REVIEW —Current Perspective—

(1*S*,2*R*)-1-Phenyl-2-[(*S*)-1-aminopropyl]-*N,N*-diethylcyclopropanecarboxamide (PPDC), a New Class of NMDA-Receptor Antagonist: Molecular Design by a Novel Conformational Restriction Strategy

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Received November 30, 2000

ABSTRACT—We have found that milnacipran, a clinically useful antidepressant due to its inhibition of the re-uptake of serotonin (5-HT) and noradrenaline, is also a non-competitive NMDA-receptor antagonist. Based on the cyclopropane structure of milnacipran, conformationally restricted analogs were designed and synthesized. Of these analogs, (1*S*,2*R*)-1-phenyl-2-[(*S*)-1-aminopropyl]-*N,N*-diethylcyclopropanecarboxamide (PPDC) is 30-fold stronger than milnacipran as an NMDA-receptor antagonist with virtually no inhibitory effect on the neurotransmitter re-uptake. PPDC was identified as a new class of NMDA-receptor antagonist because it has a mode of action different from that of the previous antagonists; it selectively binds the GluR ϵ 3/GluR ζ 1 and GluR ϵ 4/GluR ζ 1 subtype receptors in an agonist-independent allosteric manner. Functional assays of PPDC with the *Xenopus* oocytes system and cultured mouse neurons under voltage-clamp conditions confirmed that it acts as a potent NMDA-receptor antagonist. PPDC effectively protected against NMDA-induced neurotoxicity in both cultured mouse cerebral cortex and delayed neuronal death in a gerbil ischemic model. It was also active in a reserpine-treated mouse Parkinsons disease model. Thus, PPDC may be a candidate for a clinically useful NMDA-receptor antagonist, since the development of previous NMDA-receptor antagonists as drugs has been hindered by various undesirable side effects.

Keywords: Conformationally restricted analog, Cyclopropane, Milnacipran, NMDA receptor, (1*S*,2*R*)-1-phenyl-2-[(*S*)-1-aminopropyl]-*N,N*-diethylcyclopropanecarboxamide (PPDC)

Introduction

The excitatory neurotransmitter L-glutamic acid (glutamate) has been shown to cause neuronal death in instances of stroke, ischemia and head trauma (1). Glutamate receptor overstimulation may also play a role in chronic neurodegenerative conditions such as Alzheimer's disease and Parkinson's disease (1, 2). A number of studies strongly suggest that the toxicity of high levels of glutamate is mediated primarily by NMDA (*N*-methyl-D-aspartic acid) receptors, one of the ionotropic glutamate receptor (iGluRs) subclasses (3). Although, at present, six NMDA-receptor subunits have been cloned and characterized in regards to their primary structures, the number of functional NMDA receptors in the central nervous system is unknown (1, 2).

There have been attempts to develop novel agents that

block the activation of NMDA receptors by glutamate or related excitatory neurotransmitters, and a large number of competitive and non-competitive NMDA-receptor antagonists have been reported (1–3). The structures of typical antagonists to the receptors are shown in Fig. 1.

Most of the potent competitive NMDA-receptor antagonists are structural analogs of 2-amino-5-phosphonopentanoate (AP5) and 2-amino-7-phosphonoheptanoate (AP7) (4): (2*R*)-4-(3-phosphonoprop-1-yl)piperazine-2-carboxylic acid [(*R*)-CPP] (5) and LY235959 (6) are among the most potent competitive antagonists of NMDA receptors.

Dissociative anesthetics, such as phencyclidine (PCP) or ketamine, have been shown to reduce the excitatory action of NMDA by blocking the ion channels opened via the agonist-receptor interaction (7, 8). Extensive studies of this type of noncompetitive NMDA-receptor antagonist with its polycyclic amine structure have revealed that these noncompetitive antagonists are agonist-dependent; namely,

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Fig. 1. Structures of NMDA-receptor antagonists.

that the compounds bind a site within the ion channel of the receptor complex (9). One of the most potent and selective noncompetitive antagonists is MK-801 (10), the pharmacology of which has been studied in detail.

