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Bioorganic & Medicinal Chemistry Letters 18 (2008) 2765–2770
Bioorganic & Medicinal Chemistry Letters

Reactive derivatives for affinity labeling in the ifenprodil site of NMDA receptors

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Received 19 March 2008; revised 7 April 2008; accepted 8 April 2008
Available online 11 April 2008

Abstract—To prepare thiol-reactive ifenprodil derivatives designed as potential probes for cysteine-substituted NR2B containing NMDA receptors, electrophilic centers were introduced in different areas of the ifenprodil structure. Intermediates and final compounds were evaluated by binding studies and by electrophysiology to determine the structural requirements for their selectivity. The reactive compounds were further tested for their stability and for their reactivity in model reactions; some were found suitable as structural probes to investigate the binding site and the docking mode of ifenprodil in the NR2B subunit.
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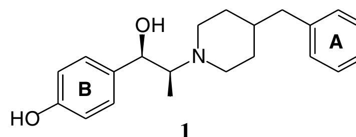
NMDA receptors (NMDARs) are a subtype of ionotropic glutamate receptors widely distributed in the vertebrate central nervous system. NMDARs play major roles in both physiological and pathological states of the CNS, including ischemic stroke, seizures, head trauma and pain. NMDARs occur as hetero-oligomers composed of NR1, NR2 (of which there are four: NR2A–NR2D), and more rarely NR3 subunits.[1,2] Because non-selective NMDAR antagonists have impeding adverse side effects, attention has focused toward drugs capable of modulating selectively certain subtypes of NMDARs. The NR2B-selective type of non-competitive antagonists has a strong potential in this regard, showing both neuroprotective and analgesic properties together with little side effects.[3–5] It has been recently shown that the N-terminal domain (NTD) of the NR2B subunit forms the binding site of ifenprodil (**1**)[6] and Ro256981,[7] two prototypic NR2B-selective antagonists, while an extensive pharmaco-chemical research had led to the description of common structural features for this family of compounds.[8–10] Our knowledge of their binding site is based on homology 3D modeling and mutational analysis: the NR2B NTD has been modeled after a periplasmic protein from *Escherichia coli* (LIVBP),[6,11,12] as well as after the glutamate binding domain of the mGluR1 receptor[7]: it is proposed to fold as two lobes separated by a large central cleft.[6] Mutating residues located in the central cleft of the NR2B NTD abolishes the high sensitivity to ifenprodil and derivatives.[6,12–14] Other biochemical evidences[6,15–17] also support the location of the ifenprodil binding site in the NTD of the NR2B subunit and in its cleft; however, the effects of the mutation of residues not located in this domain suggest alternative hypotheses.[11]



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In order to propose a docking mode of ifenprodil in the NTD of NR2B, we pursued a strategy combining cysteine-scanning mutagenesis and affinity labeling.[18] In this strategy, the formation of a covalent bond between a cysteine-reactive ligand derivative and a cysteine-substituted receptor enables to identify direct interactions between the ligand and a precise region of the receptor. In the recent years, we have applied this strategy to explore the glycine binding site (NR1 subunit) of the NMDA receptor.[19,20] Our results were obtained using full-length, membrane-inserted NR1/NR2 receptors; they provided our structural homology model

Keywords: Ifenprodil; NR2B; NMDA.
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doi:10.1016/j.bmcl.2008.04.019

with an experimental basis and established a general docking mode for antagonists at the gly_B site. These were remarkably consistent with the crystal structure of the agonist binding domain of the NR1 subunit (S1–S2 soluble fragment).[21]

This letter describes the stepwise approach toward thiol-reactive ifenprodil derivatives suitable for structural investigations of the binding site for NR2B selective inhibitors of the NMDARs. The affinity and reactivity of the derivatives should indeed be high enough for the occurrence of a covalent bond formation with a cysteine-mutated receptor while they occupy the binding site.[22]

A reliable docking of ifenprodil-like compounds being our final purpose, we have synthesized molecules consistent with their main structural features: two aryl rings, one located in a hydrophobic environment (ring A) and a ring substituted by polar groups (ring B), while a tertiary amine, at the center of the molecule, is connected to each ring by a linker.[23] A distance of 9–11 Å between the two rings, in an extended conformation of the molecule, was also suggested.[9] Electrophilic substituents were introduced in carbon positions which were selected to explore the different areas of the binding site corresponding to these components of the ligand's structure. Intermediate and final compounds were tested as ligands for the NR2B NTD site, and, for some of them, as effectors of the recombinant NR1/NR2B NMDARs. The reactive compounds were also tested for their stability and their reactivity toward thiols in model conditions. Their potential as structural probes for the ifenprodil binding site of NMDARs is discussed.

