Identification and Characterization of Novel NMDA Receptor Antagonists Selective for NR2A- over NR2B-Containing Receptors

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ABSTRACT

NR1/NR2A is a subtype of N-methyl-D-aspartate receptors (NMDARs), which are glutamate and glycine-gated Ca2+-permeable channels highly expressed in the central nervous system. A high-throughput screening (HTS) campaign using human osteosarcoma (U-2 OS) cells transiently transduced with NR1/NR2A NMDAR subunits, tested in a specifically designed fluorometric imaging plate reader (FLIPR)/Ca2+ assay, identified sulfonamide derivative series, exemplified by 3-chloro-4-fluoro-N-{[2-(phenylcarbonyl)hydrazinyl]amino}-1,3,4-thiadiazol-2-yl}thio)acetamide and thiodiazole derivative thiodiazole derivative 3-chloro-4-fluoro-N-{[2-(phenylcarbonyl)hydrazinyl]amino}-1,3,4-thiadiazol-2-yl}thio)acetamide (compound 13) as novel NR1/NR2A receptor antagonists. Compounds 1 and 13 displayed submicromolar and micromolar potency at NR1/NR2A receptor, respectively, although they did not show activity at NR2B-containing receptor up to 50 μM concentration. Addition of 1 mM glycine, but not 1 mM L-glutamate, was able to surmount compound 1 and 13 inhibitory effects in FLIPR NR1/NR2A assay. However, compounds 1 and 13 displaced a glutamate site antagonist [3H]CGP 39653 to a greater extent than the glycine site antagonist [3H]MDL 105,519, in rat brain cortex binding assay. Results of FLIPR cell-based, electrophysiological, and biochemical binding assays suggest that compounds 1 and 13 are the prototypes of novel classes of NMDAR ligands, which to the best of our knowledge are the first selective antagonists at NR1/NR2A over NR1/NR2B receptor, and might constitute useful tools able to elucidate the relative role of the NR2A subunit in physiological and pathological conditions.

Introduction

N-methyl-D-aspartate receptors (NMDARs) are ligand-gated cation-selective channels that are highly expressed in the central nervous system (Köhr, 2006). NMDAR activity is...
from ChemDiv (San Diego, CA); and compound

The majority of NMDARs are tetrameric complexes (Mayer, 2006), consisting of two glycine-binding NR1 subunits and two glutamate-binding NR2 subunits (Collingridge et al., 2009). NR1 is coded by a single gene with at least eight different splice variants; four different NR2 genes generate NR2A, NR2B, NR2C, and NR2D subunits (Pauletto and Neyton, 2007). NMDARs containing different NR2 subunits have different pharmacological and kinetic properties (Vici et al., 1998; Yuan et al., 2009).

NR2 subunits show distinct anatomical localization, providing an opportunity to target specific neuronal circuits using subunit-selective modulators. Ifenprodil has been the prototype of selective NR1/NR2B receptor antagonists (Williams, 1993), which are able to bind to the NR2B N-terminal domain (NTD) and have been used to evaluate the role of this subunit in brain function and diseases. Antagonists binding at the NR2B NTD might have therapeutic potential in pain, mood disorders, and neurodegenerative diseases (Mony et al., 2009). An involvement of NR1/NR2A receptor has been suggested in pathological conditions such as anxiety and depression (Boyce-Rustay and Holmes, 2006). Despite the importance of NMDA receptors in many neurological disorders, antagonists that are more than 10-fold selective for NR2A, NR2C, or NR2D have not yet been identified, hampering a deeper understanding of their physiological role in brain functions. To address the need for new pharmacological tools, a program was established that included a high-throughput screening (HTS) campaign on approximately 2 million compounds and was aimed at identifying NR2A selective compounds.

**Materials and Methods**

**Compound Sources.** The compounds used in this study were as follows: compounds 1 to 5, 7, 8, 10, and 13 were purchased from Enamine (Kiev, Ukraine); compounds 6, 9, and 11 were purchased from ChemDiv (San Diego, CA); and compound 12 was purchased from Asinex (Moscow, Russia). [(R)-[(1S)-1-(4-bromophenyl)-ethyl]-amino](2,3-dioxo-1,2,3,4-tetrahydro-5-quinazolinyl)methyl]phosphonic acid (NVP-AAM077) was obtained as the corresponding diamonium salt by high-performance liquid chromatography separation of diastereoisomer mixture [(1S)-1-(4-bromophenyl)-ethyl]amino](2,3-dioxo-1,2,3,4-tetrahydro-5-quinazolinyl)methyl]phosphonic acid tetrasodium hydrate (Sigma-Aldrich, Milan, Italy). Memantine hydrochloride, (+)-MK-801 hydrogen maleate, 1-(1-phenylethyl)oxepine (phencyclidine) hydrochloride, and 4-[(1R,2S)-1-hydroxy-2-methyl-3-[4-(phenylmethyl)-1-piperidinyl]propyl]phenol (Ro 25-6981) maleate were purchased from Sigma-Aldrich. All other chemicals were of analytical grade and obtained from commercial sources.