NMDA-receptor antagonists are expected to be therapeutic drugs for the treatment of epilepsy, stroke, Huntingtons disease, and/or Parkinsons disease are therefore urgently required. Although a number of studies clearly indicate that these competitive and noncompetitive antagonists are effective in experimental models of epilepsy and stroke (1–3), clinical studies of these NMDA-receptor antagonists have proven unsuccessful (1–3). Non-competitive inhibitors have produced serious behavioral effects (11, 12) and have caused neuronal vacuolization (13) in some cases, while competitive inhibitors are often inactive in vivo because of poor transport to the brain (14, 15). Since glycine acts as a co-transmitter at the NMDA receptor (1–3), glycine antagonists, which may be associated with fewer side effects, have also been considered. However, their preclinical studies suggest that brain penetration is usually insufficient. Consequently, antagonists with improved brain penetration are now being developed (16, 17). Considering these results, another type of efficient NMDA-receptor antagonist is eagerly desired.

We found (±)-(Z)-2-aminomethyl-1-phenyl-*N,N*-diethyl-

cyclopropanecarboxamide (milnacipran, **1**), an efficient antidepressant due to its competitive inhibition of the re-uptake of serotonin (5-HT) and noradrenaline in the CNS (18), to be a new class of non-competitive NMDA-receptor antagonists (19). As a lead for developing efficient NMDA-receptor antagonists, milnacipran has the advantage of penetrating into the brain sufficiently without serious side effects. Throughout clinical studies as an antidepressant (18), it demonstrated promising results. The structural features of milnacipran, which lacks an acidic functional group indispensable for competitive antagonists of the NMDA receptor, suggested that they functioned as antagonists to the receptor in a noncompetitive manner. Binding studies of milnacipran to the receptor showed that it binds to the receptor in an agonist-independent manner, which suggests that its binding site should be different from that of the known noncompetitive antagonists described above. Although the binding affinity of **1** for the NMDA receptor is not very strong, its characteristic pharmacological and structural features are an important consideration in the search for novel potent NMDA-receptor antagonists. Therefore, we undertook our medicinal chemical study with milnacipran as the lead. By increasing its specific affinity for the NMDA receptor and decreasing its inhibitory effect on the 5-HT/noradrenaline re-uptake, we succeeded in developing a new class of efficient NMDA-receptor antagonists (19–26).

Design and synthesis of conformationally restricted analogs of milnacipran

Although we first performed modifications of the functional groups of milnacipran (**1**), e.g., phenyl, diethylcarbamoyl and aminomethyl groups, the activity was not significantly improved, implying that these functional groups are important for the activity (19). Therefore, we next turned our attention to the effects of conformational changes of the molecule on the activity and designed conformationally restricted analogs of **1**, in which a new method for restricting the conformation of cyclopropane derivatives was developed (20).

It is known that as a result of free rotation about single bonds in compounds, a drug molecule can assume a variety of conformations. Only one of these conformers can bind to a receptor. If the lead compound has low binding affinity for the receptor, it may simply be that the conformation that binds to the receptor is not preferable. Therefore, the synthesis of conformationally restricted analogs of a lead compound can bring an improvement in the specific binding affinity for the receptor.

We designed the conformationally restricted analogs based on the structural feature of **1**, as shown in Fig. 2. Adjacent substituents on a cyclopropane ring mutually exert significant steric repulsion because they are fixed in

Fig. 2. Structures of the conformationally restricted analogs of milnacipran.

an eclipsed conformation. Consequently, conformations of the substituents on a cyclopropane can be restricted by the steric effect of adjacent substituents, especially when they are bulky. Because the primary amino group of **1** is essential for the binding affinity for the NMDA receptor (19), we assumed that the conformation of the aminomethyl moiety would have a considerable affect on the activity of the compound. While the aminomethyl moiety is not so bulky and may freely rotate at least to some extent, the conformers **A** and **B** would be preferable to conformer **C**, because of the serious steric repulsion for the bulky diethylcarbamoyl group in conformer **C**, as shown in Fig. 3. Introducing an alkyl group into the α -position of the amino group of **1** would prevent the rotation restricting the location of the amino function in space due to its steric repulsion with the diethylcarbamoyl group. Therefore, depending on the configuration of the alkyl group introduced, the conformation of the compounds can be restricted; conformer **B** would be predominant in **2** and *ent*-**2**, while conformer **A** would be predominant in **3** and *ent*-**3** (Fig. 3) (20).

