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Research Letter

1-Benzyl-1,2,3,4-Tetrahydro-β-Carboline as Channel Blocker of *N*-Methyl-D-Aspartate Receptors

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***N*-methyl-D-aspartate (NMDA) receptors belong to the family of ligand-gated ion channels and are important for synaptic plasticity and memory function. The NMDA receptor consists of a voltage-dependent channel permeable to Ca^{2+} and Na^{+}. In Alzheimer's disease, neuronal degeneration is thought to cause an excessive release of glutamate to the extracellular space, which may in turn mediate prolonged stimulation of the NMDA receptor complex and, as a consequence, excessive calcium influx into neuronal cells, leading to subsequent cell death. This process is called glutamate-induced excitotoxicity, and its inhibition may present an effective antidementive therapy. We found that 1-benzyl-1,2,3,4-tetrahydro-β-carboline (1a) blocked NMDA receptor–mediated, glutamate-induced excitotoxicity with an IC_{50} value of 27.4 μM, whereas the closely related 1-phenyl-1,2,3,4-tetrahydro-β-carboline (1b) had no effect. The binding modes of the reported compounds were studied by *in silico* docking simulations. We demonstrate that compounds (*S*)-1a and (*R*)-1a, but not (*S*)-1b and (*R*)-1b, have the same characteristics of potent NMDA receptor blockers, because they establish the main interactions inside the vestibule region of the receptor described previously for the high-affinity NMDA receptor blocker, MK-801.**

Key words: channel blockers, docking, excitotoxicity, NMDA receptor, β-carboline

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N-methyl-D-aspartate (NMDA) receptors belong to the family of ionotropic glutamate receptors, which are widely distributed in mammalian brain and spinal cord (1). Functional NMDA receptors consist of heterotetrameric assemblies of different subunits (2): NR1 subunits bind glycine in combination with at least one glutamate-binding NR2 subunit and, in some cases, a glycine binding NR3 subunit. At least eight splice variants of the NR1 (NR1-1a to NR1-1h) subunit, four NR2 protein (NR2A–NR2D) and NR3 (A and B) have been identified (3). The complex has been suggested to play an important role in normal brain functions such as learning and memory (4).

Several characteristics distinguish NMDA receptors from other glutamate receptors, including the requirement of glutamate and glycine agonists to activate the receptor, a high permeability of the channel for Ca^{2+} over Na^{+}, and a voltage-dependent block of the channel by Mg^{2+}. NMDA receptors have at least three regulatory domains (5). Both glutamate and glycine must be present for full activation of the NMDA receptor channel. A further site is located in the channel pore, which is blocked by Mg^{2+} under resting conditions. However, this block is removed if the cell depolarizes. This site, or at least sites close to the Mg^{2+} site, can be blocked by a large number of structurally dissimilar organic inhibitors (6,7), including ketamine (8), MK-801 (9), memantine (10), and various spider toxins and polyamine derivatives (11).

Prolonged stimulation of the receptor complex caused by high extracellular concentrations of glutamate favors excessive calcium influx into neuronal cells through activated NMDA receptors, producing cell death by mechanisms that may include irreversible overload of intracellular free Ca^{2+}, activation of calcium-sensitive proteases, and stimulation of nitric oxide synthase with the subsequent generation of toxic free radicals. This is called glutamate-induced excitotoxicity and may contribute to brain disorders and neurodegenerative diseases in the mammalian central nervous system.

Ligands that specifically antagonize the actions of the neurotransmitter glutamate at NMDA receptors may offer an effective means of novel approach to treat disorders such as Alzheimer's disease (12), cerebral ischemia and stroke (13), epilepsy (14), AIDS-associated dementia (15), Parkinson's disease (16), and Huntington's disease (17). Owing to unsatisfactory efficacy of the medicinal treatment for brain disorders and neurodegenerative diseases, a search for new drugs and understanding of structure–activity relationship