**Computational Work.** HTS concentration response curve (CRC) hits were prioritized according to their ligand efficiency (LE) and ligand lipophilicity efficiency (LLE) indexes as well as their selectivity compared with NR1/NR2B receptor. The LE formula was derived from Hopkins et al. (2004) to normalize the potency of a compound with respect to its size: $LE = \frac{pIC_{50}}{cLogP}$. Calculated partition coefficient (cLogP), obtained with the use of Daylight 4.81 software (Daylight Chemical Information Systems, Laguna Niguel, CA) was used to calculate LLE to maximize the number of receivable compounds. A cell unit of in vitro potency, according to Leeson and Springthorpe (2007): $LLE = \frac{pIC_{50}}{cLogP}$. Hit expansion was performed by fishing out derivatives from in-house repositories with the use of two-dimensional similarity searches carried out with an in-house developed tool, followed by visual compound inspection.

**cDNAs Coding for NMDAR Subunits.** cDNAs coding for human NMDAR subunits were transiently transduced in human osteosarcoma (U-2 OS) cells (LG G Standards/American Type Culture Collection, Tedsdon, UK) through BacMam vectors (Con-dreay et al., 1999) for fluorometric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA) assays or transfectected in human embryonic kidney (HEK) 293T cells (LG G Standards/American Type Culture Collection) through plasmid vectors for patch-clamp experiments: pcDNA3.1topo plasmids were used for NR1, NR2A, and NR2B subunits, whereas pcMV6-XL5 plasmids (Ori-gen, Rockville, MD) were used for the NR2D subunit. ePF-NFP-1 plasmid (Clontech, Mountain View, CA), encoding a red-shifted variant of wild-type GFP optimized for brighter fluorescence and higher expression in mammalian cells, was used to select transfected cells for patch-clamp experiments.

**FLIPR/Ca$^{2+}$ Assay in U-2 OS Cells Expressing NMDAR.** FLIPR/Ca$^{2+}$ experiments on recombinant receptors were conducted using U-2 OS cells, because they are easily transduced through BacMam vector (Con-dreay et al., 1999) and they adhere strongly to culture medium and maintained overnight (5% CO$_2$ at 37°C). Cells grown overnight were directly added with the cytoplasmic calcium indicator, Fluo-4 (Invitrogen) in the acetoxyethyl (AM) ester form (2 μM) in assay buffer at 37°C for 1 h. Assay buffer contained 145 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 g/liter D-(+)-glucose, 20 mM HEPES (pH adjusted to 7.3 with NaOH), and 2.5 mM probenecid (Sigma-Aldrich). Probenecid, an inhibitor of organic anion transport, was added to prevent intracellular Fluo-4 efflux. After Fluo-4 AM loading, cell plates were washed two or three times, for NR1/NR2A or NR1/NR2B receptor, respectively, with the assay buffer using the EMBLA 384 instrument (Molecular Devices, Sunnyvale, CA) to remove ketamine; 30 μl of assay buffer was left in each well at the end of the washing.

Cell plates were then placed into a FLIPR to monitor cell fluorescence (excitation wavelength at 488 nm, emission wavelength at 540 nm) 30 s before and until 5 min after 10 μM drug addition (Sullivan et al., 1999). Antagonist addition was able to induce a measurable decrease in fluorescence (see Supplemental Data), similarly to what was reported for other NMDARs (Hansen et al., 2010). Compounds dissolved in DMSO were 50-fold diluted with assay buffer plus 0.01% Phloric F-127 (Sigma-Aldrich) before the FLIPR/Ca$^{2+}$ experiment. For concentration response curve experiments, serial dilution (1, 3, 11 points) of compounds in DMSO was performed using a Biomek FX (Beckman Coulter, Fullerton, CA). Solution volumes of 100 mM L-glutamate and 400 mM glycine were prepared in water and stored at −20°C, then diluted at the desired concentration.
in assay buffer on the experimental day. In mode-of-action studies, a second addition of 1 mM glucose or L-glutamate was done by FLIPIR instrument, 5 min after compound addition, and fluorescence was measured for an additional 5 min.

