

Title

ONO-8590580, a novel GABA_A α 5 negative allosteric modulator enhances long-term potentiation and improves cognitive deficits in preclinical models.

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Running title

Effect of a novel GABA_A α 5 NAM in preclinical models

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Number of text pages: 32

Number of Tables: 0

Number of Figures: 8

Number of References: 58

Number of words in the Abstract: 246

Number of words in the Introduction: 591

Number of words in the Discussion: 1164

Abbreviations

JPET #247627

GABA, γ (gamma)-aminobutyric acid; NMDA, N-methyl-D-aspartic acid; NAM, negative allosteric modulator; fEPSP, field excitatory postsynaptic potential, LTP, long-term potentiation

Section assignment

Drug Discovery and Translational Medicine

Abstract

GABA_A receptors containing $\alpha 5$ subunits (GABA_A $\alpha 5$) are highly expressed in the hippocampus and negatively involved in memory processing, as shown by the fact that GABA_A $\alpha 5$ deficient mice show higher hippocampus-dependent performance than wild type mice. Accordingly, small molecule GABA_A $\alpha 5$ negative allosteric modulators (NAMs) are known to enhance spatial learning and memory in rodents. Here we introduce a new, orally-available GABA_A $\alpha 5$ NAM that improves hippocampal functions. ONO-8590580 binds to the benzodiazepine binding sites on recombinant human $\alpha 5$ -containing GABA_A receptors with a K_i of 7.9 nM, and showed functionally selective GABA_A $\alpha 5$ NAM activity for GABA-induced Cl⁻ channel activity with a maximum 44.4% inhibition and an EC₅₀ of 1.1 nM. In rat hippocampal slices, tetanus-induced long-term potentiation of CA1 synapse response was significantly augmented in the presence of 300 nM ONO-8590580. Orally administered ONO-8590580 (1-20 mg/kg) dose-dependently occupied hippocampal GABA_A $\alpha 5$ in a range of 40-90% at 1 h after intake. In the rat passive avoidance test, ONO-8590580 (3-20 mg/kg, p.o.) significantly prevented MK-801-induced memory deficit. In addition, ONO-8590580 (20 mg/kg, p.o.) was also effective in improving cognitive deficit induced by scopolamine and MK-801 in the rat 8-arm radial maze test with equal or greater activity than 0.5 mg/kg donepezil. No anxiogenic-like or proconvulsant effect was associated with ONO-8590580 at 20 mg/kg, p.o. in the elevated plus maze test or pentylenetetrazole-induced seizure test, respectively. In sum, ONO-8590580 is a novel GABA_A $\alpha 5$ NAM that enhances hippocampal memory function without an anxiogenic or proconvulsant risk.

Introduction

Because of the advent of this rapidly aging society, the number of adults with senile dementias, such as Alzheimer's disease (AD) has increased (Akagi et al., 2015). Currently approved pharmacological treatments for AD are limited to cholinesterase inhibitors as well as the N-methyl d-aspartate (NMDA) receptor antagonist, which acts on the glutamatergic pathway (Farlow et al., 2008). Limited efficacy of these drugs highlights the need for better treatment. Despite the tremendous efforts in search of disease modifying agents focusing on the β -amyloid or tau pathways, none are clinically available. Therefore, a strong cognitive enhancer with a novel mode of action is still globally desirable.

γ -Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the adult brain. In AD patients as well as transgenic AD mouse models, altered GABAergic function has been reported. Levels of GABA in cerebrospinal fluid (Samakashvili et al., 2011) and reactive astrocytes (Jo et al., 2014) are elevated in AD patients. GABA_A α 5 chloride channel, highly expressed in the hippocampus which is involved in learning and memory (Quirk et al., 1996), is upregulated in hippocampus in AD patient (Kwakowsky et al., 2018) and 5xFAD mouse, a transgenic model for AD (Wu et al., 2014). GABA_A α 5 deficient mice show improved performance in water maze test for studying spatial learning compared with wild type mice (Collinson et al., 2002). Thus, the over activation of the GABA_A α 5-mediated signaling pathway should contribute to the inhibition of neuronal activity and impairment of learning and memory in AD patients. Therefore, agents with GABA_A α 5 inhibitory activity are expected to improve cognitive disorders like AD.

Studies in molecular genetic (mice with a point mutation in the GABA_A α subunit) or pharmacological approaches suggest that GABA_A α 1 mediates the sedative effects of diazepam, whereas GABA_A α 2 and α 3 accounts for anxiolytic and myorelaxant effects, respectively (Rudolph et al., 1999; McKernan et al., 2000; Rudolph and Möhler, 2004; Atack et al., 2005). The contribution of

each subtype for the physiological function has been elucidated by the genetically modified mice functionally lacking diazepam binding site of the $\alpha 1$, $\alpha 2$, $\alpha 3$, or $\alpha 5$ subtype. These results suggest that $\alpha 1$ is responsible for the proconvulsant effect (Vergnes et al., 2001) and $\alpha 2/3$ for the anxiogenic effect (Horowski and Dorrow, 2002) of non-selective GABA_A NAMs. Whereas GABA_A positive allosteric modulators (PAMs) binding to the benzodiazepine (BZ) site such as diazepam increase the GABA response through GABA_A $\alpha 1$, $\alpha 2$, $\alpha 3$, or $\alpha 5$ ($\alpha 4$ and $\alpha 6$ are diazepam insensitive), non-selective negative allosteric modulators (NAMs) such as DMCM and FG-7142 decrease GABA response, resulting in a membrane depolarization and increased neuronal excitability (Haefely et al., 1993). Therefore, the opposing effects of PAMs and NAMs at the molecular level are reflected behaviorally in that NAMs are anxiogenic and proconvulsant effects (Haefely et al., 1993). The anxiogenic and proconvulsant liabilities of the non-selective NAMs prevent clinical application (Dorow et al., 1983). It was therefore hypothesized that a GABA_A $\alpha 5$ selective NAM should improve cognitive impairment without anxiogenic and proconvulsant effects.

