

Pharmacological Studies of Novel Agents with Serotonergic or
Glutamatergic Neuromodulatory Actions Using Mood Disorder
Model Animals

気分障害モデル動物を用いたセロトニンもしくはグルタミン酸
神経調節作用を有する新規化合物に関する薬理学的研究

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Abbreviations

Nonstandard abbreviations used in this thesis were listed in alphabetical order.

ANOVA	Analysis of variance
AUC	Area under the curve
CDP	Chlordiazepoxide
CL	Clearance
DA	Dopamine
DSP-1053	6-(2-{4-[4-Bromo-3-(2-methoxyethoxy)benzyl]piperidin-1-yl} ethyl)-2,3-dihydro-4 <i>H</i> -chromen-4-one benzenesulfonate
DSR-98776	(3-fluorophenyl)[2-(5-fluoropyridin-2-yl)]-6,7-dihydro[1,3]oxazolo [4,5- <i>c</i>]pyridin-5(4 <i>H</i>)-yl]methanone
EC ₅₀	Half maximal effective concentration
FDSS	Functional drug screening system
gp	Guinea pig
<i>K_d</i>	Dissociation constant
<i>K_i</i>	Inhibition constant
i.p.	Intraperitoneal
i.v.	Intravenous

I.A.	Intrinsic activity
IC ₅₀	Half maximal inhibitory concentration
HPLC	High-performance liquid chromatography
GDP	Guanosine 5'-diphosphate
GTP _γ S	Guanosine 5'-(γ-thio) Triphosphate, [³⁵ S]-
hum	Human
MAP	Methamphetamine
mGlu receptor	Metabotropic glutamate receptor
MPEP	2-methyl-6-(phenylethynyl)-pyridine
MPEPy	3-methoxy-5-pyridin-2-ylethynylpyridine
MTEP	3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]-pyridine
OB	Olfactory bulbectomized
PCPA	4-chloro-DL-phenylalanine methyl ester hydrochloride
p.o.	Oral, by mouth
RO ₅₀	Dose causing 50% of receptor occupancy
S.D.	Standard deviation
S.E.M	Standard error of the mean
SSRI	Selective serotonin reuptake inhibitor

SNRI	Serotonin and norepinephrine reuptake inhibitor
V _{dss}	Volume of distribution at steady state
5-HT	5-hydroxytryptamine (serotonin)
8-OH-DPAT	R-(+)-8-Hydroxy-DPAT

General Introduction

Major depressive disorder is a chronic, debilitating disease that affects approximately 14.8 million American adults, or about 6.7 percent of the US population aged 18 and older (Egede et al., 2007; Kessler et al., 2005; Waraich et al., 2005). The lifetime risk of developing major depressive disorder is approximately 15% for men and 30% for women (Kessler et al., 2005).

Major depressive disorder is associated with various dysfunctions and can have serious consequences for both the depressed individuals and families. The disorder interferes with the ability to work and pursue usual interests, and makes it extremely difficult for patients to maintain social relationships. The first onset of major depressive disorder most frequently occurs in young adulthood with recurrent episodes throughout life in the vast majority of cases (Keller et al., 1992). Estimates by the World Health Organization indicate that major depressive disorder is a leading cause of years lived with disability and that the size of the problem appears to be increasing worldwide (Murray and Lopez, 1996).

An early report by Guze and Robins (1970) indicates that 10-15% of depressed patients eventually commit suicide. This rate is supported by recent follow-up studies in wider depressed populations (Angst et al., 2002). Patients with mood disorders are

believed to have up to 10 years reduced life expectancy, mainly due to the expected increase in suicide risk (Angst et al., 2002). Considering the severity of major depressive disorder and its socioeconomic impact, effective pharmacological interventions to alleviate patients functional impairments are required.

Clinical studies have provided evidence that patients with major depressive disorder suffer from impaired monoaminergic neurotransmission, especially serotonin neurotransmission. This “monoamine hypothesis” has been supported by several studies in both healthy and depressed individuals. Indeed, early studies have shown that agents that deplete monoamines, such as reserpine, a vesicular monoamine transporter inhibitor, could lead to depression in a small percentage of individuals (Freis, 1954). In addition, normal male subjects show significantly elevated scores on the depression scale after ingesting a tryptophan-free amino acid mixture which cause marked depletion of plasma tryptophan, a chemical precursor of 5-HT, indicating a mood lowering effect of decreased brain 5-HT levels in normal males (Young et al., 1985). Moreover, patients who experience remission following treatment with antidepressants are vulnerable to relapse under depleted monoamines levels (Delgado et al., 1994; Miller et al., 1996). These findings led to the hypothesis that reduced levels of monoamine neurotransmitters, particularly serotonin, could play an important role in depression.

Strong evidence of abnormal serotonergic neurotransmission in patient with major depressive disorder comes from the discovery of prototypical antidepressants. During the 1950s, two classes of antidepressants, i.e. tricyclic antidepressants and monoamine oxidase inhibitors were discovered serendipitously. In 1957, it was reported that although G 22355 (imipramine) was ineffective in schizophrenia despite its closest structural resemblance to chlorpromazine, this drug acted as antidepressant in clinical use due to its serotonin/norepinephrine reuptake inhibitory activity (Kuhn, 1957, 1958). In the same year, two independent groups presented their findings on the antidepressant effect of iproniazid, an isonicotinic acid hydrazide for tuberculosis chemotherapy with monoamine oxidase inhibitory activity (Crane, 1957; Loomer et al., 1957). These findings led to the development of other monoamine oxidase inhibitors and tricyclic antidepressants as well as supported the “monoamine hypothesis” suggested by studies on induction of depressive state. However, tricyclic antidepressants generally affect other off-target receptors, including the muscarinic, histaminic and α_1 adrenergic receptors, leading to undesired side-effects and reduced tolerability. On the other hand, the use of monoamine oxidase inhibitors is limited by the irreversible inhibition of these target enzymes and the risk of hypertensive crisis when mixed with certain medications or with common tyramine-containing foods. Thus, research has been focusing on the

development of selective antidepressants with minimal side effects. Following this trend, the first selective serotonin reuptake inhibitor (SSRI), fluvoxamine, was developed by Kali-Duphar in 1971 and registered in Switzerland in 1983 (Freeman, 1991). Since the introduction of this first SSRI, a number of new SSRIs and serotonin and norepinephrine reuptake inhibitors (SNRIs) have been developed and approved worldwide. Currently, SSRIs and SNRIs are the first-line treatments for depression due to their relatively low potential for side effects.

Although SSRIs and SNRIs have dramatically expanded treatment options for major depressive disorder, there is still a significant unmet medical need for the management of this disorder, including therapeutics delayed onset and treatment resistance (Gelenberg et al., 2010; Reeves et al., 2008; Rush et al., 2006; Warden et al., 2007). In particular, the results of large-scale clinical trials, including STAR*D, have shown that 67% of patients do not achieve remission (the score in Quick Inventory of Depressive Symptomatology-Self Report is less than or equal to 5) even after standard citalopram treatment. Furthermore, about half of remitted patients required over 6-week consecutive treatment to achieve remission, indicating SSRI's delayed therapeutic onset (Fava, 2003; Rush et al., 2004; Trivedi et al., 2006).

Another shortcoming of the currently available armamentarium for treatment of

major depressive disorder is the presence of side effects, including nausea, sexual dysfunction, dizziness, sleep disorders, headache and agitation (Gelenberg et al., 2010). Studies have actually shown that patients adherence to antidepressants is directly related to the frequency and intensity of side effects (Bull et al., 2002; Olfson et al., 2006). Clearly, new effective and safe first-line treatments for major depressive disorder are necessary to address the needs of patients suffering from this debilitating disorder.

In some clinical trials, it has been reported that pindolol, a 5-HT_{1A/1B} and β adrenergic receptor partial agonist, may accelerate antidepressants onset and enhance SSRIs beneficial effects in treatment-resistant depression (Artigas et al., 1994; Blier and Bergeron, 1995; Pérez et al., 1997). This enhancement would be mediated by blockage of negative feedback inhibition in response to increased serotonin (5-HT) (Arborelius et al., 1996; Bel and Artigas, 1993; Kreiss and Lucki, 1995; Rutter et al., 1994). Further evidence to support the role of 5-HT_{1A} receptors in this response comes from the results of combination therapy with SSRIs and WAY-100635, a highly selective 5-HT_{1A} receptor antagonist (Dawson and Nguyen, 1998; Gartside et al., 1995).

On the other hand, activation of 5-HT_{1A} receptor is also considered to shorten SSRIs onset by accelerating desensitization of 5-HT_{1A} autoreceptor (Dawson and Watson, 2009). Therefore, dual-action antidepressants that can modulate 5-HT_{1A} receptor and

inhibit 5-HT reuptake could be a plausible option for treatment of major depressive disorder with faster onset and higher efficacy than conventional SSRIs (see reviews e.g. Celada et al., 2013).

There is growing evidence that the glutamatergic system plays an important role in the pathophysiology of major depressive disorder (Altamura et al., 1993; Mauri et al., 1998; Mitani et al., 2006). Especially, it has been reported that ketamine, a dissociative anesthetic with N-methyl-D-aspartate blocking effect, produces a rapid long-lasting antidepressant effect in a number of clinical studies (Krystal, 2007; Maeng and Zarate, 2007; Murrough et al., 2013; Pittenger et al., 2007). However, the use of ketamine has also been shown to be associated with cognitive impairment and psychotic symptoms (Krystal et al., 1994; Lahti et al., 1995; Malhotra et al., 1997), forcing researchers to turn their attention to alternative glutamatergic approaches. Recent studies have been focusing on the potential role of metabotropic glutamate (mGlu) receptors in depression. The mGlu receptors are divided into three groups based on structural homology, signal transduction, and pharmacology (Schoepp and Conn, 1993). Among them, Group I receptors, i.e. mGlu₁ and mGlu₅, are believed to be involved in the activation of phospholipase C, which play an important role in signal transduction pathways (Conn and Pin, 1997). Several preclinical studies have shown that inhibitors of the

representative mGlu₅ receptor, including 2-methyl-6-(phenylethynyl)-pyridine (MPEP) and 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]-pyridine (MTEP), have therapeutic potential in the treatment of depression (Kuhn et al., 2002; Palucha et al., 2005; Palucha and Pilc, 2007; Pilc et al., 2002; Swanson et al., 2005; Witkin et al., 2007). In addition, it has been suggested that mania, a critical state of bipolar disorder, is caused by malfunction in glutamatergic neurotransmission, including abnormal glutamine/glutamate ratio, as well as by the putative mechanisms of action of current drugs, including lithium and antiepileptics (Krystal et al., 2002; Nonaka et al., 1998; Öngür et al., 2008; Sanacora et al., 2003). Although bipolar disorder may be controlled or even prevented by use mood stabilizers and atypical antipsychotics, these drugs can produce severe side effects, necessitating the development of new therapeutic options. One of the plausible options for bipolar disorder with manic state is modulation of glutamatergic neurotransmission.