The synthetic pathways for these compounds can be found in Scheme 1. In compounds **4** and **5**, the hydroxyle group of ring B in ifenprodil has been replaced by a chloroacetamido group or an isothiocyanate group, respectively; moreover, one asymmetric center has been suppressed by converting, on the linker, the secondary alcohol into a ketone. Its synthesis involves first a Friedel-Crafts[24] reaction between acetanilide and 2-bromopropionyl chloride. Subsequently, the substitution of the bromide by 4-benzylpiperidine yielded precursor **2**. The thiol-reactive probes **4** and **5** were finally obtained by the deprotection of the aromatic amine affording compound **3**, followed by functionalization.

Compounds (±) **6** and (±) **7** conserved the structure of ifenprodil; they differed only by the presence of the amino function instead of the phenolic group. Their synthesis involved the reduction of ketone **2**, affording 4 diastereoisomers which were separated by column chromatography as erythro- and threo-racemic mixtures. The final deprotections yielded racemic threo-(±) **6** and erythro-(±) **7** derivatives.

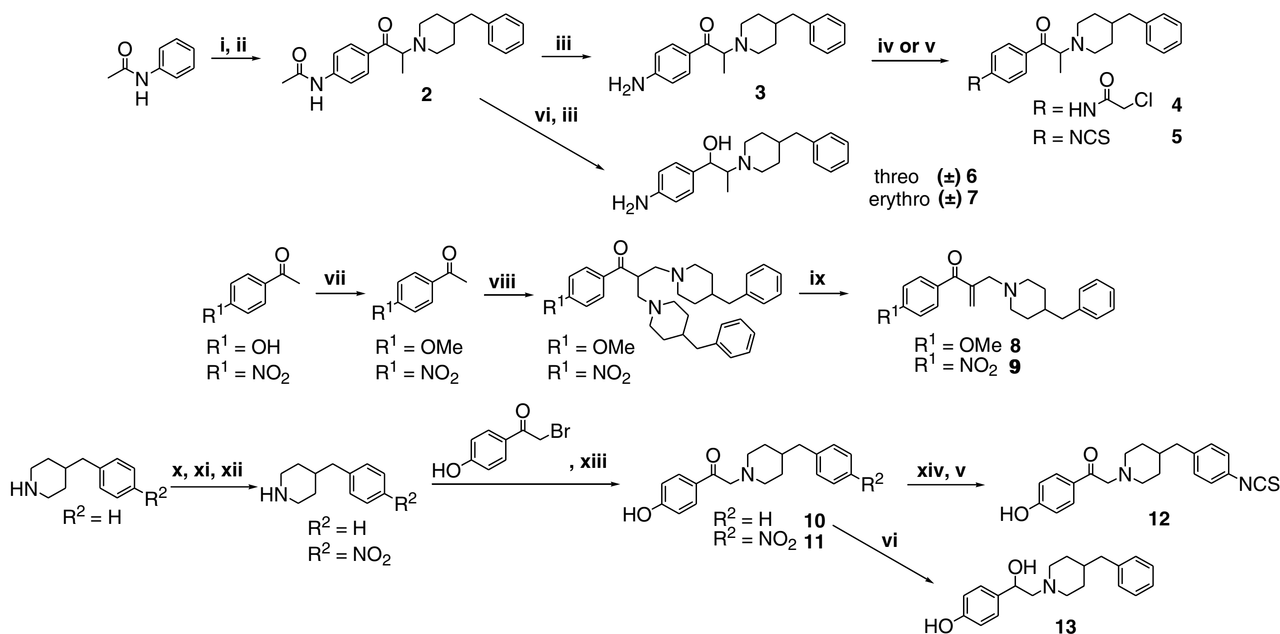
Compounds **8** and **9**, whose linker, compared to the reference ifenprodil molecule, contains a Michael acceptor together with an additional carbon atom required a different synthetic pathway. After conversion of the B ring phenol group into a methoxy derivative (to avoid reaction in the ortho position[25]), a Mannich reaction with paraformaldehyde and 4-benzylpiperidine under acidic catalysis yielded condensation products where two aminomethyl groups have been introduced.[26] Finally, the elimination of one benzylpiperidine group on silica gel produced the propenone derivatives **8** and **9**.

