8.3, H-6), 7.68 (m, 1H, H-5″), 7.45−7.12 (m, 6H), 7.00 (dd, 1H, J = 2.0, 8.3, H-6″), 3.80 (s, 2H, CH_2), 3.78 (s, 3H, OCH_3); MS (FAB) m/z 441.0 [MH^+]. Anal. ($C_{22}H_{17}ClN_2O_6$) C, H, N.

Methyl 4-Chloro-2-[[2-[3-(3-nitrophenoxy)phenyl]acetyl]amino]benzoate (10e). Treatment of **9b** in the same way as **9a** afforded the corresponding acid which, after conversion into the corresponding acid chloride, was reacted with methyl 4-chloroanthranilate as described. Silica gel chromatography (hexane−ethyl acetate, 7:3, as eluent) afforded **10e** as a pale yellow solid (1.39 g, 67%): mp 63−65 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.71 (s, 1H, NH), 8.42 (d, 1H, J = 2.2, H-6), 7.97 (dd, 1H, J = 8.2, 2.2, H-4″), 7.90 (d, 1H, J = 8.8, H-3), 7.72 (pt, 1H, J = 2.5, 2.2. H-2″), 7.51 (dd, 1H, J = 8.2, 2.5, H-4′), 7.45 (pt, 1H, J = 8.0, 7.7, H-5′), 7.24 (m, 2H, H-4, H-6″), 7.17 (s, 1H, H-2′), 7.08 (dd, 1H, J = 8.0, 2.5, H-6′), 3.82 (s, 2H, CH_2), 3.76 (s, 3H, OCH_3); MS (FAB) m/z 441 [MH^+]. Anal. ($C_{22}H_{17}ClN_2O_6$) C, H, N.

7-Chloro-4-hydroxy-3-(3-phenoxyphenyl)quinolin-2(1*H*)-one (2). Cyclization of **10a**−**e** into the corresponding quinolinones was performed following a procedure described in the literature.[20] Purification by silica gel chromatography (CH_2Cl_2−methanol, 95:5, as eluent) yielded 0.47 g (82%) of **2** as a white solid: mp 308 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.56 (s, 1H, NH), 7.91 (d, 1H, J = 8.8, H-5), 7.44−6.93 (m, 11H); MS (EI) m/z 363 [M^+]. Anal. ($C_{21}H_{14}ClNO_3$) C, H, N.

4-Hydroxy-7-nitro-3-(3-phenoxyphenyl)quinolin-2(1*H*)-one (11a). Silica gel chromatography (CH_2Cl_2−methanol, 95:5, as eluent) of crude material gave 0.68 g (63%) of a yellow solid: mp 273−275 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.88 (s, 1H, NH), 8.13 (d, 1H, J = 8.8, H-5), 8.10 (br, s, 1H, H-8), 7.94 (dd, 1H, J = 8.8, 2.2, H-6), 7.47−7.33 (m, 3H), 7.19−6.96 (m, 6H); MS (FAB) m/z 375 [MH^+]. Anal. ($C_{21}H_{14}N_2O_5$) C, H, N.

7-Chloro-4-hydroxy-3-(3-nitrophenyl)quinolin-2(1*H*)-one (11b). This product was purified by heating it in ethyl acetate and filtration of the hot suspension: yield 2.52 g (97%),

white solid; mp > 310 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.63 (s, 1H, N*H*), 8.28 (t, 1H, J = 2.2, H-2′), 8.15 (m, 1H, H-4′), 8.0 (d, 1H, J = 8.2, H-5), 7.90 (m, 1H, H-6′), 7.66 (t, 1H, J = 7.7, H-5′), 7.32 (d, 1H, J = 2.0, H-8), 7.22 (dd, 1H, J = 8.2, 2.0, H-6); MS (EI) m/z 316 [M$^+$]. Anal. ($C_{15}H_9ClN_2O_4$) C, H, N.