In this study, I evaluated the beneficial effects of two structurally-novel compounds, i.e. DSP-1053, a 5-HT reuptake inhibitor with 5-HT_{1A} receptor partial agonistic activity, and DSR-98776, a selective mGlu₅ receptor negative allosteric modulator in common rodent mood disorder models. Because modeling of human neuropsychiatric disorders in animals is extremely challenging given the subjective nature of many symptoms, and the lack of biomarkers and objective diagnostic test, I used behavioral models with high

predictive validity, including the forced swimming test, rat olfactory bulbectomy for depressive state and methamphetamine/chlordiazepoxide-induced hyperactivity for manic state. Appropriate control substances with proven efficacy in clinical use were used as positive controls. As the depression models used in this study were selected to reflect the delayed therapeutic onset of current antidepressants in human, all test compounds were sub-chronically administered over a period of 1 to 3 weeks.

Part-1

DSP-1053, a novel serotonin reuptake inhibitor with 5-HT_{1A} partial agonistic activity, displays fast antidepressant effect with minimal undesirable effects in juvenile rats

Abstract

Enhancement of serotonergic neurotransmission has been the main stream of treatment for patients with depression. However, delayed therapeutic onset and undesirable side effects are major drawbacks for conventional serotonin reuptake inhibitors. Here, I show that DSP-1053, a novel serotonin reuptake inhibitor with 5-HT_{1A} partial agonistic activity, displays fast antidepressant efficacy with minimal undesirable effects, especially nausea and emesis in animal models. DSP-1053 bound human serotonin transporter and 5-HT_{1A} receptor with the *K_i* values of 1.02 ± 0.06 and 5.05 ± 1.07 nM, respectively. This compound inhibited the serotonin transporter with an *IC*₅₀ value of 2.74 ± 0.41 nM and had an intrinsic activity for 5-HT_{1A} receptors of $70.0 \pm 6.3\%$. In rat microdialysis, DSP-1053, given once at 3 and 10 mg kg⁻¹, dose-dependently increased extracellular 5-HT levels. In the rat forced swimming test, 2-week administration of DSP-1053 (1 mg kg⁻¹) significantly reduced rats immobility time after treatment, whereas paroxetine (3 and 10 mg kg⁻¹) required 3-week administration to reduce rats

immobility time. In olfactory bulbectomy model, 1 and 2-week administration of DSP-1053 reduced both emotional scores and activity in the open field, whereas paroxetine required 2 weeks to show similar beneficial effects. Although single administration of DSP-1053 induced emesis and vomiting in the rat and *Suncus murinus*, multiple treatment with this compound, but not with paroxetine, decreased the number of vomiting episodes. These results highlight the important role of 5-HT_{1A} receptors in both the efficacy and tolerability of DSP-1053 as a new therapeutic option for the treatment of depression.

Purpose

In Part-1, I report the therapeutic benefits of DSP-1053, a structurally novel 5-HT reuptake inhibitor with 5-HT_{1A} receptor partial agonistic activity, in common rodent depression models, rat forced swimming test and olfactory bulbectomy model. I also compared DSP-1053 potential emetic effect to that of paroxetine in rats and shrews (*Suncus murinus*).

Materials and methods

Animals

All experimental procedures for the use of animals in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Sumitomo Dainippon Pharma, Co., Ltd. In part-1, rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) or Japan SLC, Inc. (Shizuoka, Japan). Shrews (*Suncus murinus*) were purchased from CLEA Japan, Inc. (Tokyo, Japan). All animals were kept in a room with controlled environmental conditions (temperature: $23 \pm 3^{\circ}\text{C}$, humidity: $55 \pm 15\%$, 12 h light-dark cycle with light on at 07:00 h) and used after a quarantine period of 7 days. The animals were given food (CE-2, Oriental Yeast Co., Ltd.) and filtered tap water *ad libitum*.

Materials

DSP-1053

(6-(2-{4-[4-Bromo-3-(2-methoxyethoxy)benzyl]piperidin-1-yl}ethyl)-2,3-dihydro-4*H*-chromen-4-one benzenesulfonate) (Figure 1) and paroxetine hydrochloride (paroxetine) were synthesized in my laboratories. The rout of synthesis of DSP-1053 has been described previously (Nishida et al., 2012). Clomipramine hydrochloride

(clomipramine), serotonin hydrochloride (5-HT), dopamine hydrochloride (dopamine), imipramine hydrochloride (imipramine), WAY-100635, pindolol, and R-(+)-8-Hydroxy-DPAT (8-OH-DPAT) were purchased from Sigma Aldrich Japan (Tokyo, Japan). All radioligands were purchased from Perkin Elmer Japan (Kanagawa, Japan). For oral (p.o.) administration in rodent models, DSP-1053 and paroxetine were dissolved in 0.5% methylcellulose. In the *Suncus murinus* model, DSP-1053 and paroxetine were dissolved in 40% polyethylene glycol. Dosing volume was determined based on each animal body weight measured in the morning of each administration day (5 mL kg⁻¹). Cell membranes expressing human serotonin transporter and 5-HT_{1A} receptor were purchased from Perkin Elmer Japan. Chinese hamster ovary cells expressing human serotonin transporter used for [³H]5-HT uptake assay were established in my Pharmacology Research Laboratories at Sumitomo Dainippon Pharma Co., Ltd..

Preparation of rat cell membranes

Five weeks old male rats (CrI:CD(SD)) were sacrificed by decapitation, and their brains were rapidly removed and dissected to obtain the cerebral cortex (for serotonin transporter binding) and hippocampus (for 5-HT_{1A} binding), which were washed in

ice-cold saline and weighed. The tissues were homogenized with a teflon-glass homogenizer in reaction buffer (50 mM Tris-HCl buffer containing 120 mM NaCl and 5 mM KCl (for serotonin transporter) or 50 mM Tris-HCl buffer containing 4 mM CaCl₂ (for 5-HT_{1A})), and the homogenates were centrifuged at 40,000-48,000×g for 10 min at 4°C. The obtained pellets were resuspended in the reaction buffer, and the homogenates were centrifuged again at 40,000-48,000×g for 10 min at 4°C. The resulting pellets were resuspended in 8-10 times their volume of reaction buffer, and the protein concentrations were measured by the method of Bradford using the Bio-Rad Protein Assay (Bio-Rad Laboratories Co., Ltd.). The cell membranes were diluted with reaction buffer to a concentration of 4 mg mL⁻¹. On the day of the experiment, the stored membranes were diluted with reaction buffer to a concentration of 447 µg mL⁻¹ (200 µg/1 assay).

Radioligand binding assay

In a total volume of 500 µL, 2.5 µL of test substance solution, clomipramine solution (2 mM), 8-OH-DPAT (2 mM) or dimethyl sulfoxide, 50 µL of [³H]citalopram or [³H]8-OH-DPAT solution, and 447.5 µL of cell membranes were mixed. Cell membranes expressing human serotonin transporter and 5-HT_{1A} receptor were diluted

with the reaction buffer to a final concentration of 1 unit/447.5 μ L beforehand. All samples were reacted at 25°C for 0.5 (for 5-HT_{1A}) or 1 h (for serotonin transporter) in an incubator. The reaction was terminated by addition of 4 mL ice-cold reaction buffer, and the cell membranes were collected by vacuum filtration through GF/B glass filters. The glass filters were then washed with 4 mL of ice-cold reaction buffer and placed in scintillation vials with scintillation fluid. After more than 3 h, the radioactivity in each sample was measured with a liquid scintillation counter for 2 min, and the calculated dpm value was used for data analysis. In the serotonin transporter binding assay, GF/B glass filters were soaked in 0.05% polyethylenimine solution for more than 15 min before use. The inhibition constant (*K_i*) was calculated in Microsoft® Office Excel 2003 (Microsoft Corporation) using the Cheng-Prusoff equation [$K_i = IC_{50}/(1 + ([L]/K_d)$], where L is the concentration of radioligand in the assay and *K_d* is the dissociation constant of the radioligand for the receptor. s

[³H]5-HT uptake assay

Phosphate buffered saline containing 0.1 mM CaCl₂ and 1 mM MgCl₂ was used as reaction buffer. One microliter of dimethyl sulfoxide or test substance and 149 μ L of human serotonin transporter-expressing cells suspension were added to 96 well assay

plates. The plates were preincubated at 37°C for 10 min. During that time, dimethyl sulfoxide or test substance (DSP-1053, paroxetine, or imipramine) was diluted in [³H]5-HT solution in another 96 well plate. After the preincubation, the prepared [³H]5-HT solution containing dimethyl sulfoxide or test substance was added to the cell suspension, and the mixture was incubated at 37°C for 10 min. Ice cold 3% formamide in 0.9% NaCl was added to each well to stop the reaction. All reaction mixtures in the 96 well plates were then filtered through a glass fiber filter plate prewashed in 200 μL of 0.3% polyethylenimine and dried under reduced pressure with manifold (Millipore). To wash the glass fiber filter, 300 μL of phosphate buffered saline was added and filtrated twice. Radioactivity in each sample was measured as described in the previous section.

Guanosine 5'-(γ-thio) Triphosphate, [³⁵S]- (GTP_γS) assay for 5-HT_{1A} receptor

To make up a total volume of 500 μL, 2.5 μL of test substance, 2 mM GTP_γS (to measure nonspecific binding), dimethyl sulfoxide (to measure basal [³⁵S]GTP_γS binding), or 20 mM 5-HT (to measure maximal [³⁵S]GTP_γS binding), 50 μL of reaction buffer (HEPES-NaOH buffer (20 mM, pH 7.4) containing 100 mM NaCl, 10 mM MgCl₂, 0.1 mM dithiothreitol, and 1 μM guanosine-5'-diphosphate sodium salt (GDP)) containing 20 nM [³⁵S]GTP_γS, and 447.5 μL of the cell membranes expressing human

5-HT_{1A} receptors (1 unit/447.5 µL) were mixed. All samples were allowed to react in an incubator set at 25°C for 20 min. The reaction was terminated by adding 4 mL of ice-cold reaction buffer, and the cell membranes were collected by vacuum filtration using GF/B glass filters. The glass filters were washed twice with 4 mL of ice-cold reaction buffer. Radioactivity in each sample was measured as described in the previous section. Intrinsic activity was expressed as relative value of the activity of 100 µM 5-HT, which was considered to be 100%.

