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

Ezio Bettini, Anna Sava, Cristiana Griffante, Corrado Carignani, Alberto Buson, Anna Maria Capelli, Michele Negri, Filippo Andreotta, Sergio A. Senar-Sancho, Lorena Guiral, and Francesca Cardullo

Screening and Compound Profiling (E.B., C.C., A.B.), Computational and Structural Science (A.M.C.), Medicinal Chemistry (F.C.), Molecular and Cellular Biology (A.S., C.G., M.N., F.A.), GlaxoSmithKline Medicines Research Centre, Verona, Italy; and Screening and Compound Profiling, GlaxoSmithKline R&D Pharmaceuticals, Tres Cantos, Spain (S.A.S.-S., L.G.)

Received July 15, 2010; accepted August 24, 2010

ABSTRACT

NR1/NR2A is a subtype of N-methyl-D-aspartate receptors (NMDARs), which are glutamate and glycine-gated Ca\(^{2+}\)-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)/Ca\(^{2+}\) assay, identified sulfonamide derivative series, exemplified by 3-chloro-4-fluoro-N-[4-[[2-(phenylcarbonyl)hydrazinyl]carbonyl]phenyl]methyl]benzenesulfonamide (compound 1) and thiodiazole derivative N-(cyclohexylmethyl)-2-[[5-[[phenyl)methy]amino]-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 ([\(^{3}\)H]MK-801, dizocilpine malate) to a greater extent than the glycine site antagonist ([\(^{3}\)H]3-[2-carboxy-2-phenylethyl]-4,6-dichloro-1H-indole-2-carboxylic acid ([\(^{3}\)H]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 (Köhrl, 2006). NMDAR activity is...
crucial to brain functions such as circuit development (McK
inney, 2010) and learning and memory (Li and Tsien, 2009),
disfunction of NMDAR activity has been implicated in a
variety of neuropathological conditions, including stroke, ep
ilepsy, schizophrenia, depression, Huntington's disease, Alz
heimer's disease, Parkinson's disease, and multiple sclerosis
(Lipton, 2006; Large, 2007; Milnerwood et al., 2010).
NMDARs are unusual ligand-gated ion channels because
their activation requires the relief of Mg²⁺ block by mem-
brane depolarization (Mayer et al., 1984) and the concomi
tant binding of two agonists: glycine (or D-serine) and L-
glutamate (Johnson and Ascher, 1987; Mothet et al., 2000).
The opening of NMDARs leads to an influx of cations, includ
ing Ca²⁺, which initiates the signal transduction cascade.

The majority of NMDARs are tetrameric complexes
(Mayer, 2006), consisting of two glycine-binding NR1 sub
units 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 originate
NR2A, NR2B, NR2C, and NR2D subunits (Paolotti and Ney
ton, 2007). NMDARs containing different NR2 subunits have
different pharmacological and kinetic properties (Vicini et
al., 1998; Yuan et al., 2009).

NR2 subunits show distinct anatomical localization, pro
viding an opportunity to target specific neuronal circuits
using subunit-selective modulators. Ifenprodil has been the
prototype of selective NR1/NR2B receptor antagonists (Wil
liams, 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
to 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 sug
gested in pathological conditions such as anxiety and depres
sion (Boyce-Rustay and Holmes, 2006). Despite the impor
tance 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-quinoxalinyl)methyl]phosphonic
acid (NVP-AAM077) was obtained as the corresponding di
aminium salt by high-performance liquid chromatography separation of diastero
isomeric mixture [(1S)-1-(4-bromophenyl)ethyl]amino(2,3-dioxo-1,2,3
4-tetrahydro-5-quinoxalinyl)methyl]phosphonic acid tetrasodium hydrate (Sigma-Aldrich, Milan, Italy).
Memantine hydrochloride, (±)-MK-801 hydrogen maleate, 1-(1-phenylethyl)cyclohexylpiperidine (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 selectiv
ity 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 = pIC₅₀/number of nonhydrogen atoms.
Calculated partition coefficient (cLogP), obtained with the use of
Daylight 4.81 software (Daylight Chemical Information Systems,
Laguna Niguel, CA) was used to calculate LE to maximize the
potential of acceptable mammalian cell unit in vitro potency, ac
according to Leeson and Springthorpe (2007): LLE = pIC₅₀/cLogP.