7-Chloro-4-hydroxy-3-[3-(2-nitrophenoxy)phenyl]quinolin-2(1*H*)-one (11c). Silica gel chromatography using 5% methanol in CH_2Cl_2 as eluent gave 0.18 g (70%) of a pale yellow solid: mp 273 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.59 (s, 1H, N*H*), 10.49 (br, s, 1H, O*H*), 8.04 (d, 1H, J = 7.3, H-3″), 7.93 (d, 1H, J = 8.8, H-5), 7.68 (pt, 1H, J = 7.3, 6.8, H-5″), 7.47 (t, 1H, J = 7.8, H-5′), 7.35−7.18 (m, 5H), 7.10 (s, 1H, H2′), 7.06 (d, 1H, J = 7.8, H-6″); MS (FAB) m/z 409 [MH]$^+$. Anal. ($C_{21}H_{13}ClN_2O_5$) C, H, N.

7-Chloro-4-hydroxy-3-[3-(3-nitrophenoxy)phenyl]quinolin-2(1*H*)-one (11d). Crude material was purified by silica gel chromatography (CH_2Cl_2−methanol, 9:1, as eluent) to give 0.98 g (97%) of **11d** as a pale yellow solid: mp 264−266 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.58 (s, 1H, N*H*), 10.6 (br, s, 1H, O*H*), 7.95 (dd, 1H, J = 8.0, 2.2, H-4″), 7.94 (d, 1H, J = 8.8, H-5), 7.79 (t, 1H, J = 2.2, H-2″), 7.66 (t, 1H, J = 8.2, H-5″), 7.54 (dd, 1H, J = 8.2, 2.5, H-6′), 7.48 (t, 1H, J = 8.0, H-5′), 7.31 (d, 1H, J = 1.9, H-8), 7.27 (d, 1H, J = 7.7, H-4′), 7.2 (dd, 1H, J = 8.8, 1.9, H-6), 7.13 (br, s, 1H, H-2′), 7.09 (dd, 1H, J = 8.0, 2.5, H-6″); MS (FAB) m/z 409 [MH]$^+$. Anal. ($C_{21}H_{13}ClN_2O_5$) C, H, N.

7-Amino-4-hydroxy-3-(3-phenoxyphenyl)quinolin-2(1*H*)-one (12). Compound **11a** (0.29 g, 0.76 mmol) was suspended in methanol (80 mL) containing hydrochloric acid (10%, 0.8 mL) and hydrogenated at atmospheric pressure over 5% palladium on carbon (100 mg) at room temperature for 12 h. After filtration and evaporation, the residue obtained was purified by silica gel chromatography using 10% methanol in CH_2Cl_2 as eluent to give 0.2 g (70%) of **12** as a white solid: mp 240−242 °C; ^{1}H NMR (200 MHz, MeOH-d_4) δ 7.70 (d, 1H, J = 8.8, H-5), 7.43−7.27 (m, 3H), 7.14−7.01 (m, 6H), 6.60 (dd, 1H, J = 8.8, 2.2, H-8), 6.47 (d, 1H, J = 2.2, H-6); MS (FAB) m/z 345 [MH]$^+$. Anal. ($C_{21}H_{16}N_2O_3$) C, H, N.

7-Chloro-4-hydroxy-7-isothiocyanato-3-(3-phenoxyphenyl)quinolin-2(1*H*)-one (13a). To a solution of **12** (60 mg, 0.17 mmol) and $NaHCO_3$ (37 mg, 0.35 mmol) in aqueous 50% THF (4.5 mL) was added dropwise thiophosgene (0.03 mL, 0.35 mmol), and the mixture was stirred for 15 min. The resulting suspension was diluted with aqueous 5% $NaHCO_3$ solution (10 mL). After evaporation of the THF, the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL). The extract was washed with aqueous 5% $NaHCO_3$ solution (10 mL) and water (10 mL), dried ($MgSO_4$), and evaporated to leave a yellow oil which was purified by silica gel chromatography (CH_2Cl_2−methanol, 95:5, as eluent) to give 57 mg (87%) of **13a** as a gray solid: mp 310 °C (dec); ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.59 (s, 1H, N*H*), 10.45 (br, s, 1H, O*H*), 7.96 (d, 1H, J = 9.5, H-5), 7.45−7.34 (m, 3H), 7.23−6.95 (m, 8H); IR (neat) 2129 (NCS); MS (EI) m/z 386 [M$^+$]. Anal. ($C_{22}H_{14}N_2O_3S\cdot 0.3H_2O$) C, H, N.