The BZ binding site, an allosteric site on the GABA_A receptor, has the potential to obtain GABA_A subtype selective PAMs and NAMs (Atack, 2011). Approximately 124,000 compounds (Charles River) were screened in both receptor binding and FLIPR functional assays in human GABA_A $\alpha 5\beta 3\gamma 2$ expressing HEK293 cell line. As a result, a novel structural class of BZ site ligand with potential to selectively modulate GABA_A $\alpha 5$ function was identified. In this study, the efficacy of ONO-8590580, a GABA_A $\alpha 5$ NAM with a novel chemotype which was obtained following lead optimization from hit compounds, was evaluated.

Materials and Methods

Compounds

ONO-8590580, 1-(cyclopropylmethyl)-5-fluoro-4-methyl-N-[5-(1-methyl-1H-imidazol-4-yl)-2-pyridinyl]-1H-benzimidazol-6-amine (Fig. 1, purity: $\geq 95\%$), was synthesized in ONO pharmaceutical Co., Ltd. Donepezil, (-)-scopolamine hydrobromide trihydrate (Scopolamine), (+)-MK-801 hydrogen maleate (MK-801), FG-7142, pentylenetetrazole (PTZ), and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich. L-655,708, a selective GABA_A $\alpha 5$ ligand (Quirk et al., 1996), was purchased from Santa Cruz Biotechnology, Inc. ONO-8590580 and L-655,708 used for *in vivo* studies was first dissolved in DMSO and then added to WellSolve (Celeste) and water (final composition ratio of the solution; 2% DMSO, 20% WellSolve, 78% water). Scopolamine, MK-801 and PTZ was dissolved in saline. Donepezil was dissolved in water. FG-7142 was suspended in 0.1 vol% polyoxyethylene (20) sorbitan monooleate (Tween 80, Wako) in saline.

Animals

All experimental procedures were approved by the institutional animal care and use committee of ONO pharmaceutical Co., Ltd. All efforts were made to minimise the number of animals used and their suffering. Male Sprague-Dawley rats (CrI:CD(SD), Charles River Laboratories Japan, Inc.; 6 weeks old) were used in the Long Term Potentiation (LTP) and *in vivo* receptor occupancy studies. Male Sprague-Dawley rats (CrI:CD(SD), Charles River Laboratories Japan, Inc.; 7 weeks old) were used in the passive avoidance test and elevated plus maze test. Male Wistar rats (CrIj:WI, Charles River Laboratories Japan, Inc.; 7-9 weeks old) were used for the 8-arm radial maze test. Male ICR mice (CrIj:CD1(ICR), Charles River Laboratories Japan, Inc.; 7-9 weeks old) was used for the PTZ proconvulsant test. Animals were housed in groups of less than 5 animals/cage in a temperature- and

humidity-controlled animal room (temp: $24 \pm 2^\circ\text{C}$, relative humidity: $55 \pm 15\%$) under a 12-h light/dark cycles (light on from 8:00 to 20:00). Food and water were available *ad libitum*.

Cell cultures

Human GABA_A $\alpha 1\beta 3\gamma 2$ expressing HEK293 cell line (CYL3053; Millipore), human GABA_A $\alpha 2\beta 3\gamma 2$ expressing HEK293 cell line (CYL3072; Millipore), human GABA_A $\alpha 3\beta 3\gamma 2$ expressing HEK293 cell line (CYL3068; Millipore), and human GABA_A $\alpha 5\beta 3\gamma 2$ expressing HEK293 cell line (CYL3073; Millipore) were cultured at 37°C (5% CO₂, 95% air) until confluence in D-MEM/F-12 with L-glutamine (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), 1% non-essential amino acids (Invitrogen), 400 $\mu\text{g}/\text{mL}$ Geneticin (Invitrogen), 100 $\mu\text{g}/\text{mL}$ Hygromycin B (Invitrogen), and 0.625 $\mu\text{g}/\text{mL}$ Puromycin (Clontech).

In vitro radioligand binding studies

The GABA_A $\alpha 1\beta 3\gamma 2$, $\alpha 2\beta 3\gamma 2$, $\alpha 3\beta 3\gamma 2$ and $\alpha 5\beta 3\gamma 2$ protein used for the Scintillation Proximity Assay was derived from membranes produced from the cell line expressing each complex. Membranes were prepared as previously published (Hadingham et al., 1993). Briefly, following routine culture in T175 flasks (CellBIND surface, Corning), cells were washed with Dulbecco's phosphate buffered saline (PBS, Invitrogen), scraped from the flask into PBS and pelleted by centrifugation (500g). The cells were resuspended in 10 mM potassium phosphate at pH 7.4 and homogenized (5,000 rpm, 15 s) using a Precellys tissue homogenizer (Precellys[®] 24-Dual, Bertin Technologies). The homogenates were centrifuged at 48,000g for 30 min, resuspended and washed twice. Final resuspension of membranes took place in buffer containing 10 mM potassium phosphate and 100 mM KCl. The protein concentration in membrane preparations was confirmed using a bicinchoninic acid (BCA) protein assay kit (Pierce).