In compound **12**, ifenprodil's asymmetric centers have been suppressed and a reactive-NCS function was introduced on aromatic ring A. A high yield synthesis of the nitro precursor **11** was achieved by coupling *p*-hydroxybromophenacyle[27] to 4-(4-nitrobenzyl)-piperidine, which was prepared in a three-step procedure from 4-benzylpiperidine.[28] Compound **10** was obtained from commercially available 4-benzylpiperidine using the same synthetic pathway. Ligand **12** was synthesized by the hydrogenation of the nitro compound **11** followed by treatment with thiophosgene in a tetrahydrofurane/aqueous Na_2CO_3 solution mixture. Compound **13**, designed to estimate the influence of asymmetry in ifenprodil, was obtained by the reduction of precursor **10** with sodium borohydride. Compounds **2**–**13** were fully characterized by ^1H NMR and HRMS. The purity of the reactive compounds was checked by HPLC.[29]

The affinities of compounds **2**–**13** were measured by equilibrium binding to rat brain membranes in competitions against ^3H-ifenprodil as in Ref. 30: ^3H-ifenprodil (2.501 Gbq./mmol from Perkin-Elmer Life Sciences) was incubated with the membranes for 2 h at 4 °C in 5 mM Tris–chloride buffer, pH 7.4, in the absence or presence of 10 μM Ro-084304 for the determination of the nonspecific binding. K_Is were calculated from the measured IC_{50}s[31] and from a K_D of 11 ± 1.5 nM (n = 5) for ifenprodil, that we measured following a previously published protocol[30] (Table 1).

The replacement of the *p*-hydroxyl group in ring B of ifenprodil by an amino group (compound (±) **7**) decreases its affinity by ca. one order of magnitude, while the conversion of this amine group of precursor **3** into amide **2**, chloroacetamide **4** and isothiocyanate **5** further shifts the K_I value by a factor of 1.8, 8.5, and 12, respectively: this is consistent with the magnitude of the H-bond donor property of substituents in this position.[8,10] The size and rigidity of these substituents are also likely to interfere: thus, the higher K_I value of **5** compared to **4** may be due to the fact that the isothiocyanate group, although smaller, is less flexible than the chloroacetamide group.

Similarly, the effect of substituting the *p*-position of ring A is evidenced by the K_I values of compounds **10**, **11** and **12**: the substitution of a hydrogen by a nitro-group and its transformation into an isothiocyanate group moderately influence the K_I value. Indeed, the total penalty for introducing this reactive group in this position is only a factor of 1.7; this position on ring A thus appears to be less sensitive to substitution than the corresponding position on ring B, in agreement with previous findings.[8,10]



Scheme 1. Reagents and conditions: (i) $ClCOCH(CH_3)Br$, $AlCl_3$, CS_2, reflux, 17%; (ii) 4-benzylpiperidine, Et_3N, CH_3CN, reflux, 66%; (iii) HCl 37%, reflux, 89%; (iv) $ClCOCH_2Cl$, Et_3N, CH_2Cl_2, 0 °C, 69%; (v) $CSCl_2$, $NaHCO_3$, THF/H_2O 1:1, rt, 96%; (vi) $NaBH_4$, MeOH, 40 °C, 88%; (vii) CH_3I, K_2CO_3, acetone, reflux, 100%; (viii) HCHO, 4-benzylpiperidine, HCl, EtOH, reflux, 100%; (ix) silica gel heptane/ethyl acetate 1:1, 40%; (x) $(CF_3CO)_2O$, CH_2Cl_2, rt, 100%; (xi) HNO_3, CF_3COOH, −30 °C to rt, 37%; (xii) NaOH, EtOH, rt, 61%; (xiii) DBU, THF, rt, 84%; (xiv) H_2, Pd/C, $MeOH/CH_2Cl_2$ 1:1, rt, 100%.