2-Chloro-*N*-[4-hydroxy-3-(3-phenoxyphenyl)-2-oxo-1,2-dihydroquinolin-7-yl]acetamide (13b). To an ice cooled solution of **12** (49 mg, 0.14 mmol) in dry pyridine under argon was added chloroacetic anhydride (25.6 mg, 0.15 mmol). The mixture was stirred for 1 h at 0 °C, water (1 mL) was added, and stirring was continued for 15 min. After removal of the solvent in vacuo, standard workup produced a residue, which was purified by silica gel chromatography using 5% methanol in CH_2Cl_2 to give 24.8 mg (42%) of **13b** as a white solid: mp 212−214 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.42 (s, 1H, N*H* Ar), 10.57 (s, 1H, N*H* amide), 7.86 (d, 1H, J = 8.8, H-5), 7.69 (s, 1H, H-8), 7.35 (m, 3H), 7.02 (m, 7H), 4.28 (s, 2H, CH_2); MS (FAB) m/z 421 [MH]$^+$. Anal. ($C_{23}H_{17}ClN_2O_4$) C, H, N.

7-[(2-Chloroacetyl)amino]-3-(3-phenoxyphenyl)-2-oxo-1,2-dihydroquinolin-4-yl-2-chloroacetate (13c). Amine **12** (69 mg, 0.2 mmol) was dissolved in dry THF (2 mL) with triethylamine (56 μL, 0.4 mmol). Chloroacetic anhydride (68.3 mg, 0.4 mmol) was added, and the mixture was stirred at room temperature for 1 h. The solvent was removed under vacuum, the residue was dissolved in 10 mL of ethyl acetate and washed with water (2 × 10 mL). The organic layer was dried ($MgSO_4$), filtrated, and concentrated in vacuo. The crude product was purified by silica gel chromatography (CH_2Cl_2−acetone, 7:3, as eluent) to give **13c** as a white solid (16.5 mg, 17%): mp 224−226 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 12.21 (s, 1H, N*H* Ar), 10.70 (s, 1H, N*H* amide), 7.90 (d, 1H, J = 1.5, H-8), 7.68 (d, 1H, J = 8.8, H-5), 7.47−7.32 (m, 4H), 7.17−7.00 (m, 5H), 4.62 (s, 2H, OCOCH_2), 4.37 (s, 2H, NHCOCH_2); MS (FAB) m/z 497 [MH]$^+$. Anal. ($C_{25}H_{18}Cl_2N_2O_5$) C, H, N.

4-Hydroxy-2-oxo-3-(3-phenoxyphenyl)-1,2-dihydroquinolin-7-diazonium (13d). Since the diazonium is highly hygroscopic and sensitive to light, the whole reaction procedure was carried out in obscurity under anhydrous conditions. Amine **12** (100 mg, 0.3 mmol) was dissolved in 3.5 mL of CH_2Cl_2/CH_3CN (2:1) with TFA (49 μL, 0.64 mmol). The solution was cooled to −15 °C, and isoamyl nitrite (40 μL, 0.3 mmol) was added dropwise. The reaction mixture was stirred 1 h, and during this time 10 μL of isoamyl nitrite was added again. After cooling to −78 °C the diazonium (**13d**) was precipitated by the addition of 3 volumes of precooled ether. The formed precipitate was left to decant, the supernatant was eliminated via a syringe, ether was added again, and after stirring and decantation the new supernatant was also removed. This washing procedure is repeated three times. The final precipitate was dried by evaporation without any heating to give 0.13 g (92%) of **13d** as a red solid: ^{1}H NMR (300 MHz, MeOH-d_4) δ 8.53 (d, 1H, J = 1.9, H-8), 8.47 (d, 1H, J = 9.0, H-5), 8.3 (dd, 1H, J = 8.8, 1.9, H-6), 7.48 (t, 1H, J = 8.4, 8.1, H-5′), 7.36 (m, 2H, H-6′, H-2′), 7.19−7.04 (m, 6H); ^{13}C NMR (300 MHz, MeOH-d_4) 165.6 C-2, 160.0 C1″, 159.3 C-4, 158.9 C-3′, 140.4 C-8a, 134.9 C-1′, 132.2 C-5′, 131.9 C5″ and C3″, 129.2 C-5, 127.8 C-4a, 127.4 C-4″, 125.6 C-6′, 125.4 C-6, 122.9 C-4′, 121.1 C-2″ and C-6″, 121.0 C-2′, 120.9 C-8, 120.5 C-3, 117.3 C-7; UV (methanol) λ_{max}, ϵ: 393, 2695.