Off-target radioligand binding assays and enzyme assays

To determine DSP-1053 interaction with off-target receptors and enzymes, 29 receptor binding assays and 3 enzyme assays (catechol-O-methyltransferase, monoamine oxidase-A and -B) were conducted on my behalf by Sekisui Medical Co., Ltd. The receptor binding assays were carried out using standard techniques as summarized in Table 3. As for the enzyme assays, pig catechol-O-methyltransferase, human monoamine oxidase-A, and -B activity was evaluated using S-adenosyl-L-[methyl-¹⁴C]-methionine, 5-hydroxy[side chain-2-¹⁴C]tryptamine, or beta-[ethyl-1-¹⁴C]-, PKI as labeled substrate, and the amount of radioactivity was quantitated.

Pharmacokinetics of DSP-1053

Pharmacokinetic study of DSP-1053 was carried out in male rats (CrI:CD(SD)) after intravenous (i.v.) (1 mg kg^{-1}) and p.o. (10 mg kg^{-1}) administration. At appropriate time points after dosing (0.083, 0.25, 0.5, 1, 2, 4, 6, and 24 h for i.v., 0.25, 0.5, 1, 2, 4, 6, and 24 h for p.o.), blood was sampled from two (for i.v.) or three (for p.o.) different rats. Each rat was sampled 8 or 7 times in total. Plasma concentrations of DSP-1053 were determined using high-performance liquid chromatography (HPLC)/tandem mass spectrometry and analyzed by non-compartmental analysis using WinNonlin (version 6.3; Pharsight Corporation, CA).

Rat microdialysis

Surgery

This experiment was performed using 5-6-week old male rats (CrIj:WI). A vertical guide cannula (AG-04; EICOM) was implanted in the right side of the frontal cortex (3.7 mm anterior, 3.0 mm lateral, and 1.5 mm ventral from the bregma) of the rat under pentobarbital anesthesia [80 mg kg^{-1} , intraperitoneal (i.p.)]. Microdialysis was conducted on the day after surgery. A dialysis probe (A-I-4-03; EICOM) was inserted into the guide cannula under light anesthesia with isoflurane and continuously perfused

by Ringer solution (147 mM NaCl, 4 mM KCl, 2.3 mM CaCl₂) at 2 μL min⁻¹ using a microsyringe pump. Microdialysate samples (10 μL) were continuously collected for 5 min at 20-min intervals and automatically injected into the HPLC system. DSP-1053 or vehicle was orally administered to the rats at least 3 h after the start of perfusion, that is, when stable HPLC baseline values for 5-HT and dopamine were obtained in the dialysate samples. Measurement continued for 3 h after drug or vehicle administration.

Chromatography

The collected microdialysate samples (10 μL) were separated by HPLC using a PP-ODS column (EICOM) and a mobile phase containing 0.1 M phosphate buffer (pH 6.0), 1% methanol, 50 mg L⁻¹ ethylenediamine tetraacetic acid disodium, and 500 mg L⁻¹ sodium 1-decanesulfonate at a flow rate of 0.5 mL min⁻¹. The peaks corresponding to 5-HT and dopamine were amperometrically detected using a graphite electrode set at 400 mV with an Ag/AgCl reference electrode (RE-100; EICOM). Online data acquisition was performed using PowerChrom software (Version 2.2; AD Instruments Pty Ltd.). Before performing the microdialysis, the retention time of the HPLC peak for 5-HT and dopamine was determined using a standard solution. The peak height (mV) of 5-HT and dopamine at each measurement was converted into a percentage of the average of the last 4 pre-drug baseline values (percentage of baseline).

Rat forced swimming test

Test-compounds antidepressant-like activity was assessed as previously described (Porsolt et al., 1978) with slight modifications. In the training session (Day 1), each animal (5-6-week old male rats (Crlj:WI)) was gently placed into a plastic cylinder (40 cm in height, 19 cm in diameter) containing 5.8 L of water set at $25 \pm 1^\circ\text{C}$. Fifteen minutes after the beginning of the training session, the animal was removed from the water and returned to its home cage. Test compounds dosing suspensions were administered to animals in a blind manner 15 min after the end of the training session. From Day 2 to Day 13 or Day 20, the animals were administered the dosing suspensions once a day between 7:00AM and 7:00PM. In the test session (Day 14 or Day 21), the animals were treated with the dosing suspensions as described in the training session, and the swimming test was performed for 5 min in the same manner as in the training session. In the swimming test, the behavior of each animal was horizontally recorded onto a DVD recorder using a video camera. An animal was judged to be immobile whenever it remained floating on the water without moving its body or forepaws, except for slight movements to maintain posture. The total time the animal remained immobile was defined as immobility time. An observer blinded to test compounds doses measured

the immobility time.

Olfactory bulbectomy

Surgery

The test was performed using 6-7-week old male rats (Crlj:WI). Bilateral olfactory bulbectomy was performed on rats anesthetized with pentobarbital (50 mg kg⁻¹; i.p.), essentially as previously described (Cryan et al., 1999). The head was shaved and a midline sagittal incision was made extending at 1 cm rostral to bregma. A burr hole was drilled at points 7 mm anterior to bregma and 2 mm either side of the midline at a point corresponding to the posterior margin of the orbit of the eye. The olfactory bulbs were removed by suction, and the burr holes filled with a hemostatic sponge (Spongel; Astellas Pharma Inc.). Tetracycline powder was applied to the wound prior to closure. Sham-operated rats underwent the same procedure with the dura above the bulbs punctured, but the bulbs left intact. Following surgery, the animals were allowed 7 days recovery prior to drug administration. During recovery, the general condition of each animal was monitored.

Emotional scoring

Emotional scoring was conducted, essentially as previously reported (Cairncross et al.,

1978; Gomita et al., 1983). Emotional scoring consisted of the following five tests; 1) response to a stick presented just in front of the nose, 2) response to a puff of air blown sharply onto the rat's back, 3) response to grasping the animal, 4) response to tail pinching by a forceps, 5) vocalization during scoring. In each of the tests numbered 1) to 4), responses were graded as follows: 0 (no response), 1 (slight response), 2 (moderate response), 3 (marked response), or 4 (extreme response). In test 5), vocalization was graded as follows: 0 (no vocalization), 1 (occasional vocalization), or 2 (extensive vocalization). Emotional scoring was performed in animals' home cages. The scores in each scoring system were added to give a single emotional score for each individual animal. The maximum emotional score was 18. Animals emotional scoring was performed just before the first drug administration (pre), and on the day after the open field test (post).

Open field test

The open field test was carried out, essentially as described elsewhere (Cryan et al., 1999). Each rat was placed onto the center of the open-field apparatus, and the number of line crosses over a 5-min period was recorded.

Conditioned taste aversion test in rats

The test was performed using 10-week old male rats (Slc:SD). Drinking water was

removed overnight (from 18:00 until 09:00 h) prior to the start of the experiment. On the following 2 days, water was first given for 60 min, and then *ad libitum* from 12:00 until 18:00 h. On day 3, all rats had access to a 0.5 % saccharin solution for 60 min instead of water. At 10:20 h of day 3, the animals were dosed with DSP-1053 or vehicle. Water was then made available *ad libitum* for the rest of the day and removed overnight (from 18:00 until 09:00 h). On the test day (day 4), the animals had again access to 0.5% saccharin solution for 60 min in the absence of test compound. Rats saccharin consumption was measured for each 60-min period on day 3 and 4, and saccharin intake ratio was calculated as follows.

$$(\text{Saccharin intake ratio}) = (\text{saccharin intake on day 4}) / (\text{saccharin intake on day 3})$$

Evaluation of emesis in *Suncus murinus*

Male Jic:SUN-Her, 5 weeks (body weight: 40 - 63 g) (experiment 1), or 10 weeks (body weight: 53 - 73 g) (experiment 2) of age at the initiation of dosing were used in this study.

Experiment 1

DSP-1053 (10, 30, 60, or 100 mg kg⁻¹) or 40% polyethylene glycol was orally administered to the animals, and the number of animals that vomited as well as the number of vomiting episodes were counted for 60 min. Paroxetine-induced emesis, as

evaluated under the same protocol (Mine et al., 2013), was used as reference.

Experiment 2

On the first day of the experiment, DSP-1053 (60 mg kg⁻¹), paroxetine (60 mg kg⁻¹), or 40% polyethylene glycol was orally administered to the animals and the number of vomiting episodes was counted. Animals that showed emesis on the first day in each group (9 of 19 in DSP-1053 group, 12 of 19 in paroxetine group), and 3 animals in 40% polyethylene glycol group were subsequently dosed once a day for 7 days with DSP-1053 (60 mg kg⁻¹), paroxetine (60 mg kg⁻¹), or 40% polyethylene glycol, respectively, and the number of animals that vomited as well as the number of vomiting episodes was counted for 60 min after administration on each day.

Data analysis

Scatchard plots approximated by regression line with Microsoft[®] Office Excel 2003 (Microsoft Corporation) were used to calculate *K_d* value in each binding assay. The IC₅₀ value in each binding assay and the maximal specific binding of each test substance (intrinsic activity (I.A.) of the test substance) as well as EC₅₀ values in GTPγS assay were determined by fitting logistic curve using “Dx calculation (logistic curve fitting) with measured value input function” method in Stat Prelinica Version 1.0.3.

In rat microdialysis, data are presented as time course changes in peak height and in cumulative values of percentage of baseline over 3 h after drug administration (AUC (0-180 min)). Cumulative data were analyzed with one-way analysis of variance (ANOVA) followed *post-hoc* parametric Dunnett's multiple comparison test. Differences in immobility time in the rat forced swimming test between test compounds treated groups and the vehicle treated group were analyzed with one-way ANOVA followed *post-hoc* parametric Dunnett's multiple comparison test.

In olfactory bulbectomy, the statistical significance of differences in emotional scores and the number of line crosses between each group was assessed with three-way ANOVA, and *post-hoc* individual group comparison were made with t-test and parametric Dunnett's multiple comparison test.

In rat conditioned taste aversion model, differences in water or saccharin and saccharin intake ratio between DSP-1053 treated group and the vehicle treated group were analyzed with one-way ANOVA followed *post-hoc* parametric Tukey's multiple comparison test.

In emesis test using *Suncus murinus*, data analysis was conducted as previously reported (Mine et al., 2013). In experiment 1, differences in incidence of vomiting between the vehicle treated group and compounds treated groups were analyzed by

Fisher's exact test. The number of emetic episodes was determined as average for all animals that vomited. In experiment 2, the number of emetic episodes was determined as average for all tested animals. Differences in the number of emetic episodes between Day 1 and Day 2 to Day 7 of treatment were analyzed with one-way ANOVA followed *post-hoc* parametric Dunnett's multiple comparison test.

In all animal experiments, Stat Prelinica Version 1.2 was used as analysis software.