is required. β-carbolines are widely distributed in many plants and mammals (18), and the core structure is present in various active compounds, which display a broad spectrum of biological activities (19). Previous studies found that bivalent β-carbolines are potent inhibitors of glutamate-induced excitotoxicity in transgenic cell lines expressing NMDA receptors, but several substituted monovalent β-carbolines were reported as being inactive (20). However, we found that 1-benzyl-1,2,3,4-tetrahydro-β-carboline (**1a**) displays a dose-dependent inhibition of glutamate-induced excitotoxicity, whereas the structurally similar compound 1-phenyl-1,2,3,4-tetrahydro-β-carboline (**1b**) was inactive. The binding modes of the reported compounds inside the vestibule region of NMDA receptor were studied by molecular modeling to explain these results.

Material and Methods

Chemistry

β-carboline amines **1a**/**1b** were synthesized following the sequence depicted in Scheme 1. Imines were obtained from the corresponding acids **2a**/**2b** and tryptamine by coupling with EDC/HOBt (21) in CH_2Cl_2 at room temperature, which afforded the corresponding amide **3** (**3a** in 97%; **3b** in 98%). The amides **3a**/**3b** were subjected to treatment with $POCl_3$ that promoted the Bischler-Napieralski cyclization (22,23) affording imines **4a**/**4b** (82% and 85% yields, respectively). Having prepared **4a**/**4b**, the next stage was set to reduce the imines by using $NaBH_4$ at room temperature for 12 h to produce dihydro-β-carbolines **1a** (98%) and **1b** (96%) (24). Moreover, we considered that the product obtained by reduction with $NaBH_4$ was a racemic mixture of (*S*)-**1a**/(*R*)-**1a** and (*S*)-**1b**/(*R*)-**1b**, respectively.

1-Benzyl-1,2,3,4-tetrahydro-β-carboline (1a)

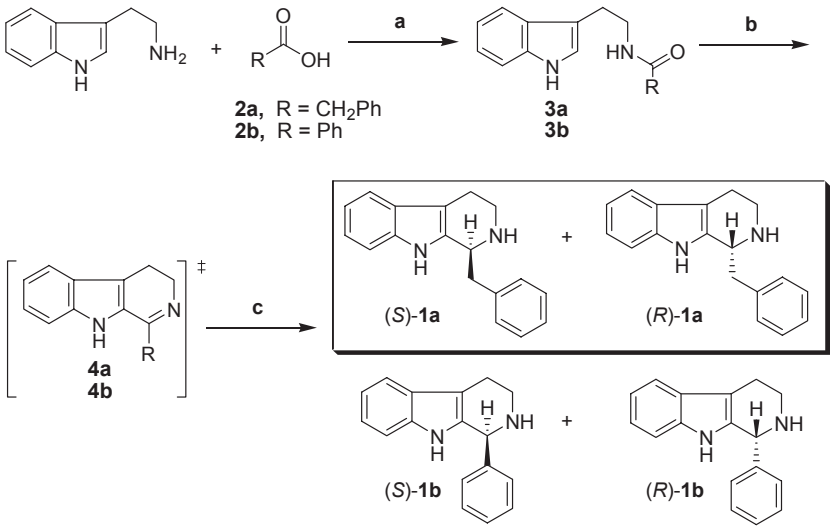
^{1}H NMR (400 MHz, $CDCl_3$), δ: 2.76–2.79 (2H, m), 3.02–3.17 (3H, m), 3.39 (1H, ddd, J 4.9, 4.9 and 12.0 Hz), 4.43 (1H, t, J 7.0 Hz), 7.10–7.14 (2H, m), 7.25 (1H, d, J 7.5 Hz), 7.32–7.40 (5H, m), 7.49 (1H, s), 7.51 (1H, d, J 7.7 Hz), 7.53 (1H, d, J 7.5 Hz). ^{13}C NMR (100 MHz, $CDCl_3$), δ: 22.3, 41.6, 42.5, 54.0, 109.3, 110.7, 118.1, 119.3, 121.6, 126.9, 127.3, 128.8, 129.3, 135.4, 135.5, 138.2. HRMS, ESI(+)-MS: *m*/*z* calcd for $[C_{18}H_{18}N_2+ H]^+$ 263.1548, found 263.1550.