7-Chloro-2-oxo-3-(3-phenoxyphenyl)-1,2-dihydroquinolin-4-yl-4-(fluorosulfonyl)benzoate (13e). To a suspension of **2** (72.8 mg, 0.2 mmol) and *p*-(fluorosulfonyl)benzoyl chloride (55.7 mg, 0.25 mmol) in dry acetonitrile was added dropwise triethylamine (35 mL, 0.25 mmol). After the mixture was stirred for 4 h at room temperature, the solvent was evaporated under vacuum and the residue was partitioned between water and ethyl acetate. The organic layer was dried ($MgSO_4$), filtered, concentrated in vacuo, and purified by silica gel chromatography using toluene−ethyl acetate (1:1) as eluent to give **13e** as a white solid (83.4 mg, 76%): mp 246−247 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.37 (s, 1H, N*H*), 8.30 (s, 4H), 7.75 (d, 1H, J = 8.5, H-5), 7.46 (d, 1H, J = 1.9, H-8), 7.35 (t, 1H, J = 8.0, H-5′), 7.25−7.19 (m, 4H), 7.06 (d, 1H, J = 7.4, H-4″), 7.02 (t, 1H, J = 1.9, H-2′), 6.9 (dd, 1H, J = 8.0, 1.7, H-4′), 6.72 (m, 2H, H-2″, H-6″); MS (FAB) m/z 550 [MH]$^+$. Anal. ($C_{25}H_{18}Cl_2N_2O_5$) C, H, N.

3-(3-Aminophenyl)-7-chloro-4-hydroxyquinolin-2(1*H*)-one (14a). To a suspension of **11b** (1.4 g, 4.42 mmol) in 150 mL of THF was added dropwise a solution of 20% $TiCl_3$ in hydrochloric acid (86 mL, 132.6 mmol) under argon. The reaction mixture was stirred at room temperature for 8−12 h. The obtained solution was cooled in an ice bath and neutralized with aqueous 15% NaOH. The resulting dark slurry was diluted with 400 mL of ethyl acetate/methanol (3:2), and the mixture was agitated vigorously until the precipitation became yellow. The suspension was filtered, and the residue was washed extensively with ethyl acetate/methanol (3:2). The filtrate was evaporated to dryness and the obtained residue purified by silica gel chromatography (CH_2Cl_2−methanol, 9:1, as eluent) to give 1.21 g (60%) of **14a** as a pale yellow solid: mp > 310 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.45 (s, 1H, N*H*), 7.87 (d, 1H, J = 8.6 Hz, H-5), 7.82 (d, 1H, J = 2.1 Hz, H-8), 7.18 (dd, 1H, J = 8.6 Hz, 2.1 Hz, H-6), 7.03 (t, 1H, J = 7.7 Hz, H-5′), 6.53 (br, s, 1H, H-2′), 6.52 (m, 1H, H-4′), 6.46 (m, 1H, H-6′); MS (FAB) m/z 287 [MH]$^+$. Anal. ($C_{15}H_{11}ClN_2O_3$) C, H, N.

Compounds **14b** and **14c** were prepared in the same way

as described for **14a** starting with the appropriate nitro derivative (**11c**, **11d**).

7-Chloro-4-hydroxy-3-[3-(2-aminophenoxy)phenyl]quinolin-2(1*H*)-one (14b). Yield 1.21 g (87%), white solid: mp 278 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.55 (s, 1H, N*H*), 7.91 (d, 1H, J = 8.3, H-5), 7.33 (t, 1H, J = 7.8, H5′), 7.30 (d, 1H, J = 1.9, H-8), 7.2 (dd, 1H, J = 8.8, 1.9, H-6), 7.3 (d, 1H, J = 7.8, H-6′), 6.85 (m, 5H), 6.54 (m, 1H, H-5″), 4.90 (br, s, 2H, NH_2); MS (FAB) m/z 379 [MH]$^+$. Anal. ($C_{21}H_{15}ClN_2O_3$) C, H, N.