Results

DSP-1053 *in vitro* binding to the serotonin transporter

DSP-1053 inhibited the binding of [³H]citalopram to human and rat serotonin transporter, with K_i values of 1.02 ± 0.06 and 0.489 ± 0.039 nM [mean \pm S.E.M. duplicate, 3 independent experiments (n=3)], respectively. In addition, in 5-HT uptake assay using Chinese hamster ovary cells expressing human serotonin transporter, DSP-1053 inhibited [³H]5-HT uptake with an IC_{50} value of 2.47 ± 0.41 (n=3). In both assays, DSP-1053 binding affinity for human serotonin transporter was lower than that of paroxetine, but higher than that of imipramine (Table 1).

DSP-1053 *in vitro* binding to the 5-HT_{1A} receptor

DSP-1053 inhibited the binding of [³H]8-OH-DPAT to human and rat 5-HT_{1A} receptor with K_i values of 5.05 ± 1.07 and 5.09 ± 1.03 nM (mean \pm S.E.M. n=3), respectively. In GTP γ S assay using Chinese hamster ovary cell membrane expressing human 5-HT_{1A} receptor, DSP-1053 displayed I.A. of $70.0 \pm 6.3\%$ (relative value of the activity of 100 μ M 5-HT) [EC_{50} ; 98.0 ± 34.9 nM] (n=3). DSP-1053 binding affinity for human 5-HT_{1A} receptor was comparable to that of pindolol or 8-OH-DPAT, but lower than that of WAY-100635. On the other hand, DSP-1053 I.A. for 5-HT_{1A} receptor was higher than

that of WAY-100635 or pindolol, but lower than that of 8-OH-DPAT (Table 2).

DSP-1053 binding to off-target receptors and enzymes

As shown in Table 3, DSP-1053 (1 μ M) showed affinity for histamine H₁ receptor with *K_i* value of 7.46 ± 1.37 nM (mean \pm S.E.M. n=3). DSP binding affinity for the other 28 tested receptors was weak (*K_i* values > 100 nM). Moreover, DSP-1053 (1 μ M) did not inhibit pig catechol-O-methyltransferase, human monoamine oxidase-A, and -B (percent inhibition; 0.00, 5.28 and 0.19 %, respectively).

Pharmacokinetics of DSP-1053

DSP-1053 reached maximum plasma levels within 1 h after p.o. administration with 7.3% bioavailability (Figure 2). DSP-1053 clearance (CL) and volume of distribution at steady state (*V_{dss}*) after injection were $57.6 \text{ mL min}^{-1} \text{ kg}^{-1}$ and 5.1 L kg^{-1} , respectively. In all DSP-1053 *in vivo* studies, dosing time was selected based on the above pharmacokinetic parameters.

Effects of DSP-1053 on extracellular 5-HT and dopamine levels in the frontal cortex of rats

Basal microdialysate levels of 5-HT and dopamine in the rat frontal cortex were 0.355 ± 0.025 and 0.305 ± 0.019 pg/10 μ l (n=17), respectively. DSP-1053 increased 5-HT extracellular levels in the rat frontal cortex. This increase reached a maximum of $180 \pm 25.9\%$ and $264 \pm 58.0\%$ (mean \pm S.E.M.) of baseline value 100 min after DSP-1053 administration at 3 and 10 mg kg⁻¹, respectively (Figure 3A). In addition, DSP-1053 (3 and 10 mg kg⁻¹) significantly increased cortical 5-HT cumulative value over 3 h after administration [$F(3,13) = 30.90, p < 0.05$] (Figure 3B). On the other hand, DSP-1053 didn't affect dopamine extracellular levels in the rat frontal cortex at any dose [$F(3,13) = 0.13, p > 0.05$] (Figure 3C, D).

DSP-1053 antidepressant-like effect in the rat forced swimming test

As shown in Figure 4A, DSP-1053 significantly decreased immobility time following a 2-week consecutive administration at 1 mg kg⁻¹ compared to the vehicle [$F(3,60) = 5.01, p < 0.05$]. On the other hand, animals treated with paroxetine (3 and 10 mg kg⁻¹) for 3-week had reduced immobility time compared to the animals treated with the vehicle [$F(2,51) = 4.64, p < 0.05$] (Figure 4B). Treatment with paroxetine for 2-week had no effect on rats immobility time [$F(3,60) = 1.59, p > 0.05$] (Figure 4C).

DSP-1053 antidepressant-like effect in the rat olfactory bulbectomy test

Effects on emotional scores

Figure 5A and B show the effects of 1-week and 2-week administration of DSP-1053 on emotional scores of sham-operated and olfactory bulbectomized animals. Three-way (drug, surgery and dosing period) ANOVA revealed no main effect for drug \times surgery \times dosing period, drug \times dosing period and surgery \times dosing period interaction and a significant main effect for drug \times surgery interaction [drug \times surgery \times dosing period interaction, $F(15,144) = 1.16$, $p > 0.05$; drug \times dosing period interaction, $F(15,144) = 0.17$, $p > 0.05$; surgery \times dosing period interaction, $F(15,144) = 1.84$, $p > 0.05$; drug \times surgery interaction, $F(15,144) = 22.55$, $p < 0.05$]. Olfactory bulbectomy significantly increased emotional scores [$F(15,144) = 598.83$, $p < 0.05$]. *Post-hoc* test analysis showed that in olfactory bulbectomized animals, DSP-1053 (0.3, 1 and 3 mg/kg) produced a significant decrease in emotional scores [$F(3,76) = 30.58$, $p < 0.05$] and no effect in the sham-operated animals [$F(3,76) = 0.21$, $p > 0.05$]. Figure 5C and D show the effects of 1-week and 2-week administration of paroxetine on emotional scores of sham-operated and olfactory bulbectomized animals. Three-way ANOVA revealed a significant main effect for drug \times surgery \times dosing period interaction [$F(13,125) = 5.18$, $p < 0.05$]. In 1-week administration group, sub-effect two-way (drug and surgery)

ANOVA revealed that paroxetine didn't alter emotional scores compared to the vehicle [$F(5,54) = 0.95, p > 0.05$]. On the other hand, olfactory bulbectomy significantly increased emotional scores [$F(5,54) = 453.49, p < 0.05$], and it didn't significantly affect the drug effect [drug \times surgery interaction, $F(5,54) = 1.94, p > 0.05$]. In 2-week administration group, sub-effect two-way (drug and surgery) ANOVA revealed a significant main effect for drug \times surgery interaction [$F(7,71) = 4.16, p < 0.05$]. *Post-hoc* test analysis showed that in olfactory bulbectomized animals, paroxetine (3 and 10 mg/kg) produced a significant decrease in emotional scores [$F(3,35) = 7.38, p < 0.05$] and no effect in the sham-operated animals [$F(3,36) = 1.26, p > 0.05$].

Effects on the number of line crosses

Figure 6A and B show the effects of 1-week and 2-week administration of DSP-1053 on the number of line crosses of sham-operated and olfactory bulbectomized animals. Three-way ANOVA revealed no main effect for drug \times surgery \times dosing period, drug \times dosing period and surgery \times dosing period interaction and a significant main effect for drug \times surgery interaction [drug \times surgery \times dosing period interaction, $F(15,144) = 0.87, p > 0.05$; drug \times dosing period interaction, $F(15,144) = 0.80, p > 0.05$; surgery \times dosing period interaction, $F(15,144) = 0.58, p > 0.05$; drug \times surgery interaction, $F(15,144) =$

7.25, $p < 0.05$]. Olfactory bulbectomy significantly increased the number of line crosses [$F(15,144) = 41.65$, $p < 0.05$]. *Post-hoc* test analysis showed that in olfactory bulbectomized animals, DSP-1053 (0.3, 1 and 3 mg/kg) produced a significant decrease in the number of line crosses [$F(3,76) = 7.85$, $p < 0.05$] and no effect in the sham-operated animals [$F(3,76) = 1.55$, $p > 0.05$]. Figure 6C and D show the effects of 1-week and 2-week administration of paroxetine on the number of line crosses of sham-operated and olfactory bulbectomized animals. Three-way ANOVA revealed a significant main effect for drug \times surgery \times dosing period interaction [$F(13,125) = 4.27$, $p < 0.05$]. In 1-week administration group, sub-effect two-way (drug and surgery) ANOVA revealed that paroxetine didn't alter the number of line crosses compared to the vehicle [$F(5,54) = 0.78$, $p > 0.05$]. On the other hand, olfactory bulbectomy significantly increased the number of line crosses [$F(5,54) = 43.03$, $p < 0.05$], and it didn't significantly affect the drug effect [drug \times surgery interaction, $F(5,54) = 0.76$, $p > 0.05$]. In 2-week administration group, sub-effect two-way (drug and surgery) ANOVA revealed a significant main effect for drug \times surgery interaction [$F(7,71) = 2.97$, $p < 0.05$]. *Post-hoc* test analysis showed that in olfactory bulbectomized animals, paroxetine (10 mg/kg) produced a significant decrease in the number of line crosses [$F(3,35) = 2.92$, $p < 0.05$] and no effect in the sham-operated animals [$F(3,36) = 2.58$, $p > 0.05$].

DSP-1053 potential to induce emesis in rats conditioned taste aversion test

On Day 4, DSP-1053 (100 mg kg⁻¹) significantly inhibited saccharin consumption 24 h after administration compared to the vehicle [$F(2,15) = 4.73, p < 0.05$] (Figure 7A).

Saccharine intake ratio was significantly inhibited at the doses of 60 and 100 mg kg⁻¹ [$F(2,15) = 22.77, p < 0.05$] (Figure 7B).

DSP-1053 potential to induce emesis in *Suncus murinus*

In experiment 1, DSP-1053 at the dose of 10, 30, or 60 mg kg⁻¹ induced emesis in 0, 1, or 3 out of 6 animals, respectively, while, as shown in Table 4, paroxetine at 10, 30, or 60 mg kg⁻¹ induced emesis in 0, 2, or 6 out of 6 animals, respectively (Mine et al., 2013).

In experiment 2, both DSP-1053 (60 mg kg⁻¹) and paroxetine (60 mg kg⁻¹) induced emesis in 9 and 12 out of 19 animals, respectively. Figure 8 shows the incidence of vomiting and the number of vomiting episodes during a 60 min period in each day of a 7-day consecutive administration of DSP-1053 or paroxetine. A significant reduction in the number of vomiting episodes was observed from Day 2 in DSP-1053 treated group [$F(6,56) = 5.60, p < 0.05$]. On the other hand, repeated administration of paroxetine didn't have significant effect on the number of vomiting episodes throughout the 7-day

administration period [$F(6,77) = 1.64, p > 0.05$].

Discussion

In this study, I evaluated the *in vitro* and *in vivo* profile of DSP-1053, a structurally novel 5-HT reuptake inhibitor with 5-HT_{1A} partial agonistic activity. My results show that DSP-1053 exhibits a potent fast antidepressant-like effect with minimal undesirable effects in animal models.

