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Structure-activity relationships for allosteric NMDA receptor inhibitors based on 2-naphthoic acid

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Abstract

Over-activation of N-methyl-D-aspartate (NMDA) receptors is critically involved in many neurological conditions, thus there has been considerable interest in developing NMDA receptor antagonists. We have recently identified a series of naphthoic and phenanthroic acid compounds that allosterically modulate NMDA receptors through a novel mechanism of action. In the present study, we have determined the structure-activity relationships of 18 naphthoic acid derivatives for the ability to inhibit the four GluN1/GluN2(A-D) NMDA receptor subtypes. 2-Naphthoic acid has low activity at GluN2A-containing receptors and yet lower activity at other NMDA receptors. 3-Amino addition, and especially 3-hydroxy addition, to 2-naphthoic acid increased inhibitory activity at GluN1/GluN2C and GluN1/GluN2D receptors. Further halogen and phenyl substitutions to 2-hydroxy-3-naphthoic acid leads to several relatively potent inhibitors, the most potent of which is UBP618 (1-bromo-2-hydroxy-6-phenylnaphthalene-3-carboxylic acid) with an $IC_{50} \sim 2 \mu M$ at each of the NMDA receptor subtypes. While UBP618 is non-selective, elimination of the hydroxyl group in UBP618, as in UBP628 and UBP608, leads to an increase in GluN1/GluN2A selectivity. Of the compounds evaluated, specifically those with a 6-phenyl substitution were less able to fully inhibit GluN1/GluN2A, GluN1/GluN2B and GluN1/GluN2C responses (maximal % inhibition of 60 – 90%). Such antagonists may potentially have reduced adverse effects by not excessively blocking NMDA receptor signaling. Together, these studies reveal discrete structure-activity relationships for the allosteric antagonism of NMDA receptors that may facilitate the development of NMDA receptor modulator agents for a variety of neuropsychiatric and neurological conditions.

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1. Introduction

N-methyl-D-aspartate (NMDA) receptors are a family of ionotropic L-glutamate receptors that mediate and modulate neurotransmission throughout the CNS (Traynelis *et al.*, 2010; Watkins, 1981). The activation of NMDA receptors can trigger diverse calcium-dependent intracellular responses that regulate distinct forms of synaptic plasticity such as long-term potentiation, long-term depression and experience-dependent synaptic refinement (Collingridge, 1987; Cotman *et al.*, 1988). Such NMDA receptor-mediated mechanisms are thought to play key roles in learning and memory, but also contribute to the expression of epilepsy, schizophrenia, drug addiction, mood disorders, post-traumatic stress disorder and neuropathic pain (Kalia *et al.*, 2008; Sanacora *et al.*, 2008). In addition, in cases of excessive NMDA receptor activation, the resulting elevation of intracellular calcium initiates cell death and this mechanism may be common to neuronal cell death in stroke, traumatic brain injury and various neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's, multiple sclerosis, amyotrophic lateral sclerosis (ALS), and Creutzfeldt-Jakob disease (Kalia *et al.*, 2008; Villmann *et al.*, 2007). Despite high expectations, the results from clinical studies of NMDA receptor antagonists have been largely disappointing (Kalia *et al.*, 2008; O'Collins *et al.*, 2006).

Allosteric modulators represent an alternative approach for developing NMDA receptor pharmacological agents with improved pharmacological properties (Kenakin, 2004). As found for other receptor systems, allosteric modulators can have greater subtype-selectivity, the ability to either negatively or positively modulate receptor function, and may not fully inhibit the receptor response. In examining the activity of a series of naphthoic acid and phenanthroic acid derivatives, we recently identified a family of allosteric modulators that have novel activities and a novel mechanism of action (Costa *et al.*, 2010). These compounds display varied subunit-selectivity and have potentiating activity as well as inhibitory activity. The NMDA receptor inhibitory compounds were not competitive L-glutamate or glycine site antagonists, did not block in a voltage-dependent manner as expected for channel blockers, and did not require the regulatory N-terminal domain (NTD) for activity. These may be binding at sites homologous to the 2-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor's positive and negative allosteric modulator binding sites (Balannik *et al.*, 2005; Ptak *et al.*, 2009).