7-Chloro-4-hydroxy-3-[3-(3-aminophenoxy)phenyl]quinolin-2(1H)-one (14c). Yield 0.14 g (45%), white solid: mp 242−244 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.51 (s, 1H, N*H*), 7.92 (d, 1H, J = 8.8, H-5), 7.53 (pt, 1H, J = 8.0, 7.7, H-5′), 7.30 (d, 1H, J = 1.9, H-8), 7.20 (dd, 1H, J = 8.8, 1.9, H-6), 7.10 (d, 1H, J = 7.7, H-6′), 7.0 (m, 2H, H2′,H5″), 6.9 (ddd, 1H, J = 8.0, 2.2, 1.1, H4′), 6.3 (dd, 1H, J = 8.0, 1.9, H6″), 6.24 (pt, 1H, J = 2.2, 1.9, H-2″), 6.2 (ddd, 1H, J = 8.0, 1.9, 0.8, H4″), 5.19 (br, s, 2H, NH_2); MS (FAB) m/z 379 [MH]$^+$. Anal. ($C_{21}H_{15}ClN_2O_3$) C, H, N.

7-Chloro-4-hydroxy-3-(3-isothiocyanatophenyl)quinolin-2(1*H*)-one (15a). Treatment of **14a** (0.14 g, 0.5 mmol) under the conditions described for the conversion of **12** to **13a** gave **15a** as a pale yellow solid (0.10 g, 62%): mp 227−229 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.64 (s, 1H, N*H*), 7.97 (d, 1H, J = 8.6, H-5), 7.46 (pt, 1H, J = 8.0, 7.7, H-5′), 7.41 (t, 1H, J = 1.7, H-2′), 7.40 (m, 2H, H-4′, H-6′), 7.33 (d, 1H, J = 2.0, H-8), 7.22 (dd, 1H, J = 8.6, 2.0, H-6′); IR (neat) 2108 (NCS); MS (FAB) m/z 329 [MH]$^+$. Anal. ($C_{16}H_9ClN_2O_2S$) C, H, N.

2-Chloro-*N*-[3-(7-chloro-4-hydroxy-2-oxo-1,2-dihydroquinolin-3-yl)phenyl]acetamide (15b). Treatment of **14a** (0.11 g, 0.4 mmol) under the conditions described for the conversion of **12** to **13b** gave **15b** as a white solid (52.3 mg, 36%): mp > 310 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.50 (s, 1H, N*H*, Ar), 10.31 (s, 1H, N*H* amide), 7.93 (d, 1H, J = 8.8, H-5), 7.60 (d, 1H, J = 7.7, H-4′), 7.55 (br, s, 1H, H-2′), 7.33 (t, 1H, J = 8.0, H-5′), 7.30 (d, 1H, J = 2.2, H-8), 7.20 (dd, 1H, J = 8.0, 1.4, H-6′), 7.09 (br, d, 1H, J = 7.1, H-6), 4.25 (s, 2H, CH_2); MS (FAB) m/z 363 [MH]$^+$. Anal. ($C_{17}H_{12}Cl_2N_2O_3$) C, H, N.

3-[3-(7-Chloro-4-hydroxy-2-oxo-1,2-dihydroquinolin-3-yl)anilino]ethylsulfonyl Fluoride (15c). The reactive agent ethenesulfonyl fluoride was obtained as described in the literature[25] with one difference: the procedure using triethylamine instead of MgO was successful for converting 2-chloroethanesulfonyl fluoride into ethenesulfonyl fluoride. To a solution of **14a** (0.14 g, 0,5 mmol) in DMF (0.6 mL) was first added TFA (0.038 mL, 0.5 mmol) and then ethenesulfonyl fluoride (0.062 mL, 0.75 mmol). The reaction mixture was stirred overnight, and after addition of water (5 mL) and ethyl acetate (10 mL), the organic layer was washed with water (2 × 15 mL), dried ($MgSO_4$), filtrated, and evaporated to dryness. Silica gel chromatography (toluene−ethyl acetate−methanol, 5:4:1, as eluent) of the crude product gave 0.14 g (69%) of **15c** as a white solid: mp 274−276 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.52 (s, 1H, N*H*, Ar), 10.10 (s, 1H, N*H*CH$_2$CH$_2$SO$_2$F), 7.89 (d, 1H, J = 8.0, H-5), 7.29 (d, 1H, J = 2.0, H-8), 7.20 (dd, 1H, J = 8.0, 2.0, H-6), 7.15 (t, 1H, J = 7.6, H-5′), 6.60 (m, 3H), 4.10 (m, 2H, NH-CH_2-CH$_2$-SO$_2$F), 3.62 (m, 2H, NH-CH$_2$-CH_2-SO$_2$F); MS (FAB) m/z 397 [MH]$^+$. Anal. ($C_{17}H_{14}Cl_2FN_2O_4S$) C, H, N.