My *in vitro* experiments demonstrate that DSP-1053 works as a serotonin reuptake inhibitor with relatively high partial agonistic activity for 5-HT_{1A} receptor ($70.0 \pm 6.3\%$). It is well reported that SSRIs enhancement of 5-HT neurotransmission is dampened by activation of somatodendritic or postsynaptic 5-HT_{1A} receptors (Artigas et al., 1996, Casanovas et al., 1999). However, acute increase in 5-HT levels in the rat frontal cortex has also been achieved when antidepressants are used in combination with pindolol, a weak 5-HT_{1A} receptor partial agonist or WAY-100635, a 5-HT_{1A} receptor antagonist (Hughes et al., 2007; Jerning et al., 2002; Papp et al., 2006; Pauwels et al., 1997; Watson et al., 2000). On the other hand, acute treatment with DSP-1053, which has a relatively high 5-HT_{1A} partial agonistic activity, enhanced 5-HT neurotransmission in the rat prefrontal cortex without affecting dopamine neurotransmission. It has been suggested that stimulation of 5-HT_{1A} receptors enhances dopamine neurotransmission via activation of presynaptic 5-HT_{1A} receptors expressed in the ventral tegmental area

(Lejeune et al., 1997; Millan et al., 1997). This indicates that DSP-1053 may act as antagonist for presynaptic 5-HT_{1A} receptors. On the other hand, the prolonged stimulation of 5-HT neurotransmission that occurs during chronic SSRI treatment has been reported to desensitize raphe 5-HT_{1A} autoreceptors, as assessed by single unit recordings and brain microdialysis (Arborelius et al., 1995; Blier and de Montigny, 1994; Invernizzi et al., 1994; Le Poul et al., 1995). These findings suggest that DSP-1053 acute enhancement of 5-HT release may be attributed to early desensitization of 5-HT_{1A} receptors. Like DSP-1053, vilazodone and vortioxetine, which inhibit the serotonin transporter with 5-HT_{1A} partial or full agonistic activity, have been reported to enhance 5-HT neurotransmission by acute treatment (Hughes et al., 2005; Pehrson et al., 2013). Although further studies are needed to clarify the exact contribution of 5-HT_{1A} partial activation in DSP-1053 beneficial effects, evidence highlighting the involvement of this receptor in antidepressants early onset suggests that both inhibition and activation of 5-HT_{1A} receptors would fasten SSRI efficacy.

In the rat forced swimming test, which is the most commonly used behavioral test to assess potential antidepressants efficacy (see reviews e.g. Cryan and Mombereau, 2004; Pollak et al., 2008), DSP-1053 decreased rats immobility time after 2 weeks treatment, while treatment with paroxetine, a representative SSRI, required 3 weeks for

comparable efficacy. These results indicate that DSP-1053 would have earlier onset of efficacy than SSRIs. Next, I evaluated the efficacy and onset of DSP-1053 in the rat olfactory bulbectomy model. The rat olfactory bulbectomy model is a suitable tool for investigating antidepressant onset, because olfactory bulbectomy-induced behavioral abnormalities can be reversed by chronic, but not acute, antidepressant treatment (Kelly et al., 1997; Roche et al., 2007, 2008). As shown in Figures 5, 6, DSP-1053 exerted significant antidepressant-like effects after 1-week administration, while paroxetine required 2-week treatment to show similar efficacy. This indicates that DSP-1053 has early onset of action compared to paroxetine. On the other hand, DSP-1053 improvement of hyperemotional behavior may reflect an anxiolytic-like effect as rats' suicidal behavior has been reported to be improved by anxiolytics (Takaoka et al., 1988). In the rat forced swimming test and olfactory bulbectomy model, DSP-1053 showed efficacy at a lower dose range than the doses that increased 5-HT level in the rat frontal cortex. The following two reasons might explain this discrepancy. One major difference between the olfactory bulbectomy test and rat microdialysis is the duration of drug administration. That is, chronic treatment with DSP-1053 is considered to induce further desensitization of 5-HT_{1A} receptors and enhancement of 5-HT neurotransmission compared to single administration. As for the second reason, it is

believed that activation of postsynaptic 5-HT_{1A} receptors contributes to DSP-1053 low dose antidepressant-like effect. This hypothesis may be supported by evidence showing that postsynaptic 5-HT_{1A} receptors are particularly important in antidepressant response (Blier and Ward, 2003). However, further investigation of changes in neurotransmission after chronic administration of DSP-1053 as well as evaluation of DSP-1053 effects at the efficacy pre- and post-synaptic regions would be necessary to confirm the above hypothesis.

Next, I investigated DSP-1053 potential for inducing emesis in experimental animals, because emesis is one of the common side effects of SSRIs (Brambilla et al., 2005; Gelenberg et al., 2010). As emesis is uncommon in rodents, I used the conditioned taste aversion test, which is recognized as a highly reliable tool for evaluation of behavioral alterations induced by radiation or other environmental agents that cause emesis and nausea (Rabin and Hunt, 1986). DSP-1053 significantly inhibited saccharin consumption compared to vehicle treatment, indicating this compound potential for nausea or feeling of vomiting. On Day 3, the lower saccharine consumption was observed in vehicle treated rats than Day 2. This phenomenon was also reported in previous study (Hatcher et al., 1998). Although the reason of this lower saccharine intake is not clear, the first experiment for new taste might defeat the appetite for water.

In addition, I used shrews (*Suncus murinus*) which can positively respond to various emetic stimuli including motion, X-radiation and emetogenic substances such as cisplatin and SSRI (Matsuki et al., 1992; Mine et al., 2013; Okada et al., 1995) to further evaluate DSP-1053 potential for emesis. As a result, single administration of DSP-1053 induced emesis in a dose-dependent manner, suggesting that acute treatment with DSP-1053 can produce emesis. This undesirable effect may be due to acute increase in 5-HT level following inhibition of serotonin transporter. On the other hand, the number of vomiting episodes decreased following multiple dosing with DSP-1053, but not paroxetine. This finding indicates that repeated treatment with DSP-1053 results in a fast adaptation to the feeling of nausea and therefore reduction in emetic episodes. It has been reported that enhancement of 5-HT_{1A} receptors activation can reduce vomiting and nausea in animals (Rock et al., 2014; Wolff and Leander, 1997). This beneficial effect is believed to be triggered by attenuation of 5-HT neurotransmission following somatodendritic 5-HT_{1A} receptors activation. On the other hand, DSP-1053 enhanced 5-HT neurotransmission in rat microdialysis, indicating fast desensitization of the serotonergic system play an important role in the fast adaptation to the feeling of nausea and therefore emesis. It is reported that SSRI-induced nausea typically ceases as treatment continues (Peretti et al., 2000). Although the precise mechanism of

SSRI-induced nausea is still unclear, 5-HT₃ receptors in the chemoreceptor trigger zone are considered to have an important role, as 5-HT₃ antagonists, including cisapride and ondansetron, are reported to reduce SSRI-induced gastrointestinal side effects (Bergeron and Blier, 1994). In addition, HTR3B gene polymorphisms are considered as significant predictors of paroxetine-induced nausea (Sugai et al., 2006). Moreover, chronic activation of ionotropic 5-HT₃ receptors is believed to produce significant desensitization of 5-HT₃ receptors (see review e.g. Jackson and Yakel, 1995). Taken together, these findings indicate that DSP-1053 fast onset enhancement of 5-HT neurotransmission induces fast desensitization of 5-HT₃ receptors, resulting in fast adaptation to the feeling of nausea and emesis. However, further work is needed to confirm this hypothesis.

In conclusion, this study shows that DSP-1053, a novel serotonin reuptake inhibitor with 5-HT_{1A} receptor partial agonistic activity, shows fast antidepressant-like effect and fast adaptation to the feeling of nausea and emesis in rodent and shrew models. These results highlight the important role of 5-HT_{1A} receptors in both the efficacy and tolerability of DSP-1053 as a new therapeutic option for the treatment of depression.

Figures and Tables

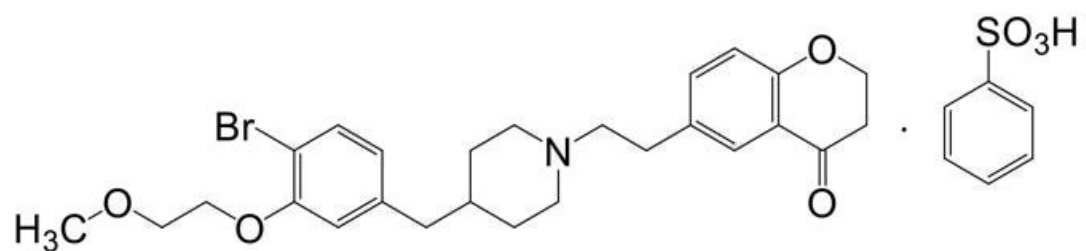


Figure 1

Chemical structure of DSP-1053

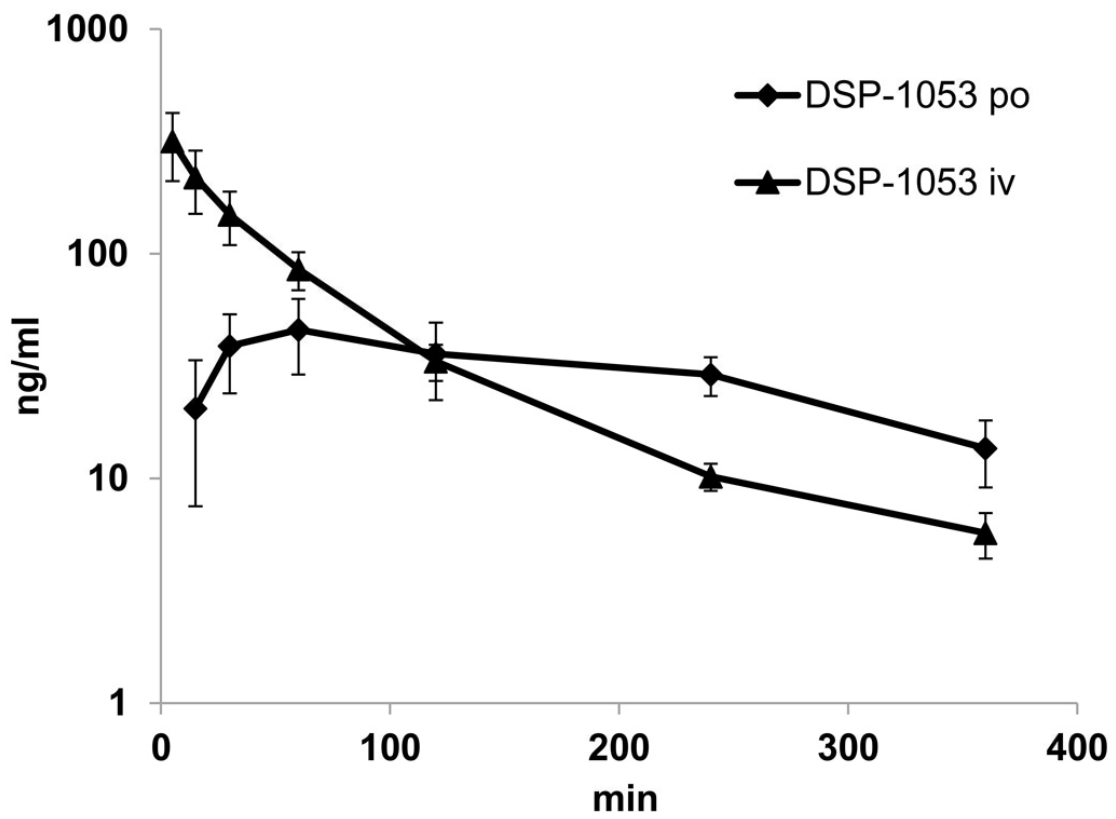


Figure 2

DSP-1053 plasma concentrations after single p.o. and i.v. administration to fed male rats. Data are the mean \pm S.D. (n=2 (for i.v.) or n=3 (for p.o.)).