The chemical structures of these agents are distinct from that of other known classes of NMDA receptor agents and they have a novel site of action. Currently, very little is known about the pharmacophore of this new pharmacological site; such information is essential for further drug design. In the present study, a variety of 2-naphthoic acid derivatives related to the prototype drug UBP618 (Costa *et al.*, 2010) were evaluated for their activity at recombinant NMDA receptors expressed in *Xenopus* oocytes in order to identify the structural features underlying the activity of these compounds.

2. Methods

2.1 NMDA receptor constructs

GRIN1a cDNA encoding the NMDAR1a subunit (GluN1a) was a generous gift of Dr. Shigetada Nakanishi (Kyoto, Japan) (Moriyoshi *et al.*, 1991). cDNA encoding the GluN2A, GluN2C and GluN2D subunits (GRIN2A, GRIN2C, and GRIN2D) were kindly provided by Dr. Peter Seeburg (Heidelberg, Germany) (Monyer *et al.*, 1992) and the GRIN2B cDNA was the generous gift of Drs. Dolan Pritchett and David Lynch (Philadelphia, USA) (Lynch *et al.*, 1995). The NTD-deleted GluN1 (GRIN1 Δ^{NTD}) construct is described elsewhere (Madry *et al.*, 2007) and the NTD-deleted GluN2 constructs (GRIN2A Δ^{NTD} and GRIN2D Δ^{NTD}) were kindly provided by Dr. Pierre Paoletti (Rachline *et al.*, 2005). Plasmids

were linearized with Not I (GRIN1a, GRIN2C, GRIN2D, and GRIN1 Δ^{NTD}), EcoR I (GRIN2A) or Sal I (GRIN2B, GRIN2A Δ^{NTD} and GRIN2D Δ^{NTD}) and transcribed *in vitro* with T7 (GRIN1a, GRIN2A, GRIN2C, and GRIN2D) and SP6 (GRIN1 Δ^{NTD} , GRIN2A Δ^{NTD} , GRIN2D Δ^{NTD} and GRIN2B) RNA polymerase using the mMessage mMachine transcription kits (Ambion, Austin, TX, USA).

2.2 GluN subunit expression and electrophysiology in *Xenopus* oocytes

Oocytes from mature female *Xenopus laevis* (Xenopus One, Ann Arbor, MI, USA) were removed and isolated using procedures approved by the University of Nebraska Medical Center's Institutional Animal Care and Use Committee in compliance with the National Institutes of Health guidelines. NMDA receptor subunit RNAs were dissolved in sterile distilled H₂O. GluN1a and GluN2 RNAs were mixed in a molar ratio of 1:1-3. 50 nl of the final RNA mixture was microinjected (15-30 ng total) into the oocyte cytoplasm. Oocytes were incubated in ND-96 solution for 1-5 days at 17°C prior to electrophysiological assay.

Electrophysiological responses were measured using a standard two-microelectrode voltage clamp model OC-725B (Warner Instruments, Hamden, Connecticut,) designed to provide fast clamp of large cells. The recording buffer contained 116 mM NaCl, 2 mM KCl, 0.3 mM BaCl₂ and 5 mM HEPES, pH 7.4. Response magnitude was determined by the steady plateau response elicited by bath application of 10 μ M L-glutamate plus 10 μ M glycine (unless stated otherwise) and held at a membrane potential of -60 mV. Response amplitudes for the four heteromeric complexes were generally between 0.1 to 3 μ A. After obtaining a steady-state response to agonist application, test compounds were bath applied (Automate Scientific 16-channel perfusion system) and the responses were digitized for analysis (Digidata 1440A and pClamp-10, Molecular Devices). Dose-response relationships were fit to a single-site with variable slope (GraphPad Prism, ISI Software), using a nonlinear regression to calculate IC₅₀ and % maximal inhibition. This uses the equation: receptor response (nA or normalized response) = response at maximal inhibition + ((response with no inhibitor - response at maximal inhibition) / (1 + 10^(logEC50-X) (Hill Slope))), where X is the logarithm of the antagonist concentration. Maximal inhibition ("bottom" of curve) was allowed to vary. This equation estimated the % maximal inhibition for low affinity compounds whose antagonist response was still approaching a plateau at the highest concentration. This was associated with about a two-fold increase in error but did not appear to significantly affect the % Maximum Inhibition estimate since this value varied according to drug structure and receptor subtype and did not correspond to having to extrapolate the % maximal inhibition.

All experiments were performed at least 4 times. IC₅₀ and % maximal inhibition values were compared between drugs using ANOVA followed by a Newman-Keuls multiple comparison test.