HTP 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 hu
man NMDAR subunits were transiently transduced in human osteosarcoma (U-2 OS) cells (LGK Standards/American Type Culture
Collection, Teddington, UK) through BacMam vectors (Con
dreay et al., 1999) for fluorimaging image plate reader (FLIPR;
Molecular Devices, Sunnyvale, CA) assays or transduced in hu
man embryonic kidney (HEK) 293T cells (LGK Standards/Ameri
can 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
gene, Rockville, MD) were used for the NR2D subunit. pEGFP-N1
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 trans
ferent cells for patch clamp experiments.

FLIPR/Ca²⁺ Assay in U-2 OS Cells Expressing NMDAR. FLIPR/Ca²⁺
experiments on recombinant receptors were conducted using U-2 OS cells, because they are efficiently transduced through
BacMam vector (Condreay et al., 1999) and they adhere strongly
to 384-well plates. Cryopreserved U-2 OS aliquots were thawed
and suspended in Dulbecco's modified Eagle's medium/Ham's F-12 me
dium (Invitrogen, Milan, Italy), and supplemented with 10% (v/v)
dialyzed fetal calf serum (Invitrogen) and 0.3 mM MgCl₂. Suspended
cells were added with 500 µM ketamine (Merial Italia, Milan, Italy),
3% (v/v) NR1 BacMam, 6% (v/v) NR2A (or NR2B) BacMam, and
seeded in black-walled clear-based 384-well plates (Greiner Bio-One
GmbH, Frickenhausen, Germany) at a density of 18,000 cells/well in
culture medium and maintained overnight (5% CO₂ 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 CaCl₂, 1 g/liter p(+)-
glucose, 20 mM HEPES (pH adjusted to 7.3 with NaOH), and 2.5 µM
probenecid (Sigma-Alrich). Probenecid, an inhibitor of organic an
ion 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 EMLBA 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 fluores
cence (excitation wavelength at 488 nm, emission wavelength at 540
nm) 30 s before and until 5 min after 10 µl/well drug addition
(Sullivan et al., 1999). Antagonist addition was able to induce a
measurable decrease in fluorescence (see Supplemental Data), si
milarly 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% (w/v) of Pluronic F-127 (Sigma-Alrich) before the FLIPR/
Ca²⁺ 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). Stock solu
tions 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 glycine or l-glutamate was done by FLIPR instrument, 5 min after compound addition, and fluorescence was measured for an additional 5 min.

FLIPR/Ca²⁺ Assay in Rat Brain Cortical Cells. Cortical neurons 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 Decree 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 Hank’s balanced salt solution supplemented with HEPES (HBSS 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 and 0.25% trypsin inhibitor (Sigma-Aldrich) and washed two times with physiological salt solution buffer (145 mM NaCl, 1 g/liter glucose, 5 mM KCl, 2.5 mM MgCl₂, and 20 mM HEPES) supplemented with 2.5 mM probe-olution wavelength at 515 to 575 nm. Camera gain and intensity were adjusted for each plate to yield a minimum baseline fluorescence of 300 s.E.M. of at least three independent experiments, each performed at least in triplicate.

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-phosphocreatine, 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, Claux, 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 refers to the range of repetitions for each data point. In all other cases, statistical data are expressed as means ± S.E.M., 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,L-(-)-2-amino-4-propyl-5-phosphono-3-pentenoic acid ([3H]CGP 39653), 3-[E]-2-carboxy-2-phenylethylidenyl-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]CGP 39653, [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 μM l-glutamate, 10 μM MDL 105,519, 1 μM (+)-MK-801, and 10 μM ifenprodil, respectively. Incubation buffers for [3H]CGP 39653, [3H]MDL 105,519, [3H]TCP, and [3H]ifenprodil single 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]CGP 39653 CRC 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]CGP 39653 (Silis 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]CGP 39653 (specific activity, 40.5 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA) was incubated for 30 min at 25°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]CGP 39653 (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 Kᵢ and B_max of [3H]CGP 39653 was assessed by elaborating saturation experiments using a one-site binding (hyperbola) equation, after checking, by use of F 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 $pIC_{50} \pm$ 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 FLIPR 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 $pIC_{50} \geq 5.0$ 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.

**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 µM Ro 25-6981 to minimize the contribution of NR2B-containing receptors (at 1 µM 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.
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.