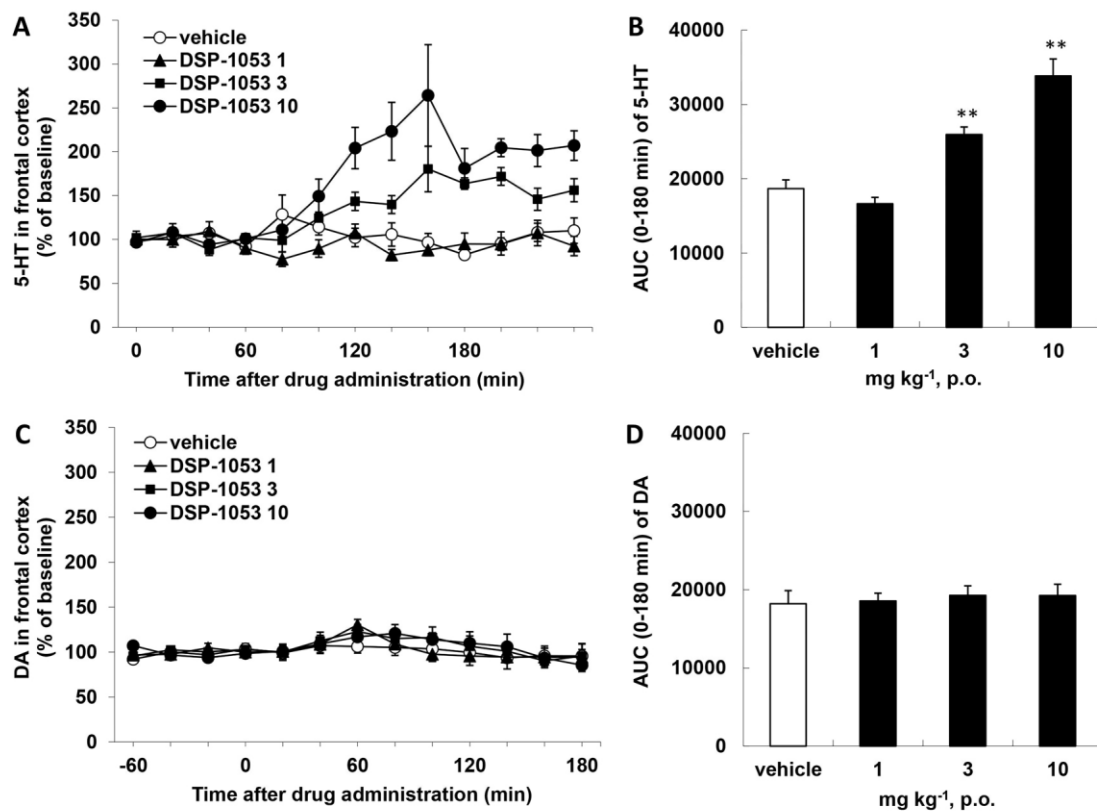


Figure 3

Effects of DSP-1053 on 5-HT and dopamine levels in rat frontal cortex. (A, C) Time-course changes in 5-HT and dopamine (DA) levels in the rat frontal cortex after DSP-1053 p.o. administration. Each point with a vertical bar represents the mean \pm S.E.M of percentage baseline value. (B, D) Effects of DSP-1053 on extracellular 5-HT and DA levels in the rat frontal cortex. Each column with vertical bar represents the mean \pm S.E.M of AUC of 5-HT or DA percent over 3 h. ** $p < 0.01$, compared to the vehicle-treated group using parametric Dunnett's multiple comparison test. Vehicle group, $n=6$; DSP-1053 1 and 3 mg kg⁻¹ groups, $n=4$; and DSP-1053 10 mg kg⁻¹, $n=3$.

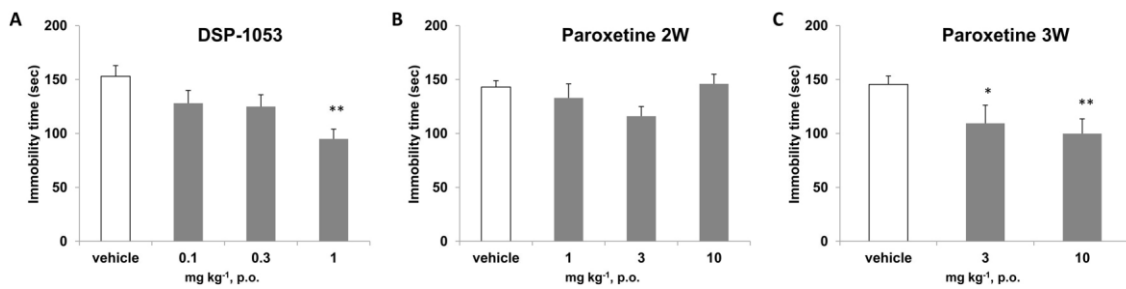


Figure 4

Effects of DSP-1053 (A) and paroxetine (B and C) on immobility time in the forced swimming test in rat. Each bar represents the mean ± S.E.M of immobility time during a 5 min test session (n=16-18 per group). * $p < 0.05$, ** $p < 0.01$, compared to the vehicle-treated group using parametric Dunnett's multiple comparison test.

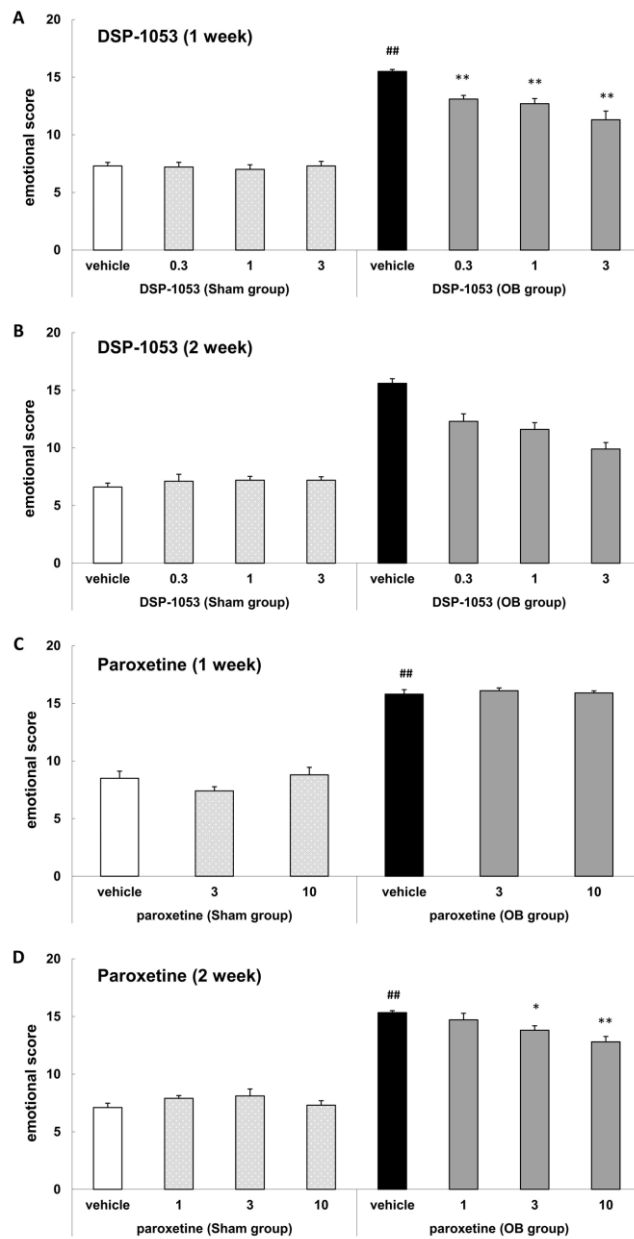


Figure 5

Effects of DSP-1053 (A, B) and paroxetine (C, D) on emotional score in sham-operated and olfactory bulbectomized (OB) rats. Each bar represents the mean \pm S.E.M (n=9-10 per group). ## $p < 0.01$ OB group vs sham-operated group (t-test with two-sided significance of 5%). * $p < 0.05$, ** $p < 0.01$ vs vehicle treated subgroup in OB group (parametric Dunnett's multiple comparison test with two-sided significance of 5%).