2.3 Compounds

Structures of compounds synthesized and tested for this report are presented in Figure 1. 1,6-Dibromo-2-hydroxy-3-naphthoic acid (UBP552), 2-amino-1,6-dibromo-3-naphthoic acid (UBP597), and 2-amino-6-bromo-3-naphthoic acid (UBP606) were synthesized according to literature procedures (Lee *et al.*, 2006; Murphy *et al.*, 1990). 1,6-Dibromo-3-naphthoic acid (UBP628), 1,6-dibromo-2-methoxy-3-naphthoic acid (UBP704), 2-hydroxy-1-iodo-3-naphthoic acid (UBP621), 6-bromo-2-hydroxy-1-iodo-3-naphthoic acid (UBP620), 2-hydroxy-6-phenyl-3-naphthoic acid (UBP617), 1-bromo-2-hydroxy-6-phenyl-3-naphthoic acid (UBP618), and 2-hydroxy-1-iodo-6-phenyl-3-naphthoic acid (UBP619) were synthesized and purified by methods to be reported elsewhere. After synthesis and purification, compound structure was verified by ¹H NMR and mass

spectroscopy. All novel compounds had elemental analyses in which the determined percentages for C, H, and N were less than 0.4% different from theoretical values. The other compounds were obtained from Sigma-Aldrich [2-naphthoic acid (UBP519), 2-hydroxy-3-naphthoic acid (UBP558), 2,3-dicarboxynaphthalene (UBP575), 6-bromo-2-oxo-2H-chromene-3-carboxylic acid (UBP608)], Alfa Aesar [3-amino-2-naphthoic acid (UBP596)], and Tokyo Chemical Industry UK Ltd [1-bromo-2-hydroxy-3-naphthoic acid (UBP573), 6-bromo-2-hydroxy-3-naphthoic acid (UBP574), 1,6-dibromo-2-naphthol (UBP644)].

3. Results

3.1 Compound activity at GluN1/GluN2A-D NMDA receptors

A series of 2-naphthoic acid derivatives were evaluated for their ability to inhibit the activation of GluN1/GluN2A-D NMDA receptors expressed in *Xenopus* oocytes using two-electrode voltage clamp at -60 mV. After obtaining a steady-state response to 10 μ M L-glutamate and 10 μ M glycine, the individual test compounds were then co-applied with the agonists.

The structures of the compounds tested are shown in Figure 1. With the exception of UBP608, these compounds are derivatives of 2-naphthoic acid (UBP519). In initial studies, the ability of a 100 μ M concentration of each compound to inhibit NMDA receptor responses was evaluated (Figure 2). 2-Naphthoic acid weakly inhibited activity at GluN2A-containing receptors by approximately 30% and very weakly inhibited the other GluN2-containing receptors (0 – 5% inhibition). 3-Substitution of 2-naphthoic acid with a 3-hydroxy group (UBP558), or a 3-amino group (UBP596), increased inhibitory activity whereas 3-carboxy substitution had no effect.

Halogen substitution at either the 1- or the 6-position of 2-hydroxy-3-naphthoic acid enhanced inhibitory activity with 1-bromo substitution (1-bromo-2-hydroxy-3-naphthoic acid, UBP573), especially enhancing activity especially at GluN2C and GluN2D-containing receptors (Figure 2). Combined 1- and 6-bromo substitution (1,6-dibromo-2-hydroxy-3-naphthoic acid; UBP552) further increased inhibitory activity (Figure 2). UBP552 is a relatively potent inhibitor with IC_{50} values of 3 to 7 μ M (Figure 3; Table 1) with a weak preference for GluN2D-containing receptors. 1-Iodo substitution of 2-hydroxy-3-naphthoic acid (resulting in UBP621), increased inhibitory activity more than 1-bromo-substitution. However, this enhancement in activity by 1-iodo substitution was not seen when activity was already enhanced by bromo- or phenyl-substitution at the 6-position (cf UBP620/UBP552 and UBP619/UBP618).

Of the 4 substituents on the naphthalene ring of UBP552, the 3-carboxy group makes the strongest contribution to activity. UBP644, which has the carboxy group substituted by hydrogen, was virtually inactive (Figure 2). The 2-hydroxy group in UBP552 was also important for high potency. Substituting a hydrogen (UBP628) for the 2-hydroxy of UBP552 reduced inhibitory activity (Figure 2) and altered subunit selectivity. UBP628 displayed a 33-fold lower potency for GluN1/GluN2D than did UBP552 but only a 3-fold lower potency at GluN1/Glu2A (Figure 3, Table 1). 2-Hydroxy substituted analogues (e.g. UBP574 and UBP552) displayed higher activity than their 2-amino substituted homologues (2-amino-6-bromo-3-naphthoic acid, UBP606 and 2-amino-1,6-dibromo-3-naphthoic acid, UBP597, respectively).