Table 2: Inhibitory effect (pIC50) in NR1/NR2A and NR1/NR2B FLIPR/Ca2⁺ assays for compounds identified through HTS and similarity searches

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₅</th>
<th>Rₓ</th>
<th>pIC50 NR1/NR2A</th>
<th>pIC50 NR1/NR2B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Phenyl</td>
<td>6.8 (21)</td>
<td>&lt;4.3 (21)</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>2,5-Dimethyl-3-furanyl</td>
<td>6.3 (14)</td>
<td>&lt;4.3 (12)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>3-Pyridinyl</td>
<td>6.2 (13)</td>
<td>&lt;4.3 (11)</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Cyclohexyl</td>
<td>6.4 (4)</td>
<td>&lt;4.3 (6)</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>Me</td>
<td>H</td>
<td>Me</td>
<td>H</td>
<td>H</td>
<td>2-F-Phenyl</td>
<td>&lt;4.3 (6)</td>
<td>&lt;4.3 (6)</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>3-Pyridinyl</td>
<td>CH₂-</td>
<td>5.4 (14)</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Tetrahydro-2-furanyl</td>
<td>CH₂-</td>
<td>5.5 (5)</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>4-Pyridinyl</td>
<td>CH₂-</td>
<td>4.9 (6)</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>4-Cl-Phenyl</td>
<td>CH₂-</td>
<td>&lt;4.3 (6)</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>2-Pyridinyl</td>
<td>CH₂-</td>
<td>4.6 (3)</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Me</td>
<td>Phenyl</td>
<td>CH₂-</td>
<td>&lt;4.3 (4)</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>H</td>
<td>H</td>
<td>Me</td>
<td>H</td>
<td>H</td>
<td>3-Pyridinyl</td>
<td>CH₂-</td>
<td>&lt;4.3 (4)</td>
</tr>
</tbody>
</table>

Fig. 2. Graph of NR1/NR2A FLIPR/Ca2⁺ mode of action experiments relative to compound 6, performed as described in Table 3.

Table 2: Inhibitory effect (pIC50) in NR1/NR2A and NR1/NR2B FLIPR/Ca2⁺ assays for compounds identified through HTS and similarity searches

Values are pIC50 means from human recombinant NR1/NR2A and NR1/NR2B FLIPR/Ca2⁺ assays. S.D. values were ≤0.3 for all tested compounds, except compounds 7 and 13, which had values of 0.4 and 0.5, respectively. Maximal inhibition was 92 to 100% for all listed compounds in NR1/NR2A assays. The number of independent experiments is indicated in parentheses.

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 et 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.

Values are pIC$_{50}$ mean ± S.D. from NR1/NR2A FLIPR/Ca$^{2+}$ assay in the presence of 1 mM glycine (Gly) or 1 mM L-glutamate (Glu), added 5 min after compound addition. 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 pIC$_{50}$ 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>
<thead>
<tr>
<th>Compound</th>
<th>pIC$_{50}$ + 1 mM Gly</th>
<th>pIC$_{50}$ + 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 ± 0.03 (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 ± 0.1 (5)</td>
</tr>
<tr>
<td>(+)-MK-801</td>
<td>7.3 ± 0.3 (4)</td>
<td>7.4 ± 0.3 (4)</td>
</tr>
</tbody>
</table>

(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 (pK$_{d}$) was 7.5 ± 0.03 and the calculated maximum binding capacity (B$_{max}$) was 5745 ± 373 fmol/mg protein (n = 3). In competition experiments, the known competitive NR1/NR2A preferring antagonist NVP-AAM077 showed a biphasic displacement curve with pIC$_{50}$ 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 pIC$_{50}$ 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 pIC$_{50}$ 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 in both FLIPR/Ca²⁺ and electrophysiology patch-clamp experiments (Table 2 and Fig. 4). Patch-clamp experiments also showed that compound 1 is inactive at NR1/NR2D receptors at 30 μM concentration, whereas the effect on receptors containing NR2C subunit has not yet been tested.

Replacing the phenyl group of sulfonamide derivative 1 with different R₅ groups (compounds 2–4) did not result in activity increase at NR2A FLIPR/Ca²⁺ assay (Table 2). Loss of activity was observed with the bis-methylated analog 5. In the shorter subseries (compounds 6–11) a drop of activity of at least 10-fold was observed, the 3-pyridinyl derivative 6 and tetrahydro-2-furanyl derivative 7 being the most active. In particular, when R₅ is a pyridinyl residue (compounds 6, 8, and 10), the 3-substitution was the preferred one. Further modification (compound 12) was detrimental for activity.