In our cysteine affinity labeling approach, one should apply to cysteine-substituted receptors only reactive ligands of well defined configuration, for a useful interpretation of their effect in terms of binding-site structure and ligand docking. Asymmetric centers generate mixtures of enantiomeric pairs. We therefore attempted to suppress the two asymmetric centers in the spacer arms while keeping as much as possible of their properties in length and polarity. With regard to the asymmetric carbon atom closest to ring B (α carbon), compounds (±) **6** and (±) **7** represent two diastereoisomeric pairs of enantiomers. Forming a ketone on this α carbon (instead of an alcohol function) shifts the K_I value from 97 to 58 nM (compounds (±) **7** and **3**). However, when there is no asymmetric center on the β carbon, the same change (from compound **13** to compound **10**) induces an opposite shift in K_I value, from 62 to 141 nM, while the suppression of the asymmetric center on the β carbon by the replacement of the linker's methyl group of ifenprodil **1** with a hydrogen atom, in **13**, produces à sixfold decrease in affinity, from a K_D value of 11.2 nM to a K_I value of 62 nM. In a racemic mixture, the presence of the less active compound decreases the apparent affinity of its isomer only modestly.[32] Similarly, the K_D for ifenprodil and the K_I values in Table 1 (compounds **2**, **3**, **4**, **5**, (±) **6**, (±) **7**, and **13**) are composite values. The maximum effect of the suppression of the two asymmetric centers of ifenprodil is a decrease in affinity by a factor of 13 (from ifenprodil to compound **10**), which is likely to confer to the achiral derivatives sufficient affinity to undergo functionalization for affinity labeling experiments. However, compounds **8** and **9** have no asymmetric center, but their high K_I values can also result from unfavourable substituents on ring B (the *p*-amino- or *p*-hydroxy-substituted compounds could not be isolated).

The stability of the reactive compounds **4**, **5**, **8**, **9** and different times, after dilution 1/10 in frog's Ringer **12**, were analyzed by HPLC: aliquots were injected, at buffer (pH 7.4) of a 10 mM stock solution in DMSO, and the time-dependent decrease of the peak area of each compound was followed. Their reactivity toward thiols was evaluated, using the same technique, by determining their half-life in the presence of excess of *N*-acetyl cysteine methyl ester (NACME). As the reaction rate varies linearly with the concentration of the reagents (pseudo-first order reaction), these were adjusted for an accurate measurement of the decay of the probes. Conditions and results can be found in Table 2. All five compounds react with thiols within minutes at the concentrations used; their stability in Ringer's buffer is appropriate for their use in receptor binding and activity assays. The compared reactivity of these compounds is consistent with their chemical structure: isothiocyanates react faster than chloroacetamides (**5** and **12** vs **4**) and, among Michael acceptors (both highly reactive), the electron attracting *p*-nitro substituent confers to **9** a 110-fold higher reactivity over **8**, in which electron-donor methoxy group is in the same position.

At submicromolar concentrations, ifenprodil selectively inhibits currents carried by NR1/NR2B receptors.[33] This high-affinity inhibition is non-competitive (toward the agonist glutamate) and does not depend on the transmembrane voltage (as expected for a binding site located outside the ionic channel). The biological activity of the reactive compounds in Table 2 was

Table 1. Binding data for ifenprodil and compounds **2–13**

Name or number	Compound	K_I[a]	Fold shift K_I^n/K_D^{ifen}
Ifenprodil (±) **1**[b]		11 ± 1.5[c]	1
2		107 ± 11	10
3		58 ± 5.3	5.3
4		493 ± 56	45
5		679 ± 34	62
(±) **6**[b]		300 ± 35	27
(±) **7**[b]		97 ± 10	8.8
8		527 ± 33	48
9		248 ± 44	23
10		141 ± 19	13
11		200 ± 14	18
12		245 ± 33	22
13		62 ± 8	16

[a] Values are given in nM ± SEM (n = 3).
[b] Ifenprodil, compounds **6** and **7** were tested as racemic erythro- or threo-derivatives (see text).
[c] K_D.

evaluated by performing two-electrode voltage-clamp measurements on Xenopus oocytes expressing wild-type NR1/NR2B receptors (ref 6). NMDA currents were induced by saturating concentrations of L-glutamate and glycine (100 μM each), and the effects of the different compounds were assessed by measuring the change in current size induced by the application of the compound during a NMDA response. Com-

Table 2. Stability in buffer and reactivity versus thiols of reactive compounds

Compound	$t_{1/2}$ for stability	Reactivity			
		[Compound] (mM)	[NACME] (mM)	$t_{1/2}$ (min)	Relative reactivity
4	>1 day	0.1	500	114	
5	20 h	0.005	0.5	3.24	704×10^3
8	5 h	0.1	10	7	814
9	5 h	0.01	0.1	62	92×10^3
12	>1 day[a]	1	100	23	2.5

[a] An apparent decrease of concentration of compound **12** results from its slow precipitation in buffer, without the formation of degradation products.