Compound activity could be further increased by 6-phenyl substitution. Replacing the 6-bromo group of either UBP574 or UBP552 with a phenyl group, yielding UBP617 and UBP618, respectively, increased potency for all NMDA receptors. Of the combination of

substitutions explored, UBP618 displayed the highest potency for NMDA receptors and did not discriminate between NMDA receptor subtypes.

The comparison made above between UBP552 and UBP628 suggests that the 2-hydroxy group has a relatively small contribution to UBP552 potency at GluN1/GluN2A receptors and a greater contribution to potency at the other NMDA receptors. The compound UBP608, incorporates some of the features of UBP552 but replaces this 2-hydroxy group with an oxygen and eliminates the 1-bromo group by incorporation of a chromene ring. UBP608, like UBP628, displays greater GluN1/GluN2A selectivity compared to the other compounds tested, with an IC_{50} of 19 μ M for GluN2A-containing receptors and 68 to 426 μ M at other NMDA receptors.

Hill coefficients for the inhibition of NMDA receptor responses were generally between 1 and 2 (see Supplemental Table 1). With some Hill coefficients above one, this suggests that there might be more than one antagonist binding site on the receptor. This is likely given the tetrameric structure of the NMDA receptor complex. However, since most Hill coefficients had a value near one, if there are multiple binding sites, they do not appear to be highly cooperative. Also, we have previously found that related chemical structures have an additional potentiation activity (Costa et al., 2010). Thus if some of the compounds studied here have a weak potentiation activity combined with their inhibitory activity, they would be likely to have an altered Hill coefficient.

3.2 Structural features underlying variations in maximal blockade

A feature that distinguishes this class of antagonists from competitive NMDA receptor antagonists is the inability of some of these compounds to fully inhibit agonist responses (while not having partial agonist activity) (Costa et al., 2010) and the present study. In our initial report the prototype compound UBP618 maximally inhibited GluN1/GluN2 receptor responses to 10 μ M L-glutamate and 10 μ M glycine by approximately 80 - 90% with GluN1/GluN2A having the lowest % maximal inhibition. In the present study, the compounds tested here also differed in their ability to maximally inhibit NMDA receptor responses (Figure 3 and Table 1). Some compounds displayed a lower maximal inhibition at GluN1/GluN2A, GluN1/GluN2B, and GluN1/GluN2C receptors than at GluN1/GluN2D receptors. UBP617 had the lowest % maximal inhibition for both GluN1/GluN2A and GluN1/GluN2B receptors (71.5% and 56.8%, respectively) yet fully inhibited GluN1/GluN2C and GluN1/GluN2D receptor responses. The closely related compounds UBP618 and UBP619 also displayed relatively low % maximal inhibition at GluN1/GluN2A and GluN1/GluN2B receptors and displayed the two lowest % maximal inhibition of GluN1/GluN2C receptors. Thus specifically those compounds with a 6-phenyl group were less able to fully inhibit NMDA receptor responses, especially at receptors containing GluN2A, GluN2B and GluN2C subunits. UBP620 was intermediate in the ability to fully inhibit NMDA receptor responses (maximal inhibition of 90% at GluN1/GluN2A and GluN1/GluN2B receptors) while UBP552 was able to fully inhibit all receptor responses.

3.3 The effects of agonist concentration and the NTD on the activity of UBP552

In our initial studies (Costa et al, 2010), we found that the inhibitory activity of naphthoic acid derivative UBP618 and a phenanthroic acid compound UBP512 were not due to competitive antagonism at either the L-glutamate or glycine site binding sites. Furthermore, the inhibitory activity of UBP608 and UBP512 was not dependent upon the NTD. Since UBP552 emerged in this study as a relatively potent antagonist, we sought to determine if the actions of UBP552 are similar to that of these previously reported prototype compounds. Thus, we tested whether UBP552 has glutamate or glycine binding site competitive antagonist activity or if the compound that acts at, or requires, the NTD. Other compounds

in this study are structurally more simple, or very similar, homologues of UBP608, UBP618, or UBP552 and thus they were not further studied.