ONO-8590580 was diluted in 100% DMSO. For SPA, 0.5 μ L of ONO-8590580 per well was added to a white-walled clear-bottomed 384-well plate (Matrix). The assay controls used to calculate % inhibition consisted of DMSO and 100 μ M flumazenil (final conc. 2 μ M), both 0.5 μ L per well, for full signal and full block respectively. Protein (10 μ L) was added to the plate at a concentration of 2.5 μ g/well followed by 9.5 μ L of PVT-WGA beads at a concentration of 0.2 mg/well. Both protein and bead were diluted to the desired concentration with 10 mM potassium phosphate, pH 7.4, containing 100 mM KCl. The reagents were pre-incubated for 30 min at room temperature with shaking before initiation of the reaction by the addition of radioligand. The ability of ONO-8590580 to inhibit the binding of either 30 nM [3 H]-Ro15-1788 (Perkin Elmer LAS) to GABA_A α 1, α 2, or α 3 or 2 nM [3 H]-Ro15-4513 (Perkin Elmer LAS) to GABA_A α 5 was measured. From the EC₅₀, the K_i was calculated from the Cheng-Prusoff equation (Cheng and Prusoff, 1973), using K_d for the binding of [3 H]-Ro15-1788 to GABA_A α 1, α 2, and α 3 of 13, 15, and 15 nM, respectively, and for the binding of [3 H]-Ro15-4513 to GABA_A α 5 of 2.0 nM. [3 H]-Ro15-1788 5 μ L was added at a concentration of 30 nM (final conc. 6 nM), giving a final reaction volume of 25 μ L and DMSO concentration of 2% v/v. The plates were sealed and the reaction mix incubated at room temperature for a minimum of 2 h with gentle agitation on a plate shaker. At the end of the incubation period the plates were centrifuged for 2 min at 1,000 rpm prior to reading on a Perkin Elmer Microbeta. For each compound, the EC₅₀ and K_i was determined, the data was entered in Activity Base (IDBS) and the curves were fitted to the mean data. Z' factors in the binding assays for GABA_A α 1, α 2, α 3, and α 5 were 0.53, 0.53, 0.50, and 0.54, respectively.

In vitro efficacy

Current recordings were performed using PatchXpress (Molecular Devices, LLC.) with HEK293 cell lines expressing human GABA_A α 1, α 2, α 3, or α 5 subunit associated with β 3 γ 2 subunits. Cells were

dissociated from poly-D-lysine treated tissue culture flasks using a 1:1 mixture of TrypLE (Invitrogen) and Dulbecco's phosphate buffered saline (Invitrogen). The dissociated cells were resuspended in D-MEM/F-12 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% non-essential amino acids (Invitrogen), and allowed to recover for 1 h at 37°C in a humidified incubator with 5% CO₂. At the end of the recovery period, cells were centrifuged at 1,000 rpm for 2 min, and the pellet was resuspended and diluted to 2.5 x 10⁶ cells/mL in an solution (137 mM NaCl, 4 mM KCl, 10 mM HEPES, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, pH 7.35). Current responses were recorded in the presence of concentration-matched DMSO (0.3% v/v). Patch pipettes were filled with the solution comprised 90 mM KCl, 50 mM KF, 11 mM EGTA, 10 mM HEPES, 1 mM MgCl₂, and 2 mM Mg-ATP (pH 7.35). The holding potential was -60 mV. Control GABA responses were first determined at its EC₂₀ concentration, and test compound was pre-applied for 1 min prior to addition of the EC₂₀ concentration of GABA (GABA_A α1; 2.4 μM, α2; 1.5 μM, α3; 2.3 μM, α5; 0.34 μM.). The test compound and/or GABA were applied for 5 s, with 2 min wash periods between applications. To calculate EC₅₀ for ONO-8590580, GraphPad Prism software (version 5.01; GraphPad Software Inc.) was used .

Long term Potentiation (LTP) in rat brain slice

LTP was measured electrophysiologically using a multi-electrode array system (MED64, Alpha MED Scientific Inc.), as described previously (Tsukamoto et al., 2003). Brain slices were prepared from rats in ice-cold artificial CSF, consisting of 124 mM NaCl, 3 mM KCl, 26 mM NaHCO₃, 2 mM CaCl₂, 1 mM MgSO₄, 1.25 mM KH₂PO₄ and 10 mM glucose, pH 7.4. Parasagittal 350 μm-thick brain slices were cut on a Vibratome sectioning system, and the hippocampal regions were placed at the center of chamber with 64 embedded recording electrodes (MED64, Alpha MED Scientific Inc.) and perfused with 95% O₂/5% CO₂-saturated artificial CSF for at least 1 h at 32°C. One of 64 planar

microelectrodes was used to stimulate the Schaffer collaterals every 30 s. Field excitatory postsynaptic potentials (fEPSPs) were evoked by the stimulation of Schaffer collateral-commissural pathway with a stimulus intensity set to produce a half-maximal slope of fEPSP. Synaptic strength was evaluated by measuring changes in the fEPSP slopes. To induce LTP, a θ -burst protocol (4 pulses delivered with a frequency of 100 Hz, repeated 10 times with an interval of 200 ms) was used.

In vivo receptor occupancy

The occupancy of the BZ binding site of rat brain GABA_A α 5 was measured using an *in vivo* [³H]-Ro15-4513 binding assay (n=3-4 in each group). ONO-8590580 was orally administered 1 h before sacrifice. [³H]-Ro15-4513 (1.11 MBq/kg) was administered via the tail vein (1 mL/kg) 10 min before sacrifice. Rats were decapitated, and the brains were rapidly removed and homogenized in ice-cold assay buffer (10 mM KH₂PO₄, 10 mM K₂HPO₄, 100 mM KCl). Aliquots of homogenate were filtered and washed over GF/B glass fiber filters (Brandel Inc). To determine the amount of non-specific [³H]-Ro15-4513 binding, a separate group of rats were pretreated for 30 min with L-655,708 (10 mg/kg, i.p.). Signal-to-background ratio was 26. The amount by which ONO-8590580 reduced the specific binding of [³H]-Ro15-4513 relative to the binding in non-treated rats was defined as the occupancy.