**FLIPIR/Ca**2+ **Assay in Rat Brain Cortical Cells.** Cortical neuronal cultures were obtained from embryonic day 18/19 Sprague-Dawley rats (Charles River Italica, Calco, Italy). Animal manipulations were performed according to Italian law (art. 7, Legislative Decreto No.116, 27 January 1992), which acknowledged the European Directive 86/609/EEC and GlaxoSmithKline policy on the care and use of laboratory animals and related codes of practice. Rat brains were dissected out and cortices were quickly isolated at 4°C in Hanks’ balanced salt solution supplemented with HEPBS buffer), containing 5 mM KCl, 0.4 mM KH2PO4, 138 mM NaCl, 0.3 mM Na2HPO4, 5.5 mM d-glucose, 26 mM phenol red, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES at pH 7.3. Cortical cells were treated with HBSS buffer added with 0.1% trypsin at 37°C for 10 min. In the last 5 min of incubation, 166 μg/ml DNase I (Sigma-Aldrich) was added. After a single wash with HBSS buffer containing 10% fetal bovine serum (Invitrogen) and two additional washes with HBSS buffer, cells were mechanically dissociated by triturating with Pasteur pipettes and placed in poly-l-lysine-coated 384-well plates (Greiner Bio-One GmbH) at a density of 100,000 cells/well in serum-free Neurobasal medium (Invitrogen) added with B27 supplement (Invitrogen), 500 μM glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37°C, 5% CO2. Addition of fresh medium was made at 3 and 10 days, and the cells were used for intracellular Ca2+ measurements at 15 days after plating. Intracellular Ca2+ measurements were made using a FLIPIR (Molecular Devices). On the assay day, cultures were washed two times with physiological salt solution buffer (145 mM NaCl, 1 g/liter glucose, 5 mM KCl, 2 mM CaCl2, and 20 mM HEPES) supplemented with 2.5 mM probenecid. Then, loading solution (physiological salt solution supplemented with 2.5 mM probenecid, 0.04% Pluronic F-127, and 2 mM Fluo-4 AM) was added. After 40 min at 37°C, cells were washed three times as described above and transferred to the FLIPIR. Ca2+ responses were measured with excitation wavelength at 470 to 495 nm and emission wavelength at 515 to 575 nm. Camera gain and intensity were adjusted for each plate to yield a minimum baseline fluorescence of 300 relative light units. To assess the effect of NMDAR antagonists on the NMDA-triggered Ca2+ response, cells were exposed first to different concentrations of test drugs (master plate at 4× concentration) for 10 min, then to a submaximal concentration of NMDA (EC50, 10–20 μM) (master plate at 5× concentration) for 3 min. Five fluorescence readings were taken to measure baseline, before compound addition.

**FLIPIR/Ca**2+ **Data Analysis.** FLIPIR/Ca2+ functional responses were measured in U-2 OS experiments as fluorescence intensity at 5 min after drug addition, normalized to the maximal response evoked at 30 μM (+MK-801) NMDAR blocker on the same plate. Iterative curve fitting and parameter estimations were carried out using a four-parameter logistic model in an Activity Base (IDBS, Guilford, UK) environment.

In rat cortical neurons, Ca2+ responses were quantified as area-under-the-curve analysis of the time-response data over baseline and adjusted for each plate to yield a minimum baseline fluorescence of 300 relative light units. To assess the effect of NMDAR antagonists on the NMDA-triggered Ca2+ response, cells were exposed first to different concentrations of test drugs (master plate at 4× concentration) for 10 min, then to a submaximal concentration of NMDA (EC50, 10–20 μM) (master plate at 5× concentration) for 3 min. Five fluorescence readings were taken to measure baseline, before compound addition.