Starting from (*R*)- or (*S*)-epichlorohydrin, four types of the conformationally restricted analogs with different stereochemistries, namely, **2a** and **2b** (Type-1), **3a** and **3b** (Type-2), *ent*-**2a** and *ent*-**2b** (Type-3), and *ent*-**3a** and *ent*-**3b** (Type-4), were stereoselectively synthesized (20, 21).

Analysis of the three-dimensional structure of the conformationally restricted analogs

X-ray crystallographic analysis clearly showed that the conformations of **2** (*ent*-**2**) and **3** (*ent*-**3**) were effectively restricted to conformers **B** and **A**, respectively, as we had hypothesized (20). The three-dimensional structure of **2b** was also analyzed by theoretical calculations to reveal that

Fig. 3. Conformational restriction of milnacipran based on the structural feature of cyclopropane ring.

conformer **B** is the most stable conformation. Furthermore, nuclear Overhauser effect (NOE) spectra-based structural analysis of **2b** suggested that conformer **B** is significantly predominant even in solution. Thus, our novel conformational restriction method based on the structural feature of the cyclopropane ring has been shown to be effective. In the design of conformationally restricted analogs, it is imperative that the conformationally restricted analogs and the lead drug molecule be as similar as possible in size, shape and molecular weight. Conformationally restricted analogs have typically been designed and synthesized by introducing cyclic moieties into lead compounds that were often rather bulky. Consequently, the chemical and physical properties could be changed. Therefore, our methodology for restricting the conformation of key functional groups due to the repulsion for the adjacent substituents by introducing only a small alkyl group, such as a methyl or ethyl group, would be useful. This method can be applied to conformational restriction of various cyclopropane derivatives.

Binding affinity of the conformationally restricted analogs for NMDA receptor

The conformationally restricted analogs with different stereochemistries, namely, Type-1, -2, -3 and -4, were evaluated in vitro for their binding affinity for the NMDA receptor of cerebral cortical synaptic membrane from rats, with [³H]MK-801 as the radioligand (22). The results, shown in Table 1, suggest that the binding affinity clearly depends on the stereochemistry of the compounds. Notably, conformationally restricted analogs with Type-1 configuration, namely, **2a** [(1*S*,2*R*)-1-phenyl-2-[(*S*)-1-amino-

Table 1. Effects of the compounds on NMDA receptor binding and 5-HT uptake

Compound	Stereochemistry	1'-Substituent	NMDA receptor binding ^a (IC ₅₀ , μ M)	5-HT uptake ^b (K _i , μ M)
($\pm$)- 1			6.3 $\pm$ 0.3	0.0085 $\pm$ 0.0006
2a (PEDC)	Type-1	Me	0.35 $\pm$ 0.08	0.014 $\pm$ 0.002
2b (PPDC)	Type-1	Et	0.20 $\pm$ 0.02	24 $\pm$ 0.9
3a	Type-2	Me	6.5 $\pm$ 0.5	2.4 $\pm$ 0.3
3b	Type-2	Et	8.2 $\pm$ 2.1	> 100
<i>ent</i> - 2a	Type-3	Me	19 $\pm$ 1.8	0.17 $\pm$.003
<i>ent</i> - 2b	Type-3	Et	8.2 $\pm$ 2.0	> 100
<i>ent</i> - 3a	Type-4	Me	31 $\pm$ 0.1	> 100
<i>ent</i> - 3b	Type-4	Et	11 $\pm$ 0.05	> 100
2c	Type-1	Pr	1.0 $\pm$ 0.05	37 $\pm$ 3
2d	Type-1	<i>i</i> -Bu	4.2 $\pm$ 0.2	> 100
2e	Type-1	Ph	17 $\pm$ 0.3	> 100
2f (PPEDC)	Type-1	CH = CH ₂	0.16 $\pm$ 0.02	0.023 $\pm$ 0.0007
2g (PPYDC)	Type-1	C $\equiv$ CH	0.29 $\pm$ 0.2	0.19 $\pm$ 0.2
2h	Type-1	C $\equiv$ N	96 $\pm$ 10	> 100
2i	Type-1	CO ₂ H	> 100	> 100
2j	Type-1	CO ₂ Me	> 100	25 $\pm$ 5
2k	Type-1	CONH ₂	27 $\pm$ 1.8	> 100