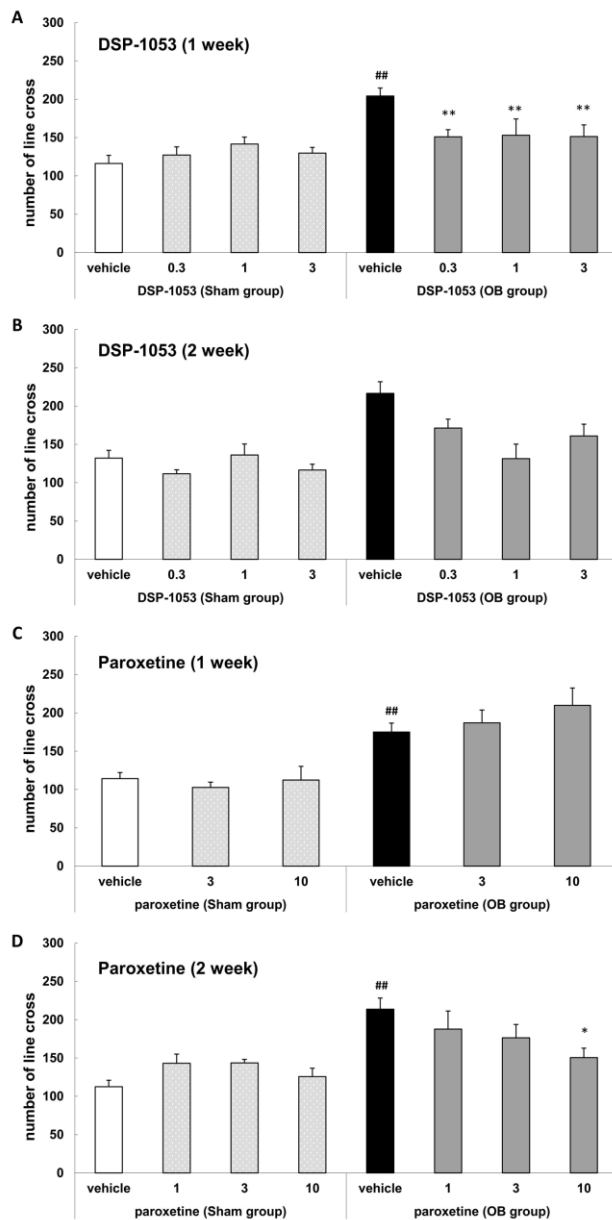


Figure 6

Effects of DSP-1053 (A, B) and paroxetine (C, D) on the number of line crosses in sham-operated and OB rats. Each bar represents the mean \pm S.E.M (n=9-10 per group). ^{##} $p < 0.01$ OB group vs sham-operated group with vehicle treatment (t-test with two-sided significance of 5%). * $p < 0.05$, ** $p < 0.01$ vs vehicle treated subgroup in OB group (parametric Dunnett's multiple comparison test with two-sided significance of 5%).

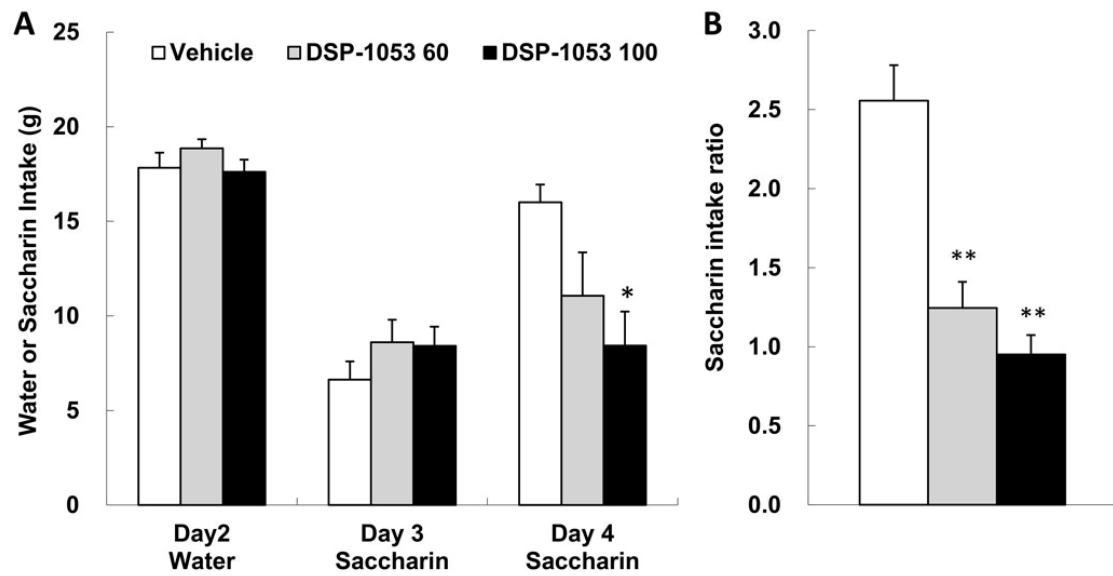


Figure 7

Effect of DSP-1053 on saccharin consumption in rat conditioned taste aversion test. (A) Each bar represents the mean \pm S.E.M of water or saccharin consumption (g) on each day (n=6). (B) Each bar represents the mean \pm S.E.M of saccharin consumption ratio of day 4/day 3. * $p < 0.05$, ** $p < 0.01$, compared with the vehicle-treated group using Turkey's test.

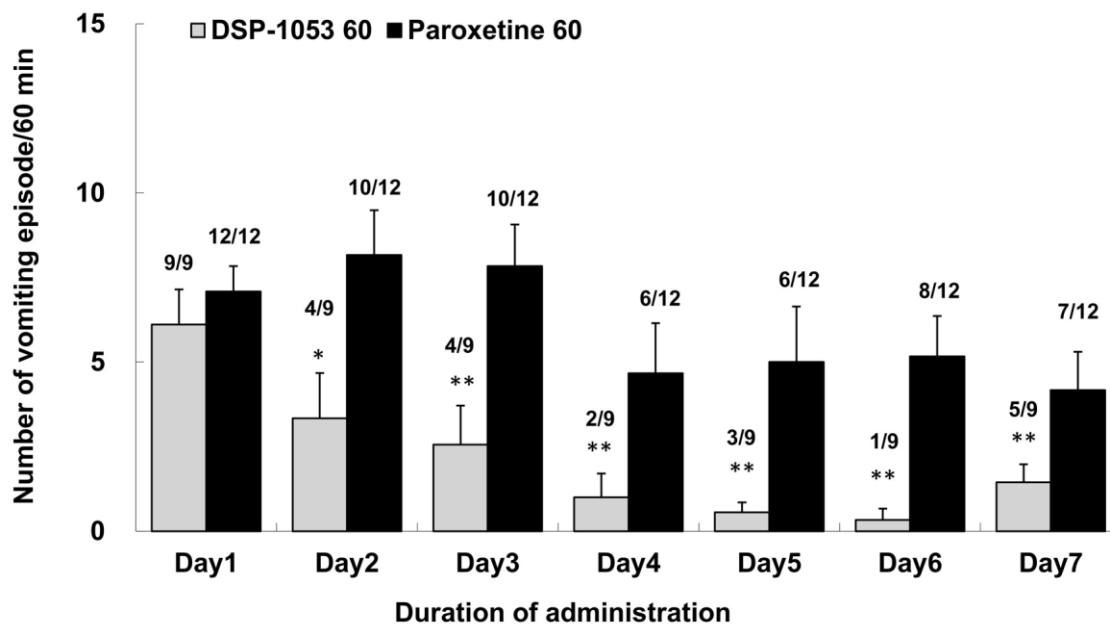


Figure 8

Effects of 7-day treatment with DSP-1053 or paroxetine on the number of vomiting episodes in *Suncus murinus*. Each column represents the mean \pm S.E.M. of the number of emetic episodes. The number above each column represents the incidence of vomiting as the number of animals that vomited / the number of animals tested. * $p < 0.05$, ** $p < 0.01$, significantly different from the number of vomiting episodes observed in Day 1 using Dunnett's multiple comparison test.

Table 1DSP-1053 *in vitro* binding to the serotonin transporter

Drug	IC ₅₀ (nM)	K _d (nM)	K _i (nM)
Human SERT binding			
DSP-1053	1.56 ± 0.09		1.02 ± 0.06
Paroxetine	0.279 ± 0.014	3.45 ± 0.19	0.183 ± 0.008
Imipramine	3.60 ± 0.07		2.36 ± 0.04
Rat SERT binding			
DSP-1053	8.84 ± 0.07	1.14 ± 0.02	0.489 ± 0.039
5-HT uptake inhibition			
DSP-1053	2.47 ± 0.41		
Paroxetine	0.181 ± 0.020	-	-
Imipramine	3.73 ± 0.27		

Table 2DSP-1053 *in vitro* binding to the 5-HT_{1A} receptor

Drug	IC ₅₀ / EC ₅₀ (nM)	K _d (nM)	K _i (nM)	I.A. (%)
Human 5-HT _{1A} binding				
DSP-1053	9.92 ± 2.11		5.05 ± 1.07	
WAY-100635	0.958 ± 0.130	0.313 ± 0.045	0.420 ± 0.060	-
Pindolol	21.6 ± 3.0		9.39 ± 1.30	
8-OH-DPAT	10.3 ± 1.5		4.47 ± 0.64	
Rat 5-HT _{1A} binding				
DSP-1053	10.7 ± 2.1	0.275 ± 0.009	5.09 ± 1.03	-
GTPγS binding				
DSP-1053	98.0 ± 34.9			70.0 ± 6.3
WAY-100635	n.d.			2.60 ± 3.88
Pindolol	n.d.	-	-	13.3 ± 3.8
8-OH-DPAT	58.0 ± 11.3			102 ± 1

Table 3DSP-1053 (1 μ M) inhibition of radioligand binding to various receptors

Target	Radioligand	Conc. (nM)	% inhibition at 1 μ M	Ki (nM) (n=3)
hum adrenergic α_{1A}	[¹²⁵ I] HEAT	0.15	57	528 \pm 45
hum adrenergic α_{1B}	[³ H] Prazosin	1.2	1	-
hum adrenergic α_{2A}	[³ H] Rawolscine	1.3	26	-
hum adrenergic α_{2B}	[³ H] Rawolscine	1.3	6	-
hum adrenergic α_{2C}	[³ H] Rawolscine	1.3	41	-
hum adrenergic β_1	[³ H] (-)-CGP-12177	0.21	14	-
hum adrenergic β_2	[³ H] (-)-CGP-12177	0.21	22	-
hum adrenergic β_3	[¹²⁵ I] (-)-Cyanopindolol	0.060	19	-
hum dopamine D ₁	[³ H] SCH-23390	0.48	4	-
hum dopamine D _{2L}	[³ H] Spiperone	2.0	35	-
hum dopamine D ₃	[³ H] R-(+)-7-OH-DPAT	0.38	65	249 \pm 21
rat GABA _A	[³ H] Flunitrazepam	0.88	8	-
rat GABA _B	[³ H] GABA	5.2	0	-
hum histamine H ₁	[³ H] Pyrilamine	5.2	93	7.46 \pm 0.20
hum histamine H ₂	[³ H] Tiotidine	5.1	37	-
hum muscarinic M ₁	[³ H] N-Methylscopolamine	0.37	9	-
hum muscarinic M ₂	[³ H] N-Methylscopolamine	0.37	21	-
hum opiate δ	[³ H] Naltrindole	0.88	10	-
hum opiate κ	[¹²⁵ I] Diprenorphine	0.71	0	-
hum opiate μ	[¹²⁵ I] Diprenorphine	0.64	2	-
gp serotonin 5-HT ₄	[³ H] GR-113808	0.55	26	-
hum serotonin 5-HT _{1B}	[³ H] 5-HT	8.9	47	-
hum serotonin 5-HT _{2A}	[³ H] Ketanserin	0.48	35	-
hum serotonin 5-HT _{2B}	[¹²⁵ I] LSD	0.29	79	117 \pm 28
hum serotonin 5-HT _{2C}	[³ H] Mesulergine	1.8	20	-
hum serotonin 5-HT ₃	[³ H] GR-65630	0.47	0	-
hum serotonin 5-HT _{5A}	[¹²⁵ I] LSD	0.020	2	-
hum serotonin 5-HT ₆	[¹²⁵ I] LSD	0.020	14	-
hum serotonin 5-HT ₇	[¹²⁵ I] LSD	0.020	73	288 \pm 35

Table 4

Incidence of vomiting and total emetic episode

Drugs	Dose (mg kg ⁻¹ , po)	Number of animals vomited/tested	Total emetic episode
Vehicle	-	0/6	-
DSP-1053	10	0/6	-
	30	1/6	2
	60	3/6	5.3 ± 1.0
Paroxetine ¹⁾	10	0/6	-
	30	2/6	6, 8
	60	**6/6	6.7 ± 0.5

Incidence of vomiting is shown as the number of shrews that vomited / the number of shrews tested. ** $p < 0.01$ vs. vehicle treatment, as analyzed by Fisher's exact test. Total emetic episodes during the 1 h observation period were calculated for animals that vomited and expressed as mean ± S.E.M. ¹⁾ Mine et al., 2013.