**Whole-Cell Patch-Clamp Electrophysiology in HEK 293T Cells Expressing NMDAR.** Whole-cell patch-clamp recording was performed on HEK 293T cells, and cells were transiently transfected with human NMDAR subunits and EGFP plasmid (see cDNA Coding for NMDAR Subunits). Electrophysiology experiments were carried out using an EPC-9 amplifier (HEKA, Lambrecht/Pfalz, Germany). Each cell was held at ~60 mV throughout the recording, performed at a sampling rate of 1 kHz (no filter was applied). Intracellular solution contained 140 mM KCl, 2.5 mM MgCl2, 11 mM EGTA, 10 mM HEPES, 4 mM Mg-ATP, 5 mM sodium-porphocreatine, and 0.2 mM Na-GTP, pH adjusted to 7.25 with KOH. Extracellular solution contained 150 mM NaCl, 5 mM KCl, 0.2 mM CaCl2, 3 μM glycine, 10 mM d-glucose, and 10 mM HEPES, pH adjusted to 7.4 using NaOH. Whole-cell voltage-clamp currents were evoked by rapidly exchanging the extracellular solution to one containing NMDA for a period of 5 s every 60 s by means of a fast-step perfusion system (RSC 160; Bio-Logic Science Instruments, Clai, France). Data were analyzed using Pulse software (HEKA) for current measurements and Prism 5 (GraphPad Software Inc.) for statistics. In CRC experiments, data were fitted to the Hill equation, with fitted parameters values given ± S.E. and where n represents the number of cells studied.

**Single Concentration Binding in Rat Brain Cortical Membranes.** Selected compounds were submitted for single concentration (10 μM) displacement binding assay in rat cortical membranes to MDS Pharma Services (Taipei, Taiwan). Tritiated ligands D-1(-E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid ([3H]TCP, 3-[E]-2-carboxy-2-phenylethyl)-4,6-dichloro-1H-indole-2-carboxylic acid ([3H]MDL 105,519), [3H]TCP, and [3H]ifenprodil binding were selected for their ability to bind glutamate site, glycine site, pore, and NR2B NTD of NMDAR, respectively. ([3H]TCP, [3H]MDL 105,519, [3H]TCP, and [3H]ifenprodil were used at 2, 0.33, 4, and 2 nM, respectively, and nonspecific binding was defined by 1 mM L-glutamate, 10 μM MDL 105,519, 1 μM (+)-MK-801, and 10 μM ifenprodil, respectively. Incubation buffers for [3H]TCP, [3H]MDL 105,519, [3H]TCP, and [3H]ifenprodil concentration binding studies were 50 mM Tris-HCl, pH 7.4, 50 mM HEPES, pH 7.7, 10 mM Tris-HCl, pH 7.4, and 50 mM Tris-HCl, pH 7.4, respectively.

**[3H]TCP, [3H]MDL 105,519, [3H]ifenprodil Binding in Rat Brain Cortical Membranes.** Cerebral cortices from male Sprague-Dawley rats (200–250 g, Charles River) were dissected immediately after animal decapitation. Cortices were weighed and homogenized in 15 volumes of ice-cold 5 mM Tris-HCl, pH 7.4 at 25°C. The homogenate was then centrifuged at 40,000g for 20 min at 4°C, and the pellet was washed five more times by resuspension and centrifugation as above. The pellet was then resuspended in 15 volumes of ice-cold 5 mM Tris-HCl containing 10 mM disodium EDTA and incubated for 20 min at 37°C. After three more resuspension and centrifugation steps in ice-cold 5 mM Tris-HCl, the final pellet was resuspended in three volumes of ice-cold 5 mM Tris-HCl buffer, and aliquots were frozen at ~80°C until use. Protein concentration was determined by protein assay (Bio-Rad Laboratories, Milan, Italy) using bovine serum albumin as standard. Binding of [3H]TCP, [3H]MDL 105,519 (Sills et al., 1991) was performed in a plate containing 96 deep wells (Whatman, Clifton, NJ) in a final volume of 0.4 ml of 50 mM Tris-HCl, pH 7.4 at 25°C, and 1% DMSO. In displacement experiments, 10 nM [3H]TCP, [3H]MDL 105,519 (specific activity, 40.5 Ci/mmole; PerkinElmer Life and Analytical Sciences, Waltham, MA) was incubated for 30 min at 24°C with 24 μg of protein in the absence or presence of competing compounds previously diluted in DMSO. Nonspecific binding was defined by 1 mM L-glutamate. In saturation experiments, increasing concentrations of [3H]TCP, [3H]MDL 105,519 (1.5–130 nM) were incubated as above. Reactions were stopped by filtration over GF/C filterplates (PerkinElmer Life and Analytical Sciences) followed by three washes with ice-cold 50 mM Tris-HCl, pH 7.4 at 25°C, using a Packard cell harvester (PerkinElmer Life and Analytical Sciences). Retained radioactivity was counted by a TopCount (PerkinElmer Life and Analytical Sciences) after the addition of 50 μl/well of MicroScint 20 (PerkinElmer Life and Analytical Sciences).