At GluN1/GluN2A receptors, increasing both glycine and L-glutamate concentrations from 10 μ M to 300 μ M reduced UBP552 inhibitory potency 2.5-fold (Table 2, Figure 4). Both agonists appear to contribute to this agonist-induced shift to larger IC₅₀ values. Increasing glycine concentration from 10 μ M to 300 μ M in the presence of 10 μ M L-glutamate increased the UBP552 IC₅₀ by 1.6-fold that was not statistically different (Table 2). Increasing glycine concentration from 10 μ M to 300 μ M in the presence of 300 μ M L-glutamate increased the UBP552 IC₅₀ value by 1.8-fold that was statistically different ($p < 0.05$). Likewise for L-glutamate, increasing L-glutamate concentration by 30-fold or 150-fold in the presence of 10 μ M glycine caused small increases in UBP552 IC₅₀ values (1.4-fold and 1.7-fold, respectively) that were not statistically significant. And increasing L-glutamate concentration from 10 μ M to 300 μ M in the presence of 300 μ M glycine increased UBP552 IC₅₀ value by 1.5 -fold ($p < 0.05$).

The less than 2-fold increases in UBP552 IC₅₀ values determined in higher agonist concentrations do not appear to represent competitive antagonist activity since such increases in IC₅₀ values are far less than expected for a competitive antagonist. We estimate that a competitive antagonist's IC₅₀ would be increased 21 to 25-fold at the glycine site, as calculated by the Cheng-Prusoff equation (Cheng *et al.*, 1973) and a glycine affinity of 2.1 μ M (Kutsuwada *et al.*, 1992) or 4.8 μ M (Buller *et al.*, 1995). Increasing L-glutamate concentration from 10 μ M to 300 μ M is estimated to increase a competitive antagonist IC₅₀ by ~23 fold using a literature average L-glutamate K_d value of 2.9 μ M (Costa *et al.*, 2009). Increasing L-glutamate from 2 μ M to 300 μ M would be expected to shift a competitive antagonist's IC₅₀ by 62-fold, whereas we observed a 1.7-fold increase.

In contrast to the results obtained with GluN1/GluN2A receptors, at GluN1/GluN2D receptors, increasing L-glutamate concentration from 0.3 μ M to 10 μ M or to 300 μ M decreased UBP552 IC₅₀ values by 46% ($p < 0.05$). This result is thus opposite to that predicted for a competitive antagonist. Increasing glycine and/or L-glutamate concentration from 10 μ M to 300 μ M had no effect on UBP552 potency. Thus, UBP552, like UBP618 (Costa *et al.*, 2010), displays an uncompetitive antagonist activity for L-glutamate at GluN1/GluN2D receptors wherein higher agonist concentration (compared to 0.3 μ M L-glutamate) increases antagonist activity. In contrast, at GluN1/GluN2A receptors, high agonist concentrations cause a small reduction in antagonist potency. In control experiments, 100 μ M UBP552 given in the absence of L-glutamate and glycine had no detectable effect on oocytes expressing GluN1/GluN2D receptors.

The role of the NTD in UBP552 activity was tested at GluN1/GluN2A and GluN1/GluN2D receptors by comparing wild-type receptors to those in which the NTD had been deleted on both the GluN1 and the GluN2 subunit (Madry *et al.*, 2007; Rachline *et al.*, 2005). UBP552 was still able to inhibit GluN1/GluN2A and GluN1/GluN2D receptor responses in the absence of the NTD (Figure 4C). However, NTD deletion increased the potency of UBP552 at inhibiting GluN1/GluN2A receptor responses and decreased the inhibitory potency at GluN1/GluN2D receptors. Thus, the NTD is not necessary for UBP552 activity, but does influence its actions.

4. Discussion

NMDA receptors are involved in a wide variety of neurological and psychiatric conditions. However, there have been relatively few successful clinical trials with NMDA receptor pharmacological agents. Nevertheless, there is still significant potential for their

development. As evidenced by other receptor systems, allosteric modulators can offer distinct advantages over orthosteric agents in terms of subtype-selectivity and the ability to either positively or negatively modulate receptor activity (Kenakin, 2004). Recently, chemically-diverse allosteric modulators have been identified for NMDA receptors that display markedly distinct patterns of activity (Bettini *et al.*, 2010; Costa *et al.*, 2010; Mosley *et al.*, 2010).