Passive avoidance test

The test was performed as described previously (Mitsui et al., 2015). The step-through type passive avoidance apparatus (BrainScience-idea. Co., Ltd.) consisted of an illuminated compartment (height 28 cm, length 25 cm, width 11 cm, 700-750 lx) connected to a darkened one (height 30 cm, length 32 cm, width 33 cm, 0.03-0.05 lux) by a guillotine door. The experiment consisted of two trials; an acquisition trial and a retention trial (n=10-15 in each group). In the acquisition trial, the rat was placed in the illuminated compartment 30 min after administration of MK-801 (0.1 mg/kg, i.p.) or saline. The guillotine door was then opened, and the rat was allowed to enter the dark compartment. The latency to

enter the dark compartment was recorded (step-through latency). Once the rat entered the dark compartment, the door was closed and an electric foot shock was immediately applied (1 s, 2.2 mA). On the following day, the rat was placed in the illuminated compartment and again allowed to enter the dark compartment by opening the guillotine door. The step-through latency was once again recorded (retention trial). The maximum cut-off time for step-through latency was set at 300 s. During the retention trial, no foot shock was applied. Impairment of learning and memory was defined by decrease in the time of step-through latency in the retention trial. ONO-8590580 or vehicle was administered 1 h before both an acquisition trial and a retention trial.

8-arm radial maze test

The test was performed as described previously (Ohta et al., 1993). Briefly, rats (n=10 in each group) were fed at a rate of approximately 13 g/day/rat (approx. 80% of normal daily feed intake). A daily training trial was carried out over 10 times in order to allow the rats to learn how to perform the radial maze test. The trial was judged complete when the rat had chosen all 8 baited arms or had spent 10 min on the maze. Entry into an arm that had not been previously visited was recorded as a correct response, and re-entry was counted as an error. The last training trial and the test trial to evaluate compounds were carried out in the same day. When a rat made no errors or only one error after the seventh choice in the last training trial, it was considered ready for compound testing. In the test trial, MK-801 and/or scopolamine were subcutaneously administered 0.5 h before the test trial. ONO-8590580, donepezil or vehicle was orally administered 1 h before the trial. A rat was placed on the central hub of the maze and allowed to visit the wells at the end of each of 8 arms. The number of errors (the number of repeat entries to arms of the maze already visited) and the latency until the bait of all 8 wells had been consumed were counted.

Elevated plus maze

Rats (n=12 in each group) were given either vehicle, ONO-8590580 (20 mg/kg, p.o.), or as a positive control, the GABA_A nonselective NAM FG-7142 (15 mg/kg, i.p.). After 1 h, rats were placed in the elevated plus maze for 5 min. Light intensity in the open arms was set at 20 lx. A video camera fitted with a polarizing lens was mounted above the maze, connected to a tracking and analyse system (EthoVision XT, Noldus Information Technology). The open and closed arms (each 10 × 50 cm) and the central area (10 × 10 cm) of plus maze were defined using the tracking system. The effect of ONO-8590580 on time spent on the open arms was assessed.

PTZ proconvulsant test

Mice (n=8 in each group) were intraperitoneally administered with either vehicle, ONO-8590580 (10 mg/kg), or FG-7142 (10 mg/kg). After 30 min, the mice were infused with 15 mg/mL PTZ solution (infusion rate 0.2 mL/min), and the time taken to reveal clonic seizures was measured, and from this the dose administrated was calculated.

Statistical analysis

Except where noted, data are expressed as mean ± standard error of the mean. Statistical analyses were performed using SAS 9.2 TS2M3 (SAS Institute Japan Inc.) and its cooperative system EXSUS Ver.7.7.1 (CAC Corporation) with Student *t* test, Wilcoxon rank-sum test, Dunnett test, or Steel test as appropriate. Tests were 2-sided and conducted at a 5% level of significance.

Results

In vitro binding of ONO-8590580 to GABA_A receptors

Inhibition of [³H]-Ro15-4513 binding showed that ONO-8590580 binds with high affinity to GABA_A α5 (K_i = 7.9 nM; Fig. 2). Inhibition of [³H]-Ro15-1788 binding showed that ONO-8590580 also binds to GABA_A α1, α2 and α3 with K_i of 140, 32 and 24 nM, respectively (Fig. 2).

Efficacy of ONO-8590580 on each subtype of GABA_A receptors

ONO-8590580 concentration-dependently inhibited the current induced by the EC₂₀ concentration of GABA in human GABA_A α5β3γ2 expressing HEK293 cell line (Fig. 3). The calculated maximum inhibition was -44.4%, the EC₅₀ and Hill slope were -1.1 nM and -0.50, respectively. ONO-8590580 was not or only slightly effective at the GABA_A α1, α2, and α3 (Fig. 3).

Effect on LTP in rat hippocampal slice

LTP was induced using a standard paradigm that involves a θ-burst stimulus (Fig. 4). In control experiments, the fEPSP slopes were enhanced after the θ-burst compared to the baseline, then returned to baseline 1 h after stimulation. In the presence of ONO-8590580 (300 nM) the slopes after the θ-burst were also enhanced compared to the baseline, but, did not return to baseline 1 h after stimulation. Therefore, ONO-8590580 significantly induced LTP after a θ-burst in the CA1.

In vivo receptor occupancy in rat hippocampus

ONO-8590580 competed the binding of [³H]-Ro15-4513, a GABA_A α5 specific binder, in the rat hippocampus after oral administration (Fig. 5). The ratio of GABA_A α5 occupancy produced by ONO-8590580 at 1, 3, 10, and 20 mg/kg was 44, 53, 71 and 89%, respectively. ED₅₀ of ONO-8590580 with 95% confidence intervals was 1.9 (1.0 2- 3.72) mg/kg.

Effect on MK-801-induced cognitive deficit in passive avoidance test

MK-801 (NMDA antagonist)-treated rodents have been validated as an animal model of cognitive dysfunction associated with dementia (van der Staay et al., 2011). It is reported that negative modulation of GABA_A $\alpha 5$ in rats partially prevents memory impairment induced by MK-801 (Timić Stamenić et al., 2015). Therefore, we evaluated the effect of ONO-8590580 on MK-801-induced cognitive deficit in passive avoidance test. The latencies in the acquisition trial did not significantly differ regardless of the treatment administered 1 h before the trial (data not shown). ONO-8590580 (3, 10, and 20 mg/kg) significantly increased the escape latency compared to control in the retention trial (Fig. 6).