**Radioligand Binding Data Analysis.** Radioligand binding data were analyzed with nonlinear regression by using Prism software. Determination of Kd and Bmax of [3H]TCP, [3H]MDL 105,519 was assessed by elaborating saturation experiments using a one-site binding (hyperbola) equation, after checking, by use of P test, that this equation fit.
better compared with two-site binding equation. Competition curves for the known competitive antagonist NVP-AAM077 were statistically analyzed for a best one- or two-site competition fit. Competition curves for the other compounds for which the site of binding within the receptor was unknown were analyzed by using a four-parameter dose-response curve. Results are expressed as mean pIC$_{50}$ ± S.E.M.

**Results**

**FLIPR/Ca$^{2+}$ Assay in U-2 OS Cells Expressing NMDAR.** Reference NMDAR antagonists binding at different sites were profiled in NR1/NR2A and NR1/NR2B FLIPR assays, obtaining pIC$_{50}$ values in line with literature data (Table 1). The preferential NR2A antagonist NVP-AAM077, binding at the glutamate site, showed pIC$_{50}$ values of 7.8 and 6.9 in NR1/NR2A and NR1/NR2B FLIPR assays, respectively (Table 1). Glycine antagonist GV196771A (Giacobbe et al., 1998; Quartaroli et al., 1999) inhibited NR2A-containing receptors with a slightly higher pIC$_{50}$ than NR2B-containing receptors (Table 1), in agreement with reported 10-fold lower affinity of the agonist glycine for NR2A- over NR2B-containing receptors (Priestley et al., 1995). NR2B NTD ligands Ro 25-6981 (Fisher et al., 1997) and CP-101,606 (traxoprodil) showed detectable potency only in NR1/NR2B-containing assay (Table 1). Pore blocker (+)-MK-801 (dizocilpine maleate) had similar potency in both assays, whereas phencyclidine and memantine pore blockers showed slight preference for NR1/NR2B receptor combination (Table 1). A cell-based HTS campaign on approximately 2 million compounds, using FLIPR/Ca$^{2+}$ methodology in transiently transduced U-2 OS cells, identified 279 compounds active with pIC$_{50}$ ≥ 5.0 on NR1/NR2A receptor in CRC experiments.

The HTS CRC hits prioritization performed with LE and LLE indexes and selectivity profile resulted in the selection of compounds 1 to 3, 6, and 13 (Fig. 1 and Table 2). These compounds were selective toward NR1/NR2A receptor with respect to NR1/NR2B subtype (Table 2). Although showing molecular weight (377–480) and polar surface area (67–104, calculated according to Ertl et al., 2000) that are high for a central nervous system drug, they were further characterized. Subsequently, similarity searches were carried out to find more potent analogs possibly maintaining good selectivity. For this purpose, 127 analogs were selected by using similarity searches, then reduced to eight compounds (4, 5, 7–12) after discarding derivatives carrying undesirable reactive chemical functionalities such as oxime or moieties unsuitable for a central nervous system drug such as carbonylate, as a result of visual compound inspection. Selected derivatives were tested in the FLIPR NR1/NR2A and NR1/ NR2B assays for pIC$_{50}$ determination. Results of this exploration have been reported together with original HTS hits in Table 2.

Selected hits completely inhibited (92–100%) human recombinant NR1/NR2A receptor in FLIPR/Ca$^{2+}$ assay. Four hits (1–4) showed pIC$_{50}$ between 6.4 and 6.8; compounds 6 and 13 exhibited pIC$_{50}$ of 5.4 in NR1/NR2A assay. All hits resulted with pIC$_{50}$ < 4.3 in FLIPR NR1/NR2B assay (Table 2).

To clarify the inhibitory mechanism of action of five selected hits (1–3, 6, and 13) in FLIPR NR1/NR2A assay, 1 mM glycine, or 1 mM L-glutamate was added to U-2 OS expressing NR1/NR2A receptor 5 min after compound addition. Only 1 mM glycine, but not 1 mM L-glutamate, was able to surmount the effect of each of the five compounds (Fig. 2 and Table 3). In the same test, NVP-AAM077 inhibitory effect was surmounted by 1 mM L-glutamate, but not by 1 mM glycine, whereas (+)-MK-801 inhibitory effect was not surmounted by any of the two agonists (Table 3).