In the present report, we have described the structure-activity relationships of a series of 2-naphthoic acid derivatives to better define this novel drug-binding site to enable the development of improved pharmacological agents. The most essential substituent was the carboxylic acid group; removal of the carboxy group from the potent compound UBP552 eliminated activity (UBP644). Antagonist potency at all NMDA receptors was also increased by 1- and 6-halogen substitution, 2-hydroxy substitution, and 6-phenyl substitution in a generally additive manner. Of this series of compounds, 1-bromo-2-hydroxy-6-phenylnaphthalene-3-carboxylic acid (UBP618) is the most active agent.

Inhibitory activity of the 2-naphthoic acid derivatives is, for the most part, similar between the different GluN1/GluN2 receptors studied. However, subtype-selectivity is, affected by substitutions ortho to the carboxy group. The hydroxy group in 1,6-dibromo, 2-hydroxy 3-naphthoic acid (UBP552) preferentially increases potency at GluN1/GluN2B-D receptors over that of GluN1/GluN2A receptors. Thus, GluN2B, GluN2C, and GluN2D may provide a hydrogen bond acceptor not present, or optimally positioned, in GluN2A. Conversely, UBP608 has a hydrogen bond acceptor at this position and has enhanced GluN1/GluN2A-selectivity over GluN2B-D.

The structure activity relationships described here are distinct from that of other classes of agents that act on NMDA receptors. They do not contain a positive charge group α to the carboxylic acid, as is common for both L-glutamate and glycine binding site agonists/antagonists. These results are thus consistent with the observation that these agents are not competitive antagonists at either the L-glutamate binding site nor the glycine binding site (Costa *et al.* 2010; present results). Likewise, the naphthoic acid modulators do not resemble NMDA receptor channel blockers which generally have a positive charge center surrounded by a hydrophobic ring system – a result consistent with the absence of any voltage-dependent blockade which would suggest a channel blocking mechanism (Costa *et al.*, 2010). The essential features of the naphthoic acid modulators are also not found in NTD-binding modulators such as ifenprodil. Indeed, these agents are not likely to be binding anywhere associated the NTD since the NTD is not necessary for naphthoic acid modulatory activity (Costa *et al.*, 2010, present results).

It is difficult to compare the structural requirements for the naphthoic acid modulators to that of the other recently described allosteric modulators of NMDA receptors the compounds QNZ46 (Hansen and Traynelis, 2011) and 3-chloro-4-fluoro-N-[(4-{{2-(phenylcarbonyl)hydrazino}carbonyl}phenyl)methyl] benzene-sulfonamide (Bettini *et al.*, 2010) – and the neurosteroid pregnanolone sulfate (Weaver *et al.*, 2000). The structures of each of these four classes of modulators described here, do however, have a negative charge group on one end that is important for activity (Mosley *et al.*, 2010; Weaver *et al.*, 2000).

The compounds tested in this report show that the modulator binding site can accept a fairly large, planar molecule that is hydrophobic but requires a negative charge on one end. Such a general structure is inconsistent with the minimal essential pharmacophore of the orthosteric binding sites and the ion channel but is consistent with the large, somewhat planar mostly hydrophobic interfaces between subunits at the level of the ligand binding domain (Furukawa *et al.*, 2005).

By analogy to AMPA receptor modulators such as GYKI-53655, these compounds may be binding in the ligand binding domain interface between GluN1 and GluN2 subunits and the linkers preceding the M1 and M4 segments (Balannik *et al.*, 2005). We found that replacing the segment 1 (S1), but not segment 2 (S2), of GluN2A with that of GluN2C reduced inhibitory activity of GluN2A-preferring UBP608 (Costa *et al.*, 2010). This site would appear then to be different from the binding site for the recently describe negative modulator QNZ46; inhibitory activity of this agent is determined by the S2 domain and not the S1 domain (Hansen *et al.*, 2011). Additionally, QNZ46's inhibitory activity is not influenced by agonist concentration (Hansen *et al.*, 2011) as is the activity of the naphthoic and phenanthroic acid modulators (present results, Costa *et al.*, 2010). Further studies will be necessary to more precisely define the site and mechanism of action for these allosteric inhibitors. Similar to that found for the allosteric AMPA receptor antagonists such as GYKI-53655, preliminary studies indicate that UBP552 has antagonist activity at AMPA receptors but not at kainate receptors.