^a Assay was done with cerebral cortical synaptic membrane of rats using [³H]MK-801 (4 nM). ^b Assay was done with cerebral cortical synaptic membrane of rats using [³H]paroxetine.

ethyl]-*N,N*-diethylcyclopropanecarboxamide, PEDC] and **2b** [(1*S*,2*R*)-1-phenyl-2-[(*S*)-1-aminopropyl]-*N,N*-diethylcyclopropanecarboxamide, PPDC], significantly inhibited the binding of [³H]MK-801 with IC₅₀ values of 0.35 and 0.20 μ M, respectively, which were about 20- and 30-fold stronger than that of **1** (IC₅₀ = 6.3 μ M). The corresponding diastereomers with Type-2 configuration, **3a** and **3b**, had weaker activities than **2a** and **2b**, and their IC₅₀ values were similar to that of **1**. The conformationally restricted analogs with Type-3, and -4 configurations, *ent*-**2a**, *ent*-**2b**, *ent*-**3a** and *ent*-**3b**, showed weaker activity than ($\pm$)-**1**.

Based on these results, we synthesized conformationally restricted analogs with Type-1 configuration, **2c-k**, which have a variety of substituents at the 1'-position and evaluated their binding affinities for the receptor. The results are also summarized in Table 1. The binding affinity was significantly affected by the substituent at the 1'-position; introduction of groups bulkier than the ethyl group clearly decreased the affinity for the receptor (22). The analogs with a functional group, such as cyano, carboxyl, carboxymethyl, or carbamoyl group, at the 1'-position (**2h-k**) were almost inactive (23). The 1'-ethynyl and -ethenyl derivatives, **2f** [(1*S*,2*R*)-1-phenyl-2-[(*S*)-1-amino-2-propenyl]-*N,N*-diethylcyclopropanecarboxamide, PPEDC] and **2g** [(1*S*,2*R*)-1-phenyl-2-[(*S*)-1-amino-2-propynyl]-*N,N*-diethylcyclopropanecarboxamide, PPYDC] bound strongly to the receptor (22, 23).

Inhibitory effects of the conformationally restricted analogs on the uptake of 5-HT

We next evaluated the inhibitory effects of the compounds on the uptake of 5-HT by nerve terminals using the cerebral cortical synaptic membrane from rats with [³H]paroxetine as a radioligand (22, 23), since milnacipran (**1**) is a potent inhibitor of 5-HT uptake (18). Unfortunately, the 1'-methyl derivative **2a** (PEDC) and -ethenyl derivative **2f** (PPEDC), which showed strong binding to the NMDA receptor as described above, also significantly inhibited 5-HT uptake (K_i = 0.014 and 0.023 μ M, respectively), similar to **1**. Surprisingly, the corresponding 1'-ethyl derivative **2b** (PPDC), which also strongly bound the NMDA receptor, was almost inactive to 5-HT uptake (K_i = 24 $\pm$ 0.9 μ M). The effect of PPDC (**2b**) was about 1500 times less than that of PEDC (**2a**) and PPEDC (**2f**), although they have the same Type-1 stereochemistry. The enantiomer of PEDC with Type-2 configuration, *ent*-**2a**, which was only a weak NMDA-receptor antagonist, considerably inhibited the 5-HT uptake (K_i = 0.17 $\pm$ 0.003 μ M). All of the 1'-ethyl derivatives had insignificant inhibitory effects on the 5-HT uptake, regardless of their stereochemistry. On the other hand, all of the 1'-methyl derivatives, except *ent*-**3a**, clearly inhibited 5-HT uptake and the potency depended on their stereochemistries: **2a** (Type-1) > *ent*-**2a** (Type-3) > **3a** (Type-2) $\gg$ *ent*-**3a** (Type-4). PPDC also lacked the inhibitor effect on the re-uptake of noradrenaline. Consequently, it was suggested that the inhibitory potency in the 5-HT