**FLIPR/Ca$^{2+}$ Assay in Rat Cortical Cells.** To confirm functional activity at native NMDAR, measurements of intracellular Ca$^{2+}$ were performed in rat cortical cultures. NMDA-evoked Ca$^{2+}$ response was completely blocked by pore blocker memantine with a pIC$_{50}$ value of 5.4 ± 0.1 (n = 5), suggesting that the NMDA-stimulated Ca$^{2+}$ influx is truly mediated by activation of NMDA receptors, whereas it was partially blocked (77 ± 3%) with pIC$_{50}$ value of 7.3 ± 0.1 (n = 6) by NR1/NR2B receptor-selective antagonist Ro 25-6981, indicating that the majority of functional NMDAR contained NR2B subunit (Fig. 3A), similarly to what was already described (Fisher et al., 1997, Zhong et al., 1994). Given the known partial antagonism of Ro 25-6981 (Fisher et al., 1997), like that of any described selective NR2B antagonist at NTD site (Paolotti and Neyton, 2007), the remaining NMDA-induced Ca$^{2+}$ response might be attributable to a mixed population of NMDAR, including both NR2A- and NR2B-containing receptors (Zhong et al., 1994). The effect of compounds 1, 6, and 13 was studied in the presence of 1 mM Ro 25-6981 to minimize the contribution of NR2B-containing receptors (at 1 mM the antagonist is expected to give approximately 95% of its maximal effect). Compounds 1, 6, and 13 partially inhibited the NMDA-induced intracellular Ca$^{2+}$ response in a concentration-dependent manner (Fig. 3B), with pIC$_{50}$ values of 6.4 ± 0.4 (n = 3), 5.4 ± 0.2 (n = 3), and 5.8 ± 0.3 (n = 3), respectively, and to a maximal level of 39 ± 1, 46 ± 6, and 66 ± 8%, respectively.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pIC$_{50}$ (NR1/NR2A)</th>
<th>pIC$_{50}$ (NR1/NR2B)</th>
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<tbody>
<tr>
<td>NVP-AAM077</td>
<td>7.8 ± 0.4 (49)</td>
<td>6.9 ± 0.4 (50)</td>
</tr>
<tr>
<td>GV196771A</td>
<td>8.0 ± 0.1 (6)</td>
<td>7.3 ± 0.2 (6)</td>
</tr>
<tr>
<td>Ro 25-6981</td>
<td>&lt;5.3 (8)</td>
<td>7.2 ± 0.1 (8)</td>
</tr>
<tr>
<td>(+)-MK-801</td>
<td>&lt;4.3 (12)</td>
<td>7.0 ± 0.4 (12)</td>
</tr>
<tr>
<td>Phencyclidine</td>
<td>5.9 ± 0.4 (19)</td>
<td>6.4 ± 0.2 (19)</td>
</tr>
<tr>
<td>Memantine</td>
<td>4.8 ± 0.3 (55)</td>
<td>5.5 ± 0.4 (61)</td>
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Fig. 1. Structures of selected HTS hits discovered through FLIPR/Ca$^{2+}$ NR1/NR2A assay.
Patch-Clamp Electrophysiology in HEK 293T Cells Expressing NMDAR. Whole-cell patch-clamp experiments were carried out with selected compounds in recombinant HEK 293T cells transiently expressing human NMDA receptor for 1 to 3 days after transfection.

As shown in Fig. 4, compound 1 inhibited the current induced by 30 μM NMDA in the presence of 3 μM glycine in a concentration-dependent manner, in HEK 293T transiently transfected with NR1/NR2A subunits. Fitting of data gave a pIC50 value of 7.0 ± 0.1 (n = 3–9) corresponding to an IC50 of 109 nM and a Hill slope of 1.4 ± 0.3 (n = 3–9). The compound at 3 μM blocked 95.1 ± 0.3% (n = 3) of the NMDA current in NR1/NR2A-transfected cells.

Compound 1 was also tested in HEK 293T transiently transfected with NR1/NR2B or NR1/NR2D subunits: it was inactive below a concentration of 30 μM on the current induced by 30 μM NMDA in the presence of 3 μM glycine in either NR1/NR2B- or NR1/NR2D-transfected cells.

Binding Studies in Rat Cortical Membranes. To gain insight into their site of action, three compounds (1, 6, and 13) were selected for single concentration (10 μM) displacement binding test in rat cortical membranes of [3H]CGP 39653, [3H]MDL 105,519, [3H]TCP, and [3H]ifenprodil, selective ligands for glutamate site (Sills at al., 1991), glycine site, pore, and NR2B NTD of NMDAR, respectively.