A potentially noteworthy finding of the present study is that specific structural features underlie the ability of this novel class of antagonists to fully block NMDA receptor responses. The compounds UBP617, UBP618, and UBP619 were the least able to fully block NMDA receptor responses at receptors containing GluN2A, GluN2B, or GluN2C subunits. Of the compounds tested, these were the only ones with a 6-phenyl substitution. Thus, while the phenyl ring increases potency, at the same time it may be preventing a complete conformational movement that corresponds to channel closing. As this class of compounds is structurally similar to compounds with allosteric potentiating activity (Costa *et al.*, 2010), it is also possible that the presence of a low affinity potentiating activity contributes to the incomplete blockade by UBP617, UBP618, and UBP619. In either case, blockers that cannot fully block NMDA responses may potentially have an improved safety profile since they would be less able to cause excessive receptor blockade.

5. Conclusions

Modifications to the 2-naphthoic acid nucleus explored in this work reveal specific structure-activity relationships that underlie the potency of these compounds at NMDA receptors. The interactions defined here generally apply to all four of the GluN1/GluN2A-D receptors. Thus, the inhibitory binding site occupied by the 2-naphthoic acid derivatives may be largely conserved among the different NMDA receptor subtypes. However, compounds UBP608 and UBP628 demonstrate that there are elements in the binding pocket that are distinct between receptor subunit subtypes. The larger phenanthroic acid derivatives that we recently reported (Costa *et al.*, 2010) displayed greater subtype selectivity – perhaps due to the ability to probe subtype-specific regions of the drug binding pocket or perhaps to the ability to additionally bind to a distinct positive allosteric modulator binding site which displays greater subtype-selectivity. Further substitutions to expand the naphthoic acid structure may further increase both affinity and subtype selectivity.

The compounds described here are negative allosteric modulators; they are not competitive antagonists and they do not require the N-terminal domain for activity. A potentially important feature of these compounds is that a specific structural modification (phenyl substitution) leads to incomplete maximal inhibition at GluN2A and GluN2B-containing receptors. Thus, it appears possible to design compounds with varied maximal antagonist activity. Such compounds may have an improved safety profile due to their inability to fully block NMDA receptor activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- A 2-naphthoic acid derivative series was found to inhibit NMDA receptor activity.
- Structure-activity relationships for optimal inhibitory activity were determined.
- Substitutions at the 3-position of 2-naphthoic acid can affect subtype-selectivity.
- 6-Phenyl substitution in 2-hydroxy-3-naphthoic acid reduces maximal inhibition.
- Inhibitory activity is allosteric and does not involve the N-terminal domain.

Figure 1.
Structure of naphthoic acid derivatives.

Figure 2.

Activity of 2-naphthoic acid derivatives for the inhibition of NMDA receptor responses. After obtaining a steady-state response to 10 μ M L-glutamate plus 10 μ M glycine, each compound was tested at 100 μ M for the inhibition of responses by GluN1/GluN2A (2A), GluN1/GluN2B (2B), GluN1/GluN2C (2C) and GluN1/GluN2D (2D) receptors. Values represent mean % inhibition $\pm$ s.e.m., $n \geq 4$.

Figure 3.

2-Naphthoic acid derivatives were tested at a range of doses for their effect on NMDA receptor activity evoked by $10\ \mu\text{M}$ L-glutamate plus $10\ \mu\text{M}$ glycine. A. A representative current trace showing the effect of increasing concentrations of UBP552 (open bars, μM) on GluN1/GluN2D receptor responses evoked by agonists (Ag; solid bars). Insert shows the scale for current (nA) and time (s). B-H. Dose-response relationships are shown for the antagonism of GluN1/GluN2A (2A), GluN1/GluN2B (2B), GluN1/GluN2C (2C), and GluN1/GluN2D receptor responses by the indicated antagonists. Results for UBP608 and UBP618 were previously reported (Costa et al., 2010) and are shown here for comparison.

Figure 4.

The effect of different L-glutamate or glycine concentrations and NTD-deletion on the inhibitory activity of UBP552. A. GluN1/GluN2A (2A) and B. GluN1/GluN2D (2D) receptor responses were evoked by the indicated concentrations of glycine and L-glutamate (Gly/Glu, μ M) and inhibited by increasing concentrations of UBP552. C. UBP552 inhibition was tested at wildtype GluN1/GluN2A and GluN1/GluN2D receptors (2A and 2D, respectively) and at these receptors with the NTD deleted on both the GluN1 and GluN2 subunits (2A^{ΔNTD}, 2D^{ΔNTD}).