2-Chloro-*N*-[2-[3-(7-chloro-4-hydroxy-2-oxo-1,2-dihydroquinolin-3yl)phenoxy]phenyl]acetamide (15d). Treatment of **14b** (0.15 mg, 0.4 mmol) with chloroacetic anhydride (0.068 g, 0.4 mmol) as described for the preparation of **13b** gave **15d** (0.053 g, 29%) as a white powder: mp 153−155 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.52 (s, 1H, N*H* Ar), 10.37 (br, s, 1H, O*H*), 9.82 (s, 1H, N*H* amide), 8.00 (m, 1H, H-3″), 7.93 (d, J = 8.5, 1H, H-5), 7.41 (pt, J = 8.0, 7.7; 1H, H-5′), 7.30 (d, J = 1.9, 1H, H-8), 7.20 (m, 2H, H-6, H-6′), 7.11 (m, 2H, H-6″, H-4″), 7.07 (br, s, 1H, H-2′), 6.98 (m, 2H, H-5″, H-4′), 4.36 (s, 2H, CH_2); MS (FAB) m/z 455 [MH]$^+$. Anal. ($C_{23}H_{16}Cl_2N_2O_5$) C, H, N.

2-[2-[3-(7-Chloro-4-hydroxy-2-oxo-1,2-dihydroquinolin-3-yl)phenoxy]phenyl]anilino]ethylsulfonyl Fluoride (15e). Compound **15e** was prepared as described for the conversion of **14a** to **15c** starting from **14b**: yield 88 mg (72%), white solid: mp 215−217 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.49 (s, 1H, N*H* amide), 10.37 (br, s, 1H, O*H*), 7.92 (d, J = 8.6, 1H, H-5), 7.37 (t, J = 8.0, 1H, H-5′), 7.30 (d, J = 1.9, 1H, H-8), 7.20 (dd, J = 8.6, 1.9, 1H, H-6), 7.10 (br, d, J = 7.7, 1H, H-6′), 7.01 (m, 1H, H-4″), 6.95 (pt, J = 2.2, 1.3, 1H, H-2′), 6.90 (dd, J = 8.3, 1.7, 1H, H-6″), 6.86 (dd, J = 8.0, 1.4, 1H, H-3″), 6.82 (dd, J = 8.0, 1.3, 1H, H-4′), 6.63 (m, 1H, H-5″), 5.48 (t, J = 6.5, 1H, NHCH$_2$CH$_2$SO$_2$F), 4.14 (m, 2H, NHCH$_2$CH_2SO$_2$F), 3.68 (m, 2H, NHCH_2CH$_2$SO$_2$F); MS (FAB) m/z 489 [MH]$^+$. Anal. ($C_{23}H_{18}ClFN_2O_5S$) C, H, N.

2-Chloro-*N*-[3-[3-(7-chloro-4-hydroxy-2-oxo-1,2-dihydroquinolin-3-yl)phenoxy]phenyl]acetamide (15f). Compound **15f** was prepared as described for the conversion of **12** to **13b** starting from **14c**: yield 58 mg (64%), white solid; mp 222−224 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.49 (s, 1H, N*H*, Ar), 10.37 (s, 1H, N*H*, amide), 7.92 (d, J = 8.5, 1H, H-5), 7.39 (m, 2H, H-5′, H-8), 7.31 (m, 3H, H-6, H-6′, H-5″), 7.19 (m, 2H, H-4′ or H-4″, H-2′), 7.05 (br, t, J = 2.5, 1H, H-2″), 76.95 (ddd, J = 8.0, 2.5, 0.5, 1H, H-4′ or H-4″), 6.78 (m, 1H, H6″), 4.22 (s, 2H, CH_2); MS (FAB) m/z 455 [MH]$^+$. Anal. ($C_{23}H_{16}Cl_2N_2O_4$) C, H, N.