Highest inhibition values were obtained with compounds 1.
1 mM glycine, but not 1 mM glutamate, ability to reverse selected HTS hits inhibitory effect in NR1/NR2A FLIPR/Ca^{2+} assays. Therefore, more detailed [3H]CGP 39653 values lower than 25% were obtained in all other binding experiments. The number of independent experiments is indicated in parentheses. Maximal inhibition was 92 to 100% for all listed compounds. The ability of 1 mM glycine to surmount inhibitory effect of compounds 1–3, 6, and 13 is reflected in pIC50 values <4.3, measured 5 min after glycine addition. Glycine (1 mM) is not able to surmount NVP-AAM077 inhibitory effect. (+)-MK-801 inhibitory effect was not surmounted by either of the two agonists.

Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>pIC50 + 1 mM Gly</th>
<th>pIC50 + 1 mM Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;4.3 (3)</td>
<td>6.5 ± 0.04 (3)</td>
</tr>
<tr>
<td>2</td>
<td>&lt;4.3 (3)</td>
<td>6.2 ± 0.3 (3)</td>
</tr>
<tr>
<td>3</td>
<td>&lt;4.3 (3)</td>
<td>6.1 ± 0.1 (3)</td>
</tr>
<tr>
<td>6</td>
<td>&lt;4.3 (3)</td>
<td>5.3 ± 0.1 (3)</td>
</tr>
<tr>
<td>13</td>
<td>&lt;4.3 (3)</td>
<td>5.3 ± 0.2 (3)</td>
</tr>
<tr>
<td>NVP-AAM077</td>
<td>7.2 ± 0.3 (5)</td>
<td>5.1 ± 0.5 (6)</td>
</tr>
<tr>
<td>(+)-MK-801</td>
<td>7.3 ± 0.3 (4)</td>
<td>7.4 ± 0.3 (4)</td>
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Fig. 3. Representative FLIPR experiments showing inhibition of NMDA-triggered intracellular Ca^{2+} response in rat cortical cultures. A, cells were first added with different concentrations of the antagonists memantine or Ro 25-6981 for 10 min, then exposed to 15 μM NMDA. B, cells were first added with different concentrations of compound 1, 6, or 13 (in presence of 1 μM Ro 25-6981) for 10 min, then exposed to 15 μM NMDA. Data are expressed as percentage of 15 μM NMDA-evoked response. (36%) and 6 (57%) in [3H]CGP 39653 assay. Displacement values lower than 25% were obtained in all other binding assays (Table 4). Therefore, more detailed [3H]CGP 39653 radioligand binding assays were performed. In saturation experiments, [3H]CGP 39653 displayed single-site, saturable binding to rat cerebral cortex membranes. The negative logarithm of the apparent dissociation constant (pKd) was 7.5 ± 0.03 and the calculated maximum binding capacity (Bmax) was 5745 ± 373 fmol/mg protein (n = 3). In competition experiments, the known competitive NR1/NR2B preferring antagonist NVP-AAM077 showed a biphasic displacement curve with pIC50 values of 8.5 ± 0.2 and 7.3 ± 0.2. The percentage of higher affinity binding sites, most likely corresponding to NR1/NR2A receptors, was 54 ± 9%. Compounds 1 and 6 partially inhibited [3H]CGP 39653 specific binding showing a maximal radioligand displacement of 44 ± 3 and 31 ± 3%, respectively, and pIC50 values of 6.5 ± 0.1 and 5.6 ± 0.1, respectively. Likewise, compound 13 inhibited only 24 ± 4% of [3H]CGP 39653 specific binding with a pIC50 value of 4.8 ± 0.4 (Table 5 and Fig. 5).

Selectivity Profile of Selected Hits. Compounds 1, 6, and 13 showed selectivity in a panel of more than 30 targets, including ion channels and seven-transmembrane receptors. The high degree of specificity of compounds 1, 6, and 13 was also demonstrated in several additional assays. In a variety of binding and functional tests, they did not interact with ion channels (transient receptor potential vanilloid 4, human
**ether-à-go-go-related gene channel, hNaV1.5 sodium channel**, receptors of nonpeptide (adenosine, adrenergic, cannabinoid, dopamine, histamine, acetylcholine, serotonin) or peptide ligands (neurokinin, opioid, vasopressin, enzymes (cyclooxygenase, phosphodiesterase), or transporter (norepinephrine, serotonin) when tested at 10 μM concentration.

**Discussion**

Novel antagonists for human recombinant NR1/NR2A receptor were discovered through a FLIPR/Ca2⁺ HTS approach (Table 2), and the potency for selected compounds was confirmed in electrophysiology patch-clamp experiments and rat brain cortical cells FLIPR/Ca2⁺ assay (Figs. 3B and 4); in addition, displacement of radioligands at different NMDAR sites was verified in rat brain cortical membranes (Fig. 5 and Tables 4 and 5).