Effect on MK-801/Scopolamine-induced cognitive deficit in 8-arm radial maze test

AD patients have both cholinergic neuron degeneration (Everitt and Robbins, 1997; Schliebs and Arendt, 2006) and hypofunction of glutamatergic neurotransmission (Hardy et al., 1987; Lin et al., 2014) in the brain. Therefore, the cognitive deficit in rats induced by the combination of scopolamine, a muscarinic antagonist, and MK-801 would be useful as a model of Alzheimer's dementia. We investigated the effect of ONO-8590580 on the MK-801/scopolamine-induced cognitive deficit in the 8-arm radial maze test, as described by Li et al. (1996). Doses of MK-801 and scopolamine for this test were 0.075 mg/kg (i.p.) and 0.2 mg/kg (i.p.) respectively, because we confirmed that each single administration at these doses increased the number of errors respectively, and that in combination there was a stronger effect than each single administration (data not shown). As a positive control and for comparison purposes, 0.5 mg/kg of donepezil (p.o.), which decreases both number of errors and running time in the scopolamine-induced cognitive deficit in 8-arm radial maze test (Sugimoto et al., 2002), was also evaluated. ONO-8590580 (20 mg/kg, p.o.) significantly decreased the number of errors and total latency compared to the control (Fig. 7). Donepezil (0.5 mg/kg, p.o.) also decreased total latency, but not the number of errors compared to the control.

Effect in elevated plus maze test and PTZ test

In the rat elevated plus maze test, the non-selective GABA_A NAM FG-7142 significantly decreased the time spent on the open arms, whereas ONO-8590580 had no effect compared to vehicle-treated animals (Fig. 8A).

In the mouse PTZ test, FG-7142 decreased the dose of PTZ required to induce clonic seizure, whereas ONO-8590580 had no effect on the threshold for PTZ-induced clonic seizure (Fig. 8B).

Discussion

The present study demonstrated that ONO-8590580, a functionally selective GABA_A α 5 NAM, significantly enhanced LTP in rat hippocampal slice and improved cognitive deficits in rats without anxiogenic-like or proconvulsant effects.

ONO-8590580 has only 3.3 to 17.7 times higher binding selectivity for GABA_A α 5 compared to GABA_A α 1, α 2 and α 3, but it shows very high functional selectivity for GABA_A α 5. This *in vitro* profile is similar to α 5IA (Dawson et al., 2006) and MRK-016 (Atack et al., 2009), but differs from RO4938581 that has both binding selectivity and function selectivity for GABA_A α 5 (Ballard et al., 2009). It is not known if compounds display different phenotypes *in vivo* depending on the presence or absence of binding selectivity to GABA_A α 5. It is reported that there are endogenous ligands to the BZ binding site, endozepines (Farzampour et al., 2015). This finding indicates that different phenotypes may be shown by each compound with and without binding selectivity. Among 82 off targets, ONO-8590580 bound only to the adenosine A3 receptor with an inhibition of 68% at a drug concentration 10 μ M, thus ONO-8590580 is highly selective for GABA_A α 5 (Eurofins Panlabs Inc., Taipei, data not shown).

The physiological properties on hippocampal-related cognitive process may involve long-term changes in synaptic efficacy like LTP (Bliss and Collingridge, 1993). Non-selective GABA_A NAMs increase LTP (Seabrook et al., 1997), whereas non-selective GABA_A PAMs impair LTP (del Cerro et al., 1992). In this study, the selective GABA_A α 5 NAM enhanced LTP after a θ -burst in rat hippocampal slice. This result is consistent with other reports showing that other GABA_A α 5 NAMs enhance LTP (Dawson et al., 2006; Atack et al., 2009; Ballard et al., 2009). Etomidate which has memory-blocking properties inhibited LTP, and this effect was reversed by L-655,708 (Martin et al., 2009). These findings indicate that GABA_A α 5 holds an important role for the enhanced LTP observed

with non-selective GABA_A NAMs. GABA_A $\alpha 5$ is predominantly localized to extrasynaptic space in the hippocampus (Farrant et al., 2005; Brickley and Mody, 2012). It has been reported that tonic inhibition mediated by GABA_A $\alpha 5$ affects the induction and maintenance of LTP (Ge et al., 2008; Martin et al., 2010; Li et al., 2012). Since ONO-8590580 inhibits the function of GABA_A $\alpha 5$, tonic inhibition may be suppressed and LTP may be consequently enhanced.

ONO-8590580 significantly improved MK-801-induced cognitive deficit in the rat passive avoidance test. MK-801 impairs contextual specificity of hippocampal immediate-early gene expression which is critical for maintenance of synaptic plasticity and memory consolidation (Kubik et al., 2014). GABA_A $\alpha 5$ is expressed in the bases of the spines and the adjacent shafts of the dendrites (Fritschy and Brünig, 2003), and modulates the excitatory input arising at the spines via NMDA receptor (Glykys et al., 2008; Brickley and Mody, 2012). Therefore, it is suggested that ONO-8590580 improved MK-801-induced cognitive impairment indirectly via enhancing the excitatory input in hippocampal CA1 spines.