This is the first evidence describing compounds with such a high degree of selectivity for NR1/NR2A compared with NR1/NR2B receptor, to the best of our knowledge. NR1/NR2A-selective molecules were described in the literature, including the competitive NMDAR antagonists perzifotol (EAA-090) or NVP-AAM077. Perzifotol was shown to be 10 times more potent at blocking NR2A- versus NR2B- or NR2C-containing NMDARs (Sun et al., 2004). NVP-AAM077 was originally described to displace [³H]CGP 39653 with nanomolar affinity, displaying greater than 100-fold preferential blockade for NR2A- compared with NR2B-containing receptors in functional assays (Auberson et al., 2002). More detailed studies have shown that NVP-AAM077 displays only approximately 10-fold preference for NR2A- over NR2B-containing receptors (Neyton and Paoletti, 2006), and the latter is the degree of selectivity observed for NVP-AAM077 in our FLIPR/Ca²⁺ experimental conditions (Table 1).

To confirm affinity and functional activity at native NMDARs, studies were performed using membranes and cell cultures, respectively, from rat brain cortex. Rat brain cortex and derived cells in culture are known to express both NR2A and NR2B subunits (Zhong et al., 1994; Takai et al., 2003). [³H]CGP39653 binding data showed that NVP-AAM077 fully displaced [³H]CGP 39653, fitting a two-site binding equation, as expected for a molecule able to interact with both NR2A- and NR2B-containing receptors (Fig. 5 and Table 5). On the other side, compounds 1, 6, and 13 only partially displaced [³H]CGP 39653 with micromolar affinities (Fig. 5 and Table 5). Partial inhibition of [³H]CGP 39653 binding, partial efficacy in rat native tissue FLIPR/Ca²⁺ assay (Fig. 3), together with the full efficacy profile at NR1/NR2A recombinant receptor (Table 2), suggest that compounds 1, 6, and 13 interact selectively with a subpopulation of NMDARs, presumably the NR2A-containing 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 Seeberg, 1994; Kendrick et al., 1996), given 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-dichlorokynurenine 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$_2^+$ assay (Table 1), because of the approximately 10-fold lower affinity for glycine of receptors containing the NR2A subunit than other NR2 subunits (Dingledine et al., 1999). It is therefore tempting to speculate that the discovered hits bind to a region of NR1/NR2A receptor that is near both glutamate and glycine binding sites. Novel hits were surr

References


HYPOTHETICAL INTERACTION MODEL OF NOVEL HITS WITH NR1/NR2A RECEPTOR

**Fig. 6.** Hypothetical interaction model of novel hits with NR1/NR2A receptor. Clamshell-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 (□) and l-glutamate (△) 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). 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.
Leeson PD and Springthorpe B (2007) The influence of drug-like concepts on deci-
303.
Lipton SA (2006) Paradigm shift in neuroprotection by NMDA receptor blockade:
McKinney RA (2010) Excitatory amino acid involvement in dendritic spine forma-
Milnerwood AJ, Gladding CM, Pouladi MA, Kaufman AM, Hines RM, Boyd JD, Ko
RW, Vasaia OC, Graham RK, Hayden MH, et al. (2010) Early increase in extra-
synaptic NMDA receptor signaling and expression contributes to phenotype onset
Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N,
Sakmann B, and Seeburg PH (1992) Heteromeric NMDA receptors: molecular and
Mothet JP, Parent AT, Wolsak H, Brady RO Jr, Linden DJ, Ferris CD, Rogawski
MA, and Snyder SH (2000) D-serine is an endogenous ligand for the glycine site of
modulators of NR2B-containing NMDA receptors: molecular mechanisms and therapeutic poten-
Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N,
Mothet JP, Parent AT, Wolsak H, Brady RO Jr, Linden DJ, Ferris CD, Rogawski
MA, and Snyder SH (2000) D-serine is an endogenous ligand for the glycine site of
modulators of NR2B-containing NMDA receptors: molecular mechanisms and therapeutic poten-
Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N,
Mothet JP, Parent AT, Wolsak H, Brady RO Jr, Linden DJ, Ferris CD, Rogawski
MA, and Snyder SH (2000) D-serine is an endogenous ligand for the glycine site of
modulators of NR2B-containing NMDA receptors: molecular mechanisms and therapeutic poten-
Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N,
Mothet JP, Parent AT, Wolsak H, Brady RO Jr, Linden DJ, Ferris CD, Rogawski
MA, and Snyder SH (2000) D-serine is an endogenous ligand for the glycine site of
modulators of NR2B-containing NMDA receptors: molecular mechanisms and therapeutic poten-