All selected hits were able to fully block (92–100%, Table 2) human recombinant NR1/NR2A receptor, the more potent compound 1 displaying submicromolar potency and higher than 300-fold selectivity compared with NR1/NR2B receptor (Table 2), suggesting that compounds 1, 6, and 13 interact selectively with a subpopulation of NMDARs, presumably the NR2A-selective receptors, at a [3H]CGP 39653 binding overlapping site or at an allosteric site modulating [3H]CGP 39653 binding.

However, the novel hits apparently do not bind to the classic glutamate site present in all NR2 subunits and recognized by [3H]CGP 39653 (Laurie and Seeburg, 1994; Kendrick et al., 1996), their NR1/NR2A-selective profile at recombinant NMDAR and the inability of glutamate to surmount their inhibitory effect at recombinant NR1/NR2A (Table 3).

Glycine site antagonist 5,7-dichlorokynurenate produced partial inhibition of [3H]CGP 39653 binding (Reynolds, 1994), and GV150526A competitively reversed the high-affinity component of [3H]CGP 39653 binding inhibition by glycine (Mugnaini et al., 2001). It is noteworthy that our FLIPR/Ca2⁺ mode of action experiments showed that glycine could surmount the inhibitory effect of compounds 1 to 3, 6, and 13 but not of NVP-AAM077, which was surmounted by 1 mM L-glutamate instead (Table 3). For the above reasons, hits 1 to 3, 6, and 13 might be glycine site antagonists; however, the tested compounds 1, 6, and 13 at 10 μM induced only 24, 13, and 14% displacement in rat native receptors, respectively, of glycine site antagonist [3H]MDL 105,519 binding (Table 4). Although [3H]MDL 105,519 displacement binding assay remains to be performed with recombinant human NR1/NR2A receptor, data obtained in rat brain cortical membranes suggest that compounds 1, 6, and 13 are acting not at the glycine site but at a site allosterically.
modulated by glycine. In addition, no glycine site antagonist has been reported in literature showing a complete selectivity for NR1/NR2A receptor over NR1/NR2B. Indeed, glycine site antagonists generally display only slight preference for NR2A-containing receptors, as it is observed for GV196771A in our FLIPR/Ca\textsuperscript{2+} assay (Fig. 2 and Table 3), and they displaced the glutamate site antagonist \textsuperscript{3}H]CGP 39653 but not the glycine site antagonist \textsuperscript{3}H]MDL 105,519 in binding assays (Tables 4 and 5). Therefore, these compounds might preferentially interact with or at the interface between both agonist binding domains (ABDs) of NR1/NR2A receptor, central to agonist/competitive antagonist binding and to activation gating. Glycine and glutamate binding sites are located within NR1 and NR2 subunit ABDs, respectively, and not between subunits (Mayer, 2006). Agonist binding results in closure of the “clamshell”-like ABD, whereas antagonist binding stabilizes the binding domain clamshell in an open conformation, and mechanisms for allosteric coupling between glycine and glutamate ABDs have been proposed (Mayer, 2006). In addition, the existence of independent NR1 and NR2 subunit activating transitions has been postulated according to single-channel recordings (Erreger et al., 2005), and novel hits might preferentially interact with NR1/NR2A having glycine and glutamate clam shell ABDs open and closed, respectively (Fig. 6).

It will be necessary to perform X-ray crystal studies on purified recombinant receptor, or part of it, to clarify the site of action of discovered hits (such as compound 1, 6, or 13) and gain insight on structural determinants of its NR1/NR2A selectivity. Binding studies on both recombinant receptors and isolated soluble ligand-binding domains, site-directed mutagenesis, and chimeric receptor studies might also be useful in clarifying the novel hits interactions with NR2A and/or NR1 domains. Compound 1 and other described hits may constitute useful tools or chemical starting points to investigate the role of NR1/NR2A receptor in physiological and pathological conditions.

Fig. 6. Hypothetical interaction model of novel hits with NR1/NR2A receptor. Clam shell-like agonist binding domains (ABD) and transmembrane domains (TMD) of one NR1 (filled figure) and one NR2A (open figure) subunit are graphically represented. The existence of four open/closed clamshell ABDs combination is postulated (states 1–4). Glycine (\textbullet) and L-glutamate (\textbullet) stabilize the clamshell ABD of NR1 and NR2A, respectively, in closed conformation (state 4). Novel hits might interact specifically with an open/closed ABDs combination (state 2).

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