4-Chloro-2-nitrobenzoic Amide (17). Compound **17** was synthesized according to a reported procedure starting from 4-chloro-2-nitrobenzoic acid (6.05 g, 30 mmol).[53] The crude product was purified by silica gel chromatography using 5% methanol in CH_2Cl_2 as eluent to give 5.86 g (97%) of **17** as a white solid: mp 171 °C (lit. 172 °C[53]); ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.20 (s, 1H, NH_2), 8.13 (d, 1H, J = 2.2, H-3), 7.86 (dd, 1H, J = 8.4, 2.2, H-5), 7.77 (s, 1H, NH_2), 7.66 (d, 1H, J = 8.4, H-6); MS (EI) m/z 200 [M$^+$]. Anal. ($C_7H_5ClN_2O_3$) C, H, N.

4-Chloro-2-nitrobenzonitrile (18). Compound **18** was prepared according to the method of Campagna et al.[27] starting from **17** (4.01 g, 20 mmol). Workup was performed by precipitation of the product with ice, filtration, and redissolution of the residue in CH_2Cl_2 (100 mL). After extraction with water (2 × 50 mL), the organic layer was dried ($MgSO_4$), filtrated, and evaporated to dryness. The crude product was purified by silica gel chromatography (hexane−ethyl acetate, 8:2, as eluent): yield 3.30 g (90%), white solid; mp 98 °C (lit. 93−97 °C[54]); ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.33 (d, 1H, J = 1.8, H-6), 7.90 (d, 1H, J = 8.0, H-3), 7.82 (dd, 1H, J = 8.0, 1H, H-5); MS (EI) m/z 182 [M$^+$]. Anal. ($C_7H_3ClN_2O_2$) C, H, N.

2-Amino-4-chlorobenzonitrile (19). Amine **19** was synthesized according to the procedure of Lamara et al.[54] using 12 equiv of a solution of 20% $TiCl_3$ in hydrochloric acid to reduce 0.91 g (5 mmol) of **18**. Purification by silica gel chromatography (hexane−ether, 6:4, as eluent) gave 617 mg (81%) of **19** as a white solid: mp 156 °C (lit. 156−158 °C[55]); ^{1}H NMR (200 MHz, DMSO-d_6) δ 7.41 (d, J = 8.4, 1H, H-6), 6.82 (d, J = 2.2, 1H, H-3), 6.59 (dd, J = 8.4, 2.2, 1H, H-5), 6.33 (s, 2H, NH_2), MS (EI) m/z 152 [M$^+$]. Anal. ($C_7H_5ClN_2$) C, H, N.

4-Amino-7-chloro-3-(3-phenoxyphenyl)quinolin-2(1*H*)-one (3). Compound **3** was prepared as described by Carling et al.[22] starting from **19** (0.31 g, 2 mmol) and crude 3-phenoxyphenylacetic chloride (4 mmol). The obtained intermediate was not isolated but directly treated with sodium hydride in DMF to yield compound **3**, which was purified by silica gel chromatography (CH_2Cl_2−methanol, 95:5, as eluent): yield 0.32 g (51%), white solid; mp 196 °C (lit. 196−197 °C[22]); the spectrocopic data are as published by Carling et al.[22] Anal. ($C_{21}H_{15}ClN_2O_2$) C, H, N.

2-Chloro-*N*-[7-chloro-2-oxo-3-(3-phenoxyphenyl)-1,2-dihydroquinolin-4-yl]acetamide (20). To a solution of **3** (0.109 g, 0.3 mmol) in THF (10 mL) with triethylamine (0.21 mL, 1.5 mmol) was added chloroacetic chloride (0.12 mL, 1.5 mmol), and the mixture was heated to reflux for 1 h. It was evaporated, and the residue was dissolved in ethyl acetate (20 mL). The organic layer was washed with water (2 × 20 mL), dried (Na_2SO_4), filtrated, and removed under vacuum. The crude product was purified on silica gel (toluene−ethyl acetate, 6:4, as eluent) to give **20** as a white solid (40 mg, 30%): mp

197−199 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 12.19 (s, 1H, N*H*, Ar), 10.19 (s, 1H, N*H*, amide), 7.62 (d, J = 8.8, 1H, H-5), 7.33−7.44 (m, 4H,), 7.26 (dd, J = 8.8, 2.2, 1H, H-6), 6.93−7.15 (m, 6H), 4.13 (s, 2H, CH_2); MS (EI) *m*/*z* 438 [M$^+$]. Anal. ($C_{23}H_{16}Cl_2N_2O_3$) C, H, N.