1-Phenyl-1,2,3,4-tetrahydro-β-carboline (1b)

^{1}H NMR (400 MHz, $CDCl_3$), δ: 2.83–2.95 (2H, m), 3.07–3.16 (1H, m), 3.33–3.39 (1H, m), 5.17 (1H, s), 7.10–7.19 (3H, m), 7.30–7.37 (5H, m), 7.56–7.60 (2H, m), 7.66 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$), δ: 22.6, 42.8, 58.1, 110.2, 110.9, 118.3, 119.4, 121.8, 127.3, 128.3, 128.6, 128.9, 134.4, 136.0, 141.9. HRMS, ESI(+)-MS: *m*/*z* calcd for $[C_{17}H_{16}N_2+ H]^+$ 249.1392, found: 249.1388.

Assay of glutamate-induced excitotoxicity

Glutamate-induced excitotoxicity was analyzed in a cell-based assay as described previously (20). Briefly, mouse fibroblast cells expressing cDNAs of either the human NR1-1a/NR2A (L12-G10 cells) or NR1-1a/NR2B subunits (L13-E6) of the NMDA receptor were seeded into 96-well microtiter plates at 1×10^4/well and grown for 24 h at 37 °C and 5% CO_2 in minimal essential medium containing 10% fetal bovine serum, penicillin/streptomycin (100 U/100 μg/mL), and 50 mM sodium pyruvate. Cells expressing NMDA receptors were protected from receptor-mediated background cytotoxicity by the addition of 100 μM ketamine to the medium. To assay synthetic compounds, cells were washed three times to remove ketamine and pre-incubated for 30 min in serum-free medium containing the test compounds (final DMSO content 0.1%). NMDA receptor–mediated excitotoxicity was then triggered by adding 10 μM of (*S*)-glutamate and glycine. Cytotoxicity was measured by determining the release of lactate dehydrogenase (LDH) from damaged cells using Roche's Cytotoxicity Detection Kit (LDH) (Roche Diagnostics GmbH, Mannhein, Germany). All tests were conducted in triplicate and are expressed as mean values ±SD. Baseline excitotoxicity (0%) was defined as the LDH release after addition of (*S*)-glutamate and glycine in the presence of 100 μM ketamine.



Scheme 1: Reagents and conditions: (a) **2a**/**2b**, tryptamine, EDC, HOBt, CH_2Cl_2, rt, 12 h. (b) **3a**/**3b**, $POCl_3$, benzene, reflux, 2–3 h. (c) **4a**/**4b**, $NaBH_4$, rt, 12 h.

Conversely, 100% excitotoxicity was defined as the LDH release induced by (*S*)-glutamate/glycine in the absence of ketamine. IC_{50} values were calculated using the GraphPad Prism 5.0 software package (GraphPad, La Jolla, CA, USA).

Molecular modeling

The homology model of the ion channel of NMDA receptor (M1–M2–M3 regions of NR1 and NR2A units) was constructed by using SWISS-MODEL (25) and the X-ray crystal structure of the potassium channel KcsA (accession code in Protein Data Bank: 1BL8) as a template. The sequence alignment was performed by using ICM program (Internal Coordinate Modeling; ICM, version 3.4–8, La Jolla, CA, USA: Molsoft LLC, 2006). The resulting alignment was verified by analyzing previous information related to the alignment of NR1 and NR2A against KcsA (26). The homology model was optimized by minimization and molecular dynamics protocols to remove bad contacts, by using NAMD2 (27) program with the force field CHARMM27 (28). During these processes, backbone atoms were restrained, and cyclic hydrogen bonds between asparagines at the entrance of the pore were imposed by constraints (29).