In AD patients the presence of not only cholinergic neuron degeneration in the nasal forebrain but also hypofunction of NMDA receptor-mediated neurotransmission in cortical and hippocampus regions in postmortem and imaging studies of the brains (Hardy et al., 1987; Bi and Sze, 2002; Lin et al., 2014). Blockage of NMDA receptor-mediated neurotransmission has been reported to prevent induction of LTP in the hippocampus (Bashir et al., 1991; Seabrook et al., 1997). MK-801 is known to induce memory deficit in animals (Suryavanshi et al., 2014). In addition, the decrease in NMDA receptors in the hippocampus and entorhinal cortex of AD patients has been reported to correlate with the disease neuropathological progression as assessed by post-mortem examination (Kravitz et al., 2013). Thus, we used a rat model with cognitive deficit induced by co-treatment model of muscarinic receptor antagonist scopolamine (0.2 mg/kg) and the NMDA receptor antagonist MK-801 (0.075 mg/kg), as a

model reflecting a part of Alzheimer's dementia in the 8-arm radial maze test. The effective dose of donepezil on the total latency was 0.5 mg/kg in this model, but it did not improve number of errors. Donepezil at this dose significantly improves cognitive deficit induced by scopolamine in 8-arm radial maze test (Sugimoto et al., 2002) and it also improves cognitive deficit induced by lesions of the medial septum in the water maze test more effectively at the dose of 0.5 mg/kg than 2 mg/kg (Ogura et al., 2000). Therefore 0.5 mg/kg of donepezil is an appropriate dose to investigate the efficacy of donepezil. The present study in which ONO-8590580 but not donepezil significantly decreased the number of errors may suggest that ONO-8590580 could be more potent for the treatment of AD patients. The data showing that ONO-8590580 has not anxiogenic-like or proconvulsant effects are in agreement with the behavioral phenotype of $\alpha 5^{-/-}$ mice (Collinson et al., 2002).

The receptor occupancy was 44, 53, 71 and 89% at 1, 3, 10 and 20 mg/kg (p.o.), respectively. The effective doses of ONO-8590580 were 3 to 20 mg/kg (p.o.) in passive avoidance test and 20 mg/kg (p.o.) in 8- arm radial maze test. Anxiogenic-like and proconvulsant effects were not observed at 20 mg/kg (p.o.) and 15 mg/kg (i.p.), respectively. From these results, the target level of occupancy for clinical studies might be 53 to 89%.

In the brain of AD patients, reactive astrocytes increase (Jo et al., 2014) and phenotypically switch from GABA-negative to GABA-producing (Oh and Lee, 2017). Released GABA from reactive astrocytes to extracellular space activates the extrasynaptic GABA_A receptor, GABA_A $\alpha 5$, and induces tonic inhibition (Kim et al., 2017; Farrant and Nuzzer, 2005). Tonic inhibition may suppress the activity of excitatory neurons in hippocampus, decrease LTP, and impair learning and memory (Ge et al., 2008; Martin et al., 2010; Li et al., 2012). In accordance with previous findings (Dawson et al., 2006; Atack et al., 2009; Ballard et al., 2009), this study confirmed a GABA_A $\alpha 5$ NAM increases LTP and improves learning and memory. In addition, GABA_A $\alpha 5$ expression level in hippocampus

increases in AD patients (Kwakowsky et al., 2018). These results suggest that astrocytic GABA and extrasynaptic GABA_A $\alpha 5$ are important players in the pathogenesis of AD, and ONO-8590580, a GABA_A $\alpha 5$ NAM, is an attractive agent to treat AD. Since reactive astrocytes have also been described in patients with Parkinson's disease, stroke, epilepsy, brain trauma and other neurodegenerative diseases (Luchetti et al., 2011; Brichta et al., 2013), ONO-8590580 could also be considered for the treatment of such neurodegenerative diseases as well as AD. Future work is needed to explore these exciting possibilities.

Development of another chemotype of GABA_A $\alpha 5$ NAM, $\alpha 5IA$, was discontinued because of preclinical renal toxicity (Atack, 2010), and MRK-016 was poorly tolerated in the elderly volunteers (Atack, 2011). Clinical trial of RG1662 was conducted in subjects with Down syndrome (Costa and Scott-McKean, 2013), but there is no information in AD patients.

We have shown in this study that ONO-8590580, a novel GABA_A $\alpha 5$ NAM, will improve cognitive impairment without anxiogenic or proconvulsant side effects. These findings indicate that ONO-8590580 could be beneficial for the pre-clinically evaluating cognitive disorders like AD.

Authorship Contributions

Participated in research design: Kawaharada, Yasuhiro, Clark, Maidment, Katsumata, and Kaneko.

Conducted experiments: Kawaharada, M Nakanishi, N Nakanishi, Hazama, Clark, and Maidment.

Contributed new reagents or analytic tools: Higashino, Lewis, and Chambers.

Performed data analysis: Kawaharada, M Nakanishi, N Nakanishi, Hazama, Clark, and Maidment.

Wrote or contributed to the writing of the manuscript: Kawaharada, Katsumata, and Kaneko

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Footnotes

This research was funded by ONO Pharmaceutical Co., Ltd (Osaka, Japan).

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Legends for Figures

Figure 1. Chemical structure of ONO-8590580.

Figure 2. *In vitro* binding affinity of ONO-8590580 at human recombinant GABA_A receptors. HEK-293 cell lines stably expressing human GABA_A α 1, α 2, α 3, and α 5 β 3 γ 2 were used in a scintillation proximity assay (SPA). Affinity at GABA_A α 1, α 2, and α 3 was measured using a [³H]-Ro15-1788 binding assay, whereas affinity at GABA_A α 5 was measured using [³H]-Ro15-4513.

Figure 3. Efficacy of ONO-8590580 on human recombinant GABA_A receptors. HEK-293 cell lines stably expressing human GABA_A α 1, α 2, α 3, (n=3) and α 5 β 3 γ 2 (n=5) were used in a patch-clamp study. The ability of ONO-8590580 to attenuate or potentiate the current induced by the EC₂₀ concentration of GABA was assessed by whole-cell patch-clamp electrophysiology. Data are expressed as the mean and standard error of the mean.

Figure 4. Effect of ONO-8590580 (300 nM) on LTP in rat hippocampal slice. The fEPSPs in response to stimulation of the Schaffer collateral-commissural pathway were recorded. To produce LTP, a θ -burst protocol (four pulses delivered with a frequency of 100 Hz, repeated 10 times with an interval of 200 ms) was used. Data are expressed as the mean and standard error of the mean (n=7-8 slices). * p <0.05 compared to the control group (Student *t* test).