/noradrenaline uptakes did not correlate with that in NMDA receptor activation in these compounds. It should be notable that the conformation of milnacipran binding to the NMDA receptor was different from that binding to the 5-HT and noradrenaline transporters.

Voltage-clamp study of the conformationally restricted analogs

We tested the blocking effect of the conformationally restricted analogs with the *Xenopus* oocytes system expressing rat NMDA receptors and cultured mouse neurons under voltage-clamp conditions (24). The *Xenopus* oocytes injected with rat total mRNA produced NMDA receptors in addition to other neurotransmitter receptors. As shown in Fig. 4, the blocking effects of the compounds on the channel were co-related with their binding affinities for the receptor. PPDC was the most effective blocker to the NMDA receptor channel with an IC_{50} of $1.69 \mu\text{M}$ and had no effect on other glutamate receptor classes. Furthermore, it showed no blocking effects on responses to different neurotransmitter receptors such as ACh_{MI} , GABA_{A} and $5\text{HT}_{2\text{C}}$. Studies on elementary currents of NMDA receptors on cultured mouse hippocampal neurons showed that PPDC decreased the mean open time of NMDA receptors without decreasing their elementary conductance. These results suggested that PPDC is an open channel blocker of NMDA receptors.

Based on these results, we performed further evaluations of PPDC as an NMDA-receptor antagonist described below.

Investigation of the PPDC binding site on the receptor

We next investigated the PPDC binding sites on the receptor. Single NMDA receptors have multiple binding sites that are targets for endogenous as well as exogenous compounds, five of which, i.e., glutamate, glycine, Zn^{2+} ,

polyamine and Mg^{2+} sites, are known.

PPDC blocked NMDA receptors without competing with NMDA and glycine and also blocked NMDA receptors with open ion channels in a voltage-dependent manner. Among the known binding sites on NMDA receptors, only the Mg^{2+} site might be possible as the binding site for PPDC, since the Mg^{2+} site is within the open channel of NMDA receptors, and the binding of Mg^{2+} to this site occludes the open channel. Under physiological conditions, due to the relatively high extracellular Mg^{2+} -concentration, NMDA receptors are blocked on resting membrane potentials. The depolarization induced by the activation of non-NMDA receptors reverses the electric field across the open channel and repels the occluding Mg^{2+} (27).

MK-801 is well known to bind to the Mg^{2+} site blocking NMDA receptors, and both PPDC and MK-801 blocked NMDA receptors with similar blocking rate constants of $2.8 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ at -100 mV (PPDC) (24) and $2.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ at -60 mV (MK-801) (28). However, the unblocking process of PPDC was different from that of MK-801. In voltage-gated K^+ and Na^+ channels, open channel blockers can be swept out by the flux of permeating ions flowing from the opposite side where open channel blockers are applied. Similarly the outward flux of permeating ions through NMDA receptor channels immediately reversed the MK-801 block, whereas when the MK-801 was applied to extracellular solutions, the inward flux did not. In contrast, the recovery from the PPDC block was virtually independent of the direction of the flux but depended on membrane potentials (K. Yoshii, unpublished results). It appears that no permeating ions involved in outward flux interact with PPDC. It is likely that the PPDC binding sites are different from the MK-801 binding sites and hence different from Mg^{2+} sites. The binding of PPDC to this site within NMDA channel pores may elicit conformational changes in the pore and close it. Alternatively,