After the optimization of the model, the binding of the enantiomers of compounds **1a** and **1b** [compounds (*R*)-**1a**, (*S*)-**1a**, (*R*)-**1b**, and (*S*)-**1b**] was studied by using the ICM docking method (30). The characteristics of the ICM docking method have been described elsewhere (31). The structures of the inhibitors were sketched by using the Molecular Editor of ICM software. Protein and inhibitor structures were converted into ICM objects. During protein conversion process, hydrogens were added and optimized. Meanwhile, during ligand conversions, 2D representations were converted into 3D ones, partial charges were assigned, and rotatable bonds were identified. IcmPocketFinder (32) was used for identifying putative pockets with a tolerance value of 4.6. Initial ligand position and orientation, and box position and size, were kept according to the values suggested by the program. The 20 better docking poses for each ligand were analyzed by examining their relative total energy scores. The best docked position was determined by considering the total energy value.

Results and Discussion

In a previous study, Rook *et al.* (20) identified N2- or N9-connected, homobivalent β-carbolines as potent inhibitors of NMDA receptor–mediated excitotoxicity in a cell-based assay. In the same study, it was reported that monovalent, N2- or N9-substituted compounds were generally inactive. In our investigation, compound **1a** inhibited glutamate-induced excitotoxicity with an IC_{50} of 27.4 ± 1.3 and 27.9 ± 3.9 μM for NR1/NR2A and NR1/NR2B, respectively (Table 1), whereas compound **1b** was inactive. Thus, the potency of compound **1a** to block NMDA receptor's channel activity was somewhat weaker than that of memantine (IC_{50} 5.6 and 5.5 μM against NR1/NR2A and NR1/NR2B, respectively) and that of N9-connected bivalent β-carbolines described by Rook *et al.* (20) (IC_{50} 1.4 and 2.9 μM against NR1/NR2A and NR1/NR2B, respectively). Furthermore, several 1-substituted-β-carbolines were synthesized (R = Et; ^{i}Pr; $-C_{11}H_{23}$; $-C_{15}H_{31}$; $-C_7H_{14}(CH{=}CHCH_2CH{=}CH)C_5H_{11}$; 2-hydroxyphenyl; and 3-pyridyl) and evaluated on NMDA receptor inhibitory activity and found to be inactive. Bivalent β-carbolines have a rather high molecular weight and must contain quartenary nitrogens to possess high NMDA receptor inhibitory activity, which may violate their development as neuroprotective drugs with clinically relevant *in vivo* activity. On the other hand, compound **1a** reported here is the first monovalent, not permanently charged compound displaying considerable inhibitory activity at the NMDA receptor. Compound **1a** may therefore present a valuable lead compound to further develop efficient neuroprotective drugs that act by preventing neuronal damage owing to excessive NMDA receptor activity. The difference in NMDA receptor-blocking activity of compounds **1a** and

Table 1: *N*-methyl-D-aspartate receptor inhibitory activities

Compounds	L12-G10 (NR1/NR2A) IC_{50} (μM) ± SD [excitotoxicity (%) at 25 μM]	L13-E6 (NR1/NR2B) IC_{50} (μM) ± SD [excitotoxicity (%) at 25 μM]
Memantine	5.6 ± 1.3 (11.4 ± 0.3)	5.5 ± 1.1 (11.3 ± 2.1)
1a	27.4 ± 1.3 (53.5 ± 5.5)	27.9 ± 3.9 (53.4 ± 9.2)
1b	n.d. (105.2 ± 12.1)	n.d. (98.1 ± 12.1)

n.d., not determined.