Figure 5. Receptor occupancy of ONO-8590580 on rat hippocampal GABA_A α 5. ONO-8590580 at 1, 3, 10, and 20 mg/kg was orally administered 1 h before sacrifice. [³H]-Ro15-4513 was intravenously administered 10 min before sacrificing the rats. Data are expressed as the mean and standard error of the mean (n=3-4).

Figure 6. Effect on MK-801-induced cognitive deficit in rat passive avoidance test. On day 1 (acquisition trial), MK-801 (0.1 mg/kg, s.c.) was administered to rats 30 min before the trial. Retention

trial was conducted 24 h after acquisition trial. Vehicle or ONO-8590580 was orally administered 1 h before the acquisition and retention trial. Data are represented by box plot (n=10-15 in each group). ### $p < 0.001$ compared to Normal group (Wilcoxon rank-sum test). * $p < 0.05$, ** $p < 0.01$ compared to the control group (Steel test).

Figure 7. Effect on MK-801/scopolamine-induced cognitive deficit in the rat 8-arm radial maze test. Vehicle, ONO-8590580, or donepezil was administered orally 1 h before the trial. MK-801 (0.05 mg/kg) and scopolamine (0.15 mg/kg) were administered subcutaneously 0.5 h before the test trial. The number of errors and the latency until the bait of all 8 wells had been consumed were counted. Data are expressed as the mean and standard error of the mean (n=10 in each group). ** $p < 0.01$ compared to the control group (Dunnett test). \$ $p < 0.05$ compared to the control group (Student *t* test).

Figure 8. No anxiogenic-like effect in the rat elevated plus maze test (A) and no proconvulsant effect in the mouse PTZ test (B). (A) Vehicle (p.o.), ONO-8590580 (20 mg/kg, p.o.), or FG-7142 (15 mg/kg, i.p.) was administered 1 h before the test. One h after administration, rats were given a 5-min trial on the elevated plus maze, and the time spent on the open arm was calculated. (B) Vehicle (i.p.), ONO-8590580 (10 mg/kg, i.p.), or FG-7142 (10 mg/kg, i.p.) was administered 30 min before the test. Mice were infused intravenously with PTZ via the tail vein. Data are expressed as the mean and standard error of the mean (n=12 in each group in elevated plus maze test, n=8 in each group in PTZ test). \$ $p < 0.05$ compared to the control group (Student *t* test).

Figures

Figure 1.

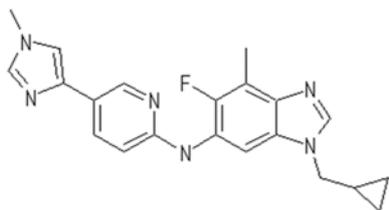


Figure 2.

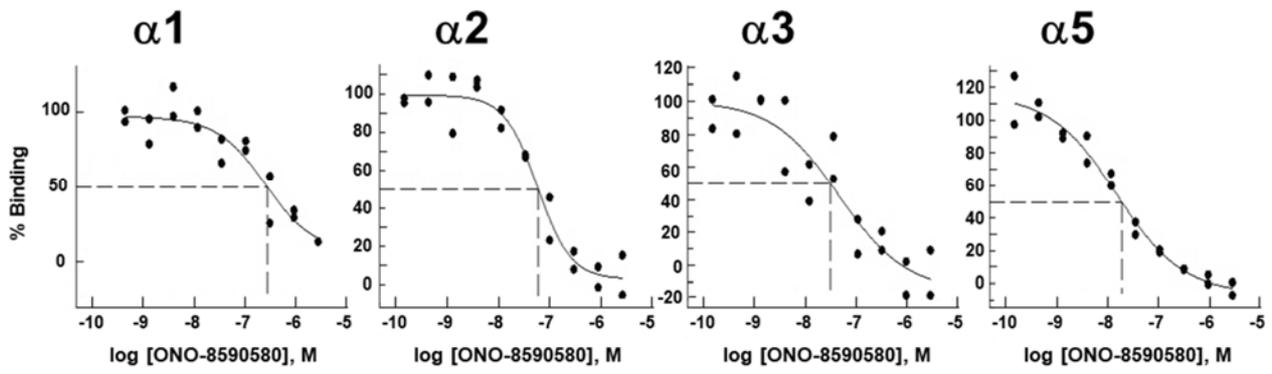


Figure 3.

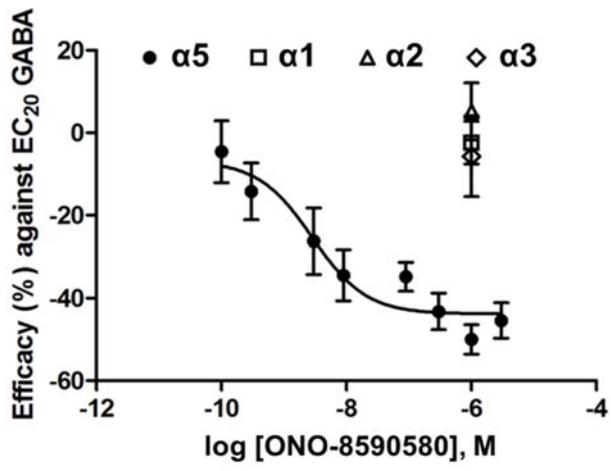


Figure 4.