Fig. 4. Assay was performed with *Xenopus* oocytes injected with rat total mRNA. Suppression curves of the conformationally restricted analogs for NMDA responses. Whole cell currents evoked by $30 \mu\text{M}$ NMDA supplemented with $10 \mu\text{M}$ glycine were recorded in the absence and presence of various concentrations of the compounds. The ratio of residual current was plotted as a function of the concentration of these compounds. PPDC (2b, ●), PEDC (2a, ○), PPEDC (2f, □), ent-2a (◇), ent-2b (◆), 3a (▽), 3b (▼), ent-3a (△), ent-3b (▲). Inset: the residual currents where horizontal bars show the application of the agonists (N) and $3 \mu\text{M}$ PPDC (B). Holding potential, -50 mV . In this and following figures, each point represents the mean $\pm$ S.D. obtained from five oocytes unless otherwise noted.

PPDC blocks NMDA receptors by binding to a site outside the ion channel pore as an allosteric open channel blocker. Although NMDA receptors are voltage-insensitive, membrane potential may change the affinity of the PPDC binding sites. In both cases, PPDC binding will prevent the binding of MK-801 to Mg^{2+} sites. PPDC thus is not only a neuroprotective drug but also a useful tool for investigating the properties of NMDA receptor channel pores.

NMDA receptor subtype selectivity of PPDC

NMDA receptors are composed of GluR ϵ (NR2)- and GluR ζ (NR1)-subunits. The GluR ϵ subunit contains the glutamate binding site, while the GluR ζ subunit bears the glycine binding site, and mammalian NMDA receptors have shown to be heterooligomeric combinations of GluR ζ subunits and at least one of four GluR ϵ subunits (GluR ϵ 1 – 4) (1, 2). Subtype-selective NMDA-receptor antagonists may retain therapeutic activity without the side effects observed in the previous antagonists (29).

PPDC was evaluated with CHO cell lines expressing four subtypes (GluR ϵ 1/GluR ζ 1, GluR ϵ 2/GluR ζ 1, GluR ϵ 3/GluR ζ 1, GluR ϵ 4/GluR ζ 1) of mouse NMDA receptors which indicated that it is an antagonist selective for the GluR ϵ 3/GluR ζ 1- and GluR ϵ 4/GluR ζ 1-subtypes (S. Uchino et al., unpublished results). These results demonstrate that PPDC is a new class of NMDA-receptor antagonist, since the previous antagonists are selective for GluR ϵ 1/GluR ζ 1, GluR ϵ 2/GluR ζ 1, and/or GluR ϵ 3/GluR ζ 1, or non-selective (S. Uchino et al., unpublished results; and refs. 30 and 31).

Other pharmacological effects of PPDC

The binding study of PPDC with rat cortical membrane showed that it binds to the receptor in an agonist-independent manner, similar to the parent compound milnacipran (R. Tsujita, unpublished results).

The neuroprotective effects of PPDC were evaluated both in vitro and in vivo. Thus, PPDC effectively protected against NMDA-induced neurotoxicity in cultured mouse cerebral cortex (24) and also the delayed neuronal death in the gerbil ischemic model when it was i.p.-administered prior to as well as after occlusion (R. Tsujita, unpublished results).

We also evaluated PPDC as an anti-Parkinsonian agent with a reserpine-treated mouse model. I.p.-treatment of PPDC clearly improved the locomotor activity of mice and its co-administration with L-DOPA significantly enhanced the activity (R. Tsujita, unpublished results).

Conclusion

It has been shown that PPDC selectively binds the GluR ϵ 3/GluR ζ 1- and GluR ϵ 4/GluR ζ 1-subtype receptors in an agonist-independent allosteric manner. These find-

ings demonstrate that PPDC is a new class of NMDA-receptor antagonist. PPDC functioned as a potent NMDA-receptor antagonist in several in vitro and in vivo models. Therefore, PPDC, due to a mode of action different from that of other antagonists, may be a candidate for clinically useful drugs, while development of previous NMDA-receptor antagonists as drugs has been hindered by various dose-limiting side-effects, e.g., neurotoxicity, psychotic reactions and sedation.

Further pharmacological and structure-activity relationship studies on PPDC are now in progress.

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