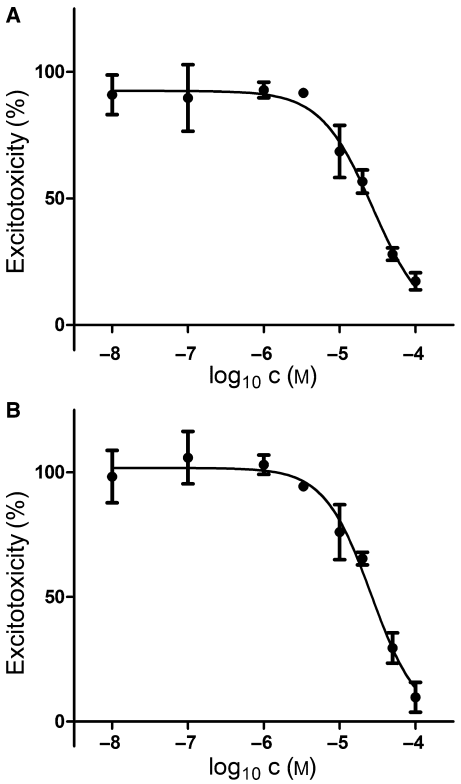


Figure 1: Inhibition of *N*-methyl-D-aspartate receptor–mediated cell toxicity by compound **1a**. Different concentrations of compound were applied to L12-G10 cells (A) and L13-E6 cells (B). Compound **1a** had IC_{50} values of 27.4 ± 1.3 μM and 27.9 ± 3.9 μM, respectively.

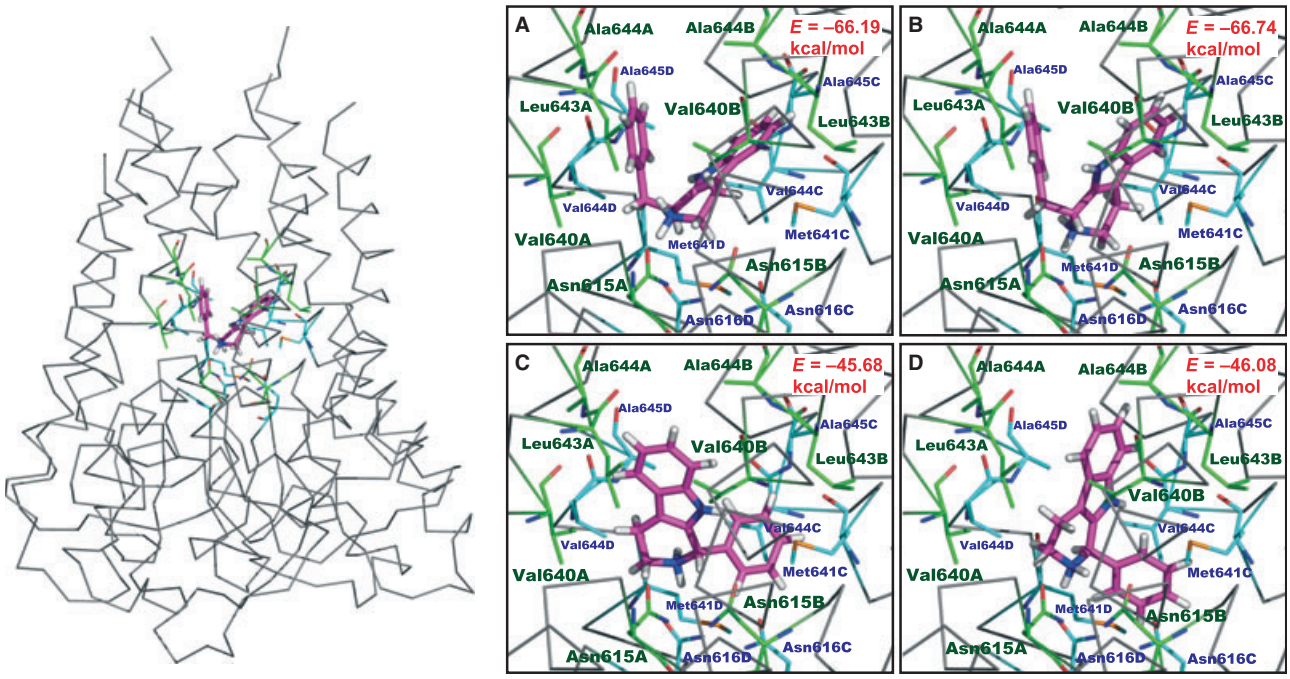


Figure 2: (left) Location of the docked blockers in the vestibule region of the *N*-methyl-D-aspartate (NMDA) receptor. (right) Predicted binding conformations of compounds (*S*)-**1a** (A), (*R*)-**1a** (B), (*S*)-**1b** (C), and (*R*)-**1b** (D). Residues that interact with blockers in the pore and vestibule region of the NMDA receptor are represented in green (NR2A, chains A and B) and cyan (NR1, chains C and D). Binding energies (kcal/mol) obtained from ICM scoring function are shown for each compound in red letters.