Table 1

Compound	GluN1/GluN2A		GluN1/GluN2B		GluN1/GluN2C		GluN1/GluN2D	
	IC ₅₀ (μM)	% Max. Inh.	IC ₅₀	% Max. Inh.	IC ₅₀	% Max. Inh.	IC ₅₀	% Max. Inh.
UBP608 ^a	18.6 ± 1.4	106 ± 0.5	89.5 ± 3.5	100 ^b	68.3 ± 9.1	100 ^b	426 ± 40 ^j	100 ^b
UBP618 ^a	1.76 ± 0.24 ^{CH}	83.0 ± 4.0 ^{CHI}	2.44 ± 0.11 ^{CEH}	88.0 ± 2.0	2.00 ± 0.08 ^{CEH}	87.3 ± 2.0 ^{C-IC-IC-i}	2.4 ± 0.26 ^H	92.0 ± 0.7
UBP617	11.0 ± 5.2	71.5 ± 6.4 ^{C-iHI}	97.8 ± 20.9	56.8 ± 5.2 ^{C-IC-IC-IC-iI}	41.8 ± 8.4 ^H	105.5 ± 4.4	49.6 ± 6.0 ^{DH}	98.6 ± 3.2
UBP619	5.48 ± 2.02 ^{C-i}	78.8 ± 2.5 ^{CHI}	5.65 ± 1.10 ^{CEH}	82.1 ± 3.1	4.95 ± 0.10 ^{CEH}	77.3 ± 5.0 ^{EH}	2.8 ± 0.14 ^{EH}	97.0 ± 2.2
UBP620	3.17 ± 0.30 ^{CH}	90.5 ± 7.4	9.06 ± 1.02 ^{CEH}	90.6 ± 10.7	5.93 ± 1.56 ^{CEH}	96.1 ± 2.3	5.36 ± 0.60 ^{EH}	107.1 ± 4.3
UBP628	19.3 ± 5.9	104.3 ± 4.3	88.2 ± 25.7	84.4 ± 12.5	84.1 ± 7.1	103.0 ± 7.1	109.7 ± 11.0	112.4 ± 21.3
UBP552	6.29 ± 0.88 ^{C-i}	105.6 ± 4.3	7.30 ± 1.21 ^{CEH}	108.3 ± 2.0	5.04 ± 0.16 ^{CEH}	101.5 ± 0.9	3.35 ± 0.18 ^{EH}	103.6 ± 2.2

Compound potency (IC₅₀ ± s.e.m., μM) at NMDA receptor subtypes and extent of maximal inhibition (% Max. Inh.) ± s.e.m., n ≥ 4.

^aCompounds UBP608 and UBP618 were previously reported (Costa et al., 2010) and are shown here for comparison.

^b% Maximal inhibition constrained to 100% to improve IC₅₀ estimate

^{c-i} statistically different from UBP608 (c, C), UBP618 (d, D), UBP617 (e, E), UBP619 (f, F), UBP620 (g, G), UBP628 (h, H), and UBP552 (i, I) with p values < 0.05 (c-i), p < 0.01 (C-D), and p < 0.001 (C-I).

^j extrapolated value, thus excluded from ANOVA.

Table 2

<u>[Gly] μM</u>	<u>[L-Glu] μM</u>	<u>GluN1/GluN2A</u>	<u>GluN1/GluN2D</u>
		<u>IC₅₀ (μM)</u>	<u>IC₅₀ (μM)</u>
10	0.3	NT	6.26 $\pm$ 0.58
10	2.0	5.05 $\pm$ 0.73 ^A	NT
10	10	6.29 $\pm$ 0.88 ^A	3.35 $\pm$ 0.18 ^b
10	300	8.58 $\pm$ 0.84 ^a	3.35 $\pm$ 0.82 ^b
300	10	10.02 $\pm$ 1.93 ^a	3.73 $\pm$ 0.75 ^b
300	300	15.48 $\pm$ 1.73	4.85 $\pm$ 0.27

UBP552 inhibitory potency (IC₅₀) at GluN1/GluN2A and GluN1/GluN2D receptor responses evoked by different agonist concentrations.

^A a = statistically different from the 300 μ M/300 μ M condition; A, $p < 0.01$

^a $p < 0.05$

^b = different from the 10 μ M/ 0.03 μ M condition, $p < 0.05$; NT = not tested.