Reactivity and Stability of the Affinity Probes. Stability. Ligands were initially dissolved in DMSO at a concentration of 10 mM. At zero time, the test compounds were diluted in HEPES/KOH (50 mM, pH 7.5) or frog's Ringer (10 mM HEPES, 115 mM NaCl, 0.9 mM $CaCl_2$, 1 mM KCl, pH 7.2), incubated at room temperature, and 100 μL aliquots were injected at various times for HPLC analysis, using a Zorbax reverse-phase SB C-18 column (250 mm × 4.6 mm internal diameter) fitted with a Bondasorb C-18 precolumn. Elution was effected using a gradient of 50 to 100% of A (CH_3CN, TFA 0.05% (v/v)) in B (H_2O, TFA 0.05% (v/v)) in 20 min running at a flow rate of 1 mL/min. Peaks were monitored at 320 nm.

Reactivity. The reactivity of the probes toward cysteine were evaluated similarly: at zero times 10 μL of the stock solution of the ligand was mixed with frog's Ringer containing 10 μL of a freshly prepared 1 M stock solution of *N*-acetyl-L-cysteine methyl ester (Fluka) in DMSO to a final volume of 1 mL. The reaction was followed as in the stability studies.

Pharmacology. DCKA Binding Assay. The affinities of the newly described ligands for the NMDA receptor glycine-site were determined by competition against [^{3}H]-5,7-dichlorokynurenic acid ([^{3}H]-DCKA, DuPont NEN, 573.5 Gbq/mmol) binding in rat brain membranes homogenate, which was prepared according to Danysz et al.[28] and kept frozen at −80 °C for no longer than 10 weeks. For binding, the stock membrane suspension was thawed and further washed with binding buffer (HEPES-KOH, 50 mM, pH 7.5) as described by Jimonet et al.[29] An aliquot of final suspension (ca. 0.1 mg of proteins) was incubated 30 min at 4 °C with 20 nM [^{3}H]-DCKA in the absence or in the presence of 1 mM glycine, for total and nonspecific binding, respectively, or in the presence of increasing concentrations of the affinity probes (whose dilutions were prepared on the day of the experiment[56] out of a 1 mM stock solution in DMSO). To detect ligand−receptor reaction, samples containing the affinity marker at the concentration of its IC_{50} were incubated for the regular 30 min and for 2.5 h. The assays were terminated by rapid filtration (Whatman GF/B filters), and filter-bound radioactivity was determined by liquid scintillation spectrometry. Dose−response curves were fitted to the logistic equation by nonlinear least-squares regression analysis as in Bowen et al.[57] K_I values were calculated using the equation of Cheng and Prussof with a K_D value of 35 nM for DCKA, as determined in these conditions in a preliminary saturation experiment (data not shown). The values reported here are the mean (and standard error) of at least three experiments.

Electrophysiology. The potency of our ligands as antagonists were determined electrophysiologically in *Xenopus laevis* oocytes expressing the cloned NMDA receptor 1_A/2B subunit combination[5,30] superfused with frog's Ringer supplemented with 1 μM glycine and 100 μM glutamate, for the control membrane current response, plus increasing concentrations of analogue, for its suppression by the antagonist. A minimal washing time of 4−6 min with frog's Ringer was performed on the oocyte between each stimulation. IC_{50} values for each antagonist were calculated, assuming a competitive inhibition, from five-point concentration−inhibition curves using the sigmoidal equation in the Kaleidagraph graphic software. To assess the reversibility of the antagonistic effect, pulses of 1 mM glycine were incorporated in the washing procedure, at least for the high-affinity ligands, including L-701.324. The values given are the mean (and standard error) of at least three experiments.

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