1b was surprising, because the only structural difference between the compounds was a methylene group. To evaluate this interesting observation, we studied the structure of the complexes by molecular modeling (Figure 1).

The NMDA receptor is thought to exist as a tetramer containing two NR1 and two NR2 subunits. The binding modes of compounds (*R*)-**1a**, (*S*)-**1a**, (*R*)-**1b**, and (*S*)-**1b** were examined by applying docking simulations inside the model containing NR1 and NR2A. The optimal binding modes of these compounds are shown in Figure 2. All the studied blockers were docked in the vestibule inside the channel, with the protonated amino group positioned near the side chain of residues Asn616 of NR1 and Asn615 of NR2A. The studied drugs bind in the position that overlaps with the proposed binding site of memantine and MK-801 in NMDA receptor (29). The residues Asn616 of NR1 units and Asn615 of NR2A units have been denoted as Asn 0 in previous papers because these residues are at the tip of the helices of M2 regions and are critical for calcium permeability and magnesium blockade (33). In a recent work, Tikhonov (29) identified that residues at positions +25, +28, and +29 contribute to the binding energy of MK-801. In addition, potent NMDA receptor blockers (such as memantine and MK-801) possess a 'V'-like shape with the protonated nitrogen at the vertex, and two hydrophobic wings (34). Residues at positions +25, +28, and +29, which are mainly hydrophobic (Val640, Leu643, and Ala644 in NR2A, and Met641, Val644, and Ala645 in NR1), are able to establish van der Waals interactions with the 'hydrophobic wings' of the blocker. According to our docking simulations, compounds (*R*)-**1a** and (*S*)-**1a** possess a 'V'-like shape, and the groups 1,2,3,4-tetrahydro-β-carboline and benzyl are the 'wings', which interact with the above-mentioned residues (Figure 2A,B). However, compounds (*R*)-**1b** and (*S*)-**1b** do not establish these interactions. Compound (*S*)-**1b** has an orientation where 1,2,3,4-tetrahydro-β-carboline scaffold interacts with some of the residues at positions +25, +28, and +29, but the phenyl substituent locates between Met641 and Val644 from an NR1 unit, chain C (Figure 2C). Meanwhile, compound (*R*)-**1b** has an orientation where 1,2,3,4-tetrahydro-β-carboline scaffold is located in the center of the vestibule, and the phenyl substituent locates close to Met641 from a NR1 unit, chain C (Figure 2D).

The energy values obtained from ICM scoring function are also shown in Figure 2. These values were around −66 kcal/mol for compounds (*R*)-**1a** and (*S*)-**1a** and around −46 kcal/mol for compounds (*R*)-**1b** and (*S*)-**1b**. These values reflect that compounds (*R*)-**1a** and (*S*)-**1a** have better binding affinities. As the difference between energy values for the two isomers (*R*)-**1a** and (*S*)-**1a** is only 0.55 kcal/mol, the obtained energy values do not help to suggest which isomer is the best candidate.

Conclusions

The 1-benzyl-1,2,3,4-tetrahydro-β-carboline (**1a**) described in this study was identified as an inhibitor of the NMDA receptor, whereas the similar compound 1-phenyl-1,2,3,4-tetrahydro-β-carboline (**1b**) was inactive. It is worth noting that activity profiles between compounds **1a** and **1b** were different owing to the presence of an additional methylene group in the molecular structure of compound **1a**.

Docking simulations of the reported compounds explain why compound **1a** is a NMDA receptor blocker and compound **1b** does not have this activity. The results suggest that rational modifications of the phenyl group in the scaffold 1-methyl-1,2,3,4-tetrahydro-β-carboline based on the described NMDA receptor model provide a basis for the development of NMDA receptor blockers with advantage over the described bivalent structures (20) in having a smaller size and no permanent charge, and thus a reasonable bioavailability.

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