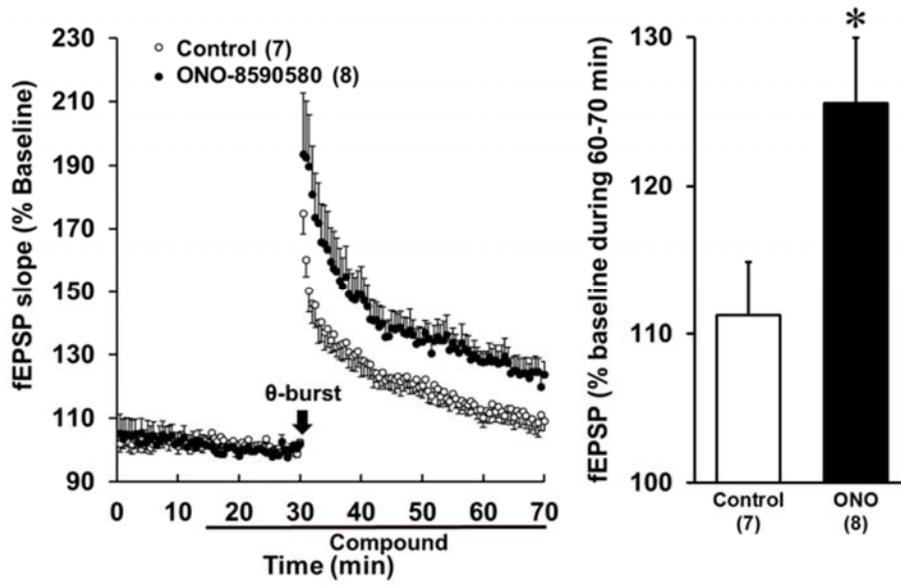


Figure 5.

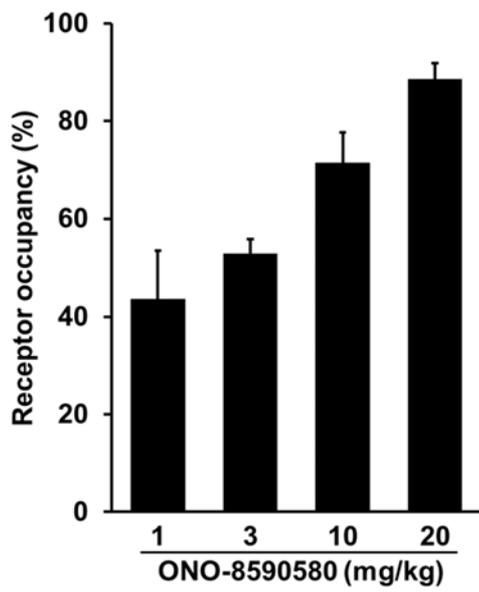


Figure 6.

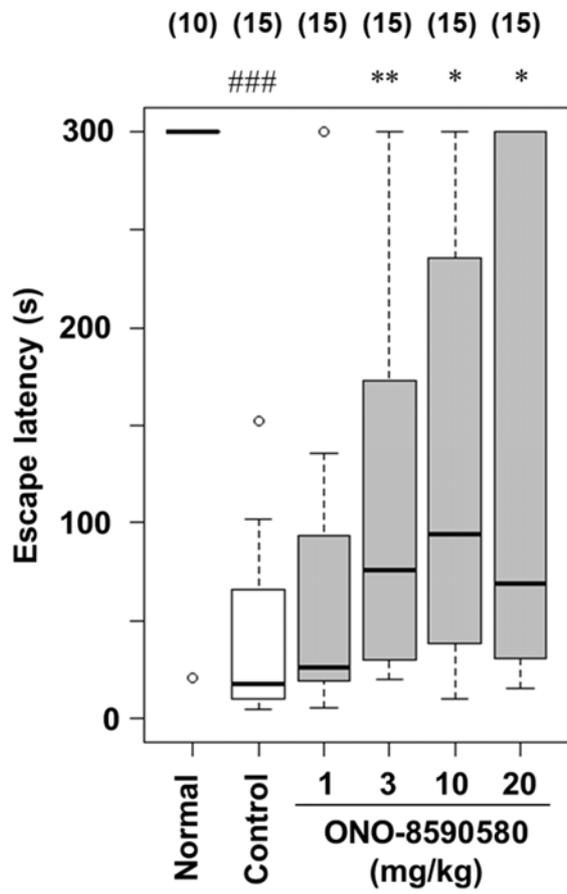


Figure 7.

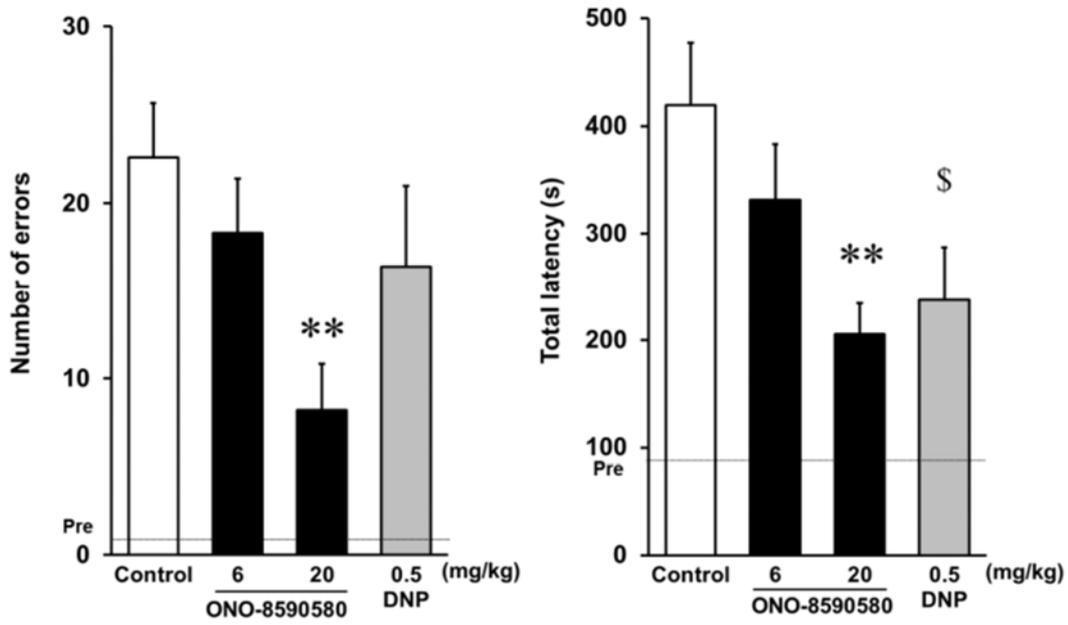


Figure 8.