Table 3. Activity of thiol-reactive ifenprodil derivatives at wild-type NR1/NR2B receptors

Compound	IC_{50} (μM)	$IC_{50}^{compound}/IC_{50}^{ifenprodil}$
1 (Ifenprodil)	0.11	1
4	14 ± 7 (n = 5)	130
8	70 ± 10 (n = 3)	640
12	16 ± 6 (n = 4)	145

pound **5**, which displayed the highest K_I value, showed only very weak inhibition of NR1/NR2B receptors (8% current inhibition with 10 μM of **5**). Compounds **4**, **8**, and **12** were much better at inhibiting NR1/NR2B receptors, with IC_{50}s of 14, 70, and 16 μM, respectively (Table 3). For comparison, in the same test, the IC_{50} for ifenprodil was found to be of 0.11 μM, a value very similar to what has been previously reported.[6] When tested by electrophysiology, the compounds appear less potent compared to binding studies, as their activity is decreased by at least two orders of magnitude compared to ifenprodil. Moreover, compounds **4** and **12** show similar IC_{50}s, whereas their K_I values are twofold different. However, a general trend is followed: the derivatives which have the lowest K_I value also have the lowest IC_{50}. Surprisingly, compound **9** was found to be inactive, since it had no effect on NMDA responses at 10 μM. The example of compound **9** suggests the existence of NR2B-NTD compounds functionally 'silent', that is, compounds unable to modulate the receptor activity in spite of their ifenprodil-competitive binding.

To ascertain that the inhibitory effects seen with the compounds were specific to the NR2B subunit and engaging the NR2B NTD, we repeated some of the above experiments on oocytes expressing either wild-type NR1/NR2A receptors or NR1/NR2B receptors truncated for the entire NR2B-NTD (NR1/N2B ΔNTD; see Ref. 14). As expected for compounds targeting selectively the NR2B NTD, compounds **4**, **8**, and **12** have little effects on NR1/NR2A receptors with EC_{50}s estimated to be >100 μM (25% [n = 3] and 11% [n = 3] inhibition for compounds **4** and **8** at 100 μM). The IC_{50} for compound **12** was shifted from 16 (NR2B, n = 4) to 150 μM (NR2A, n = 5) and became voltage dependent, as expected if the observed inhibition on NR1/NR2A receptors is mostly due to channel block by the compound.[6] When the NTD segment was truncated from the NR2B subunit, similar rightward shifts were observed for compounds **12** (IC_{50} = 570 μM, [n = 4], voltage dependent) and **4** (IC_{50} = 230 μM [n = 3]); compound **8** was found inactive, as with the NR2A subunit (no effect up to 100 μM). Altogether, these results show that the synthesized compounds have a common and subunit-specific target on NMDARs, the NR2B NTD.

In summary, we have synthesized ifenprodil-derived compounds to design NR2B selective ligands with a reactive group in different parts of the molecule. These ligands were assayed for their ability to be recognized by the ifenprodil binding site and to produce the expected NR2B-NTD mediated allosteric antagonism of NMDAR mediated currents. Characterized as stable in buffer but reactive with thiol groups, these compounds, like ifenprodil, present a pharmacological profile consistent with their specific binding to the modulatory NTD of the NR2B subunit. Although these compounds displayed a decreased potency compared to ifenprodil, they appear to be valuable tools for a further characterization of the ifenprodil binding site, its topological analysis, and the docking of cognate ligands.

Acknowledgments

We wish to thank the Roche Company for the kind gift of Ro-084304 as well as the Centre National de la Recherche Scientifique, the Université Louis Pasteur, the Institut National de la Santé et de la Recherche Médicale, the Agence Nationale de la Recherche and the Ministère de la Recherche et de la Technologie for their material and financial support.

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29. HPLC retention time on a Analytical C18 Acclaim (4.6 × 300 mm) column using a 30 min linear gradient from 0% to 50% acetonitrile in 0.1% TFA water solution at 1 ml/min, for compounds **4** and **12**, and 20 min similar linear gradient for compounds **8** and **9**: 22.7, 25.5, 20.6 and 19.9 min, respectively. For compound **5**, the linear gradient was as for compounds **4** and **12**, except for the acetonitrile concentration, from 20% to 70%; retention time: 24.7 min.
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