Part-2

DSR-98776, a novel selective mGlu₅ receptor negative allosteric modulator with potent antidepressant and antimanic activity

Abstract

Modulation of monoaminergic systems has been the main stream of treatment for patients with mood disorders. However, recent evidence suggests that the glutamatergic system plays an important role in the pathophysiology of these disorders. This study pharmacologically characterized a structurally novel metabotropic glutamate 5 (mGlu₅) receptor negative allosteric modulator, DSR-98776, and evaluated its effect on rodent models of depression and mania. First, DSR-98776 *in vitro* profile was assessed using intracellular calcium and radioligand binding assays. This compound showed dose-dependent inhibitory activity for mGlu₅ receptors by binding to the same allosteric site as 2-methyl-6-(phenylethynyl)-pyridine (MPEP), a known mGlu₅ inhibitor. The *in vivo* therapeutic benefits of DSR-98776 were evaluated in common rodent models of depression and mania. In the rat forced swimming test, DSR-98776 (1-3 mg kg⁻¹) significantly reduced rats immobility time after treatment for 7 consecutive days, while paroxetine (3 and 10 mg kg⁻¹) required administration for 2 consecutive weeks to reduce

rats immobility time. In the mouse forced swimming test, acute administration of DSR-98776 (10-30 mg kg⁻¹) significantly reduced immobility time. This effect was not influenced by 4-chloro-DL-phenylalanine methyl ester hydrochloride-induced 5-HT depletion. Finally, DSR-98776 (30 mg kg⁻¹) significantly decreased methamphetamine/chlordiazepoxide-induced hyperactivity in mice, which reflects this compound antimanic-like effect. These results indicate that DSR-98776 acts as an orally potent antidepressant and antimanic in rodent models and can be a promising therapeutic option for the treatment of a broad range of mood disorders with depressive and manic states.

Purpose

In Part-2, I report the *in vitro* profile of a structurally novel mGlu₅ receptor negative allosteric modulator, DSR-98776, using intracellular calcium and radioligand binding assays. Furthermore, I evaluate the antidepressant and antimanic efficacy of this compound in the rat and mouse forced swimming test and in the mouse methamphetamine/chlordiazepoxide-induced hyperactivity model.

Materials and methods

Animals

In part-2, all animals were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and used after a quarantine period of 7 days. The rats and mice were housed in the same condition as part-1.

Chemical reagents

DSR-98776

((3-fluorophenyl)[2-(5-fluoropyridin-2-yl)]-6,7-dihydro[1,3]oxazolo[4,5-c]pyridin-5(4H)-yl)methanone, Figure 1), paroxetine hydrochloride (paroxetine), and methamphetamine were synthesized in my laboratories. DSR-98776 was synthesized through amide-condensation reaction between 3-fluorobenzoyl chloride and 2-(5-fluoropyridin-2-yl)-4,5,6,7-tetrahydrooxazolo[4,5-c]pyridine which was prepared from 4-fluoropicolinic acid according to the published procedure (Burdi et al., 2010) except that Burgess' reagent was used instead of POCl₃ in oxazole formation step. Total yield from 4-fluoropicolinic acid to DSR-98776 was 54%. PCPA (4-chloro-DL-phenylalanine methyl ester hydrochloride), chlordiazepoxide hydrochloride (chlordiazepoxide), lithium chloride (lithium), and carbamazepine were

purchased from Sigma Aldrich Japan (Tokyo, Japan). All radioligands were purchased from Perkin Elmer Japan (Kanagawa, Japan). For oral administration, DSR-98776, paroxetine, lithium, and carbamazepine were dissolved in 0.5% methylcellulose. For intraperitoneal (i.p.) administration, PCPA, chlordiazepoxide and methamphetamine were dissolved in saline. Dosing volume was determined based on each animal body weight measured in the morning of each administration day (5 mL kg⁻¹ for rats and 10 mL kg⁻¹ for mice).

Intracellular calcium assay

The intracellular calcium assay was conducted using HEK 293 cells stably expressing cloned human mGlu receptors, i.e. mGlu₁, ₂, ₄, ₅, ₆, and ₈ receptors. The cells were cultured in DMEM (high glucose) supplemented with 10% dialyzed fetal bovine serum and 200 µg mL⁻¹ G418 (culture medium), and transiently expressed apoaequorin as previously described (Stables et al., 1997). Gα16 subunit (for mGlu₂ receptor) or G-protein chimera protein (for mGlu₄, ₆, and ₈ receptors) was also expressed concomitantly with apoaequorin. The cells were plated at a density of 1500 (for mGlu₂ and ₅ receptors), 2000 (for mGlu₄ and ₆ receptors), 4000 (for mGlu₈ receptor) and 5000 (for mGlu₁ receptor) cells/50 µL/well into black 384-well plates with clear bottoms and

incubated overnight at 37°C in the presence of 5% CO₂. The following day, 10 µL of assay buffer (1x Hanks supplemented with 20 mM HEPES and 0.1% BSA) containing 30 nM of coenlenterazine-h (for mGlu₁, 2, 4, 5, and 8 receptors) or 3 µM of ViviRen (for mGlu₆ receptor) was loaded into each well containing the cells. After 4 h incubation at R.T., 10 µL of assay buffer containing each test-compound at the indicated concentrations was applied to the cells. Two minutes later, assay buffer containing 20 µM (for mGlu₂ receptor), 10 µM (for mGlu₄ receptor), 2 µM (for mGlu₅ and 8 receptors), 100 µM (for mGlu₁ receptor) or 200 µM (for mGlu₆ receptor) of glutamate was added, and the fluorescence produced was measured by a functional drug screening system (FDSS7000 Hamamatsu Photonics K.K., Japan). As numerical treatment, 2 s was integrated for 1 min evaluation.

Radioligand binding assay

In a total volume of 200 µL, 1 µL of each test-substance (glutamate, MPEP or DSR-98776) or DMSO, 50 µL of assay buffer [15 mM Tris-HCl (pH7.6) containing 120 mM NaCl, 100 mM KCl, 25 mM CaCl₂, and 25 mM MgCl₂ for allosteric binding or 20 mM Tris-HCl (pH7.2) containing 2 mM CaCl₂ and 2 mM MgCl₂ for orthosteric binding] containing a radioligand (2 nM of [³H]-MPEP for allosteric binding or 37.5 nM

of [³H]-quisqualic acid for orthosteric binding), and 149 μL of assay buffer containing HEK293 cell membranes (0.15 μg μL⁻¹) stably expressing cloned human mGlu₅ receptor were mixed. The reaction was started by addition of the cell membranes. To evaluate the binding affinity for both the allosteric and orthosteric sites, the samples were reacted at 25°C for 1 h in a dry block bath. GF/B glass filters coated for 1 h with 0.1% polyethyleneimine and dried under low pressure were used to terminate the reaction by filtration under vacuum. The glass filters were washed with 200 μL of ice-cold 50 mM Tris-HCl (pH7.6) and placed in scintillation vials with 2 mL of ACS II scintillation fluid (GE health care bioscience). Measurement of radioactivity was conducted as described in part-1.

Off-target radioligand binding assays and enzyme assays

To determine DSR-98776 interaction with off-target receptors, transporters, channels, and enzymes, 43 binding assays and 2 enzyme assays (catechol-O-methyltransferase and monoamine oxidase-A) were conducted on my behalf by Eurofines Panlabs Inc. The binding assays were carried out using standard techniques as summarized in Table 1. As for the enzyme assays, pig catechol-O-methyltransferase activity was evaluated using 3.0 mM catechol + S-adenosyl-L-[methyl-³H]-methionine as substrate and the

amount of the produced [³H]-guaiacol was quantitated. Monoamine oxidase-A activity was evaluated using 50 μM kynuramine as substrate and the amount of the formed 4-hydroxyquinoline was quantitated.

***Ex vivo* inhibition of [³H]-3-methoxy-5-pyridin-2-ylethynylpyridine (MPEPy) binding**

One hour after administration of MPEP or DSR-98776 to 5-week old male CD1 (ICR) mice (n=4 per group) and 7-week old male SD rats (n=6 per group), the animals were sacrificed, and the brain was rapidly removed and immediately dissociated on ice into the forebrain (for mice) or frontal cortex (for rats). The prepared tissues were homogenized in ice-cold 50 mM Tris-HCl (pH7.5) containing 0.9% NaCl (assay buffer) using a motor-driven glass-Teflon homogenizer, and subsequently diluted with the assay buffer to 25 mg mL⁻¹. In a total volume of 52 μL, 1 μL of DMSO for measurement of total binding or 10 mM of MPEP for measurement of non-specific binding, 1 μL of assay buffer [50 mM Tris-HCl (pH7.5) containing 0.9% NaCl] containing 52 nM of [³H]-MPEPy, and 50 μL of the prepared tissue membranes were mixed. The reaction was started by addition of the tissue membranes and allowed to proceed for 1 h at R.T. GF/B glass filters coated for 1 h with 0.5% polyethyleneimine and dried under low

pressure were used to terminate the reaction by filtration under vacuum. The glass filters were washed with 200 μ L of ice-cold 50 mM Tris-HCl (pH7.5) and placed in scintillation vials with 2 mL of ACS II scintillation fluid (GE health care bioscience). Measurement of radioactivity was conducted as described in part-1.

Rat forced swimming test

The test was performed using 7-week old male Wistar rats. Test-compounds antidepressant-like activity was assessed as described in part-1 except for conducting swimming test on day 7 or 14.

Mouse forced swimming test

The test was performed using 5-week old male CD1 (ICR) mice. Test compounds antidepressant-like activity was assessed using an automatic behavior analysis system, SCANET AQ (Melquest Ltd., Toyama, Japan). One hour after dosing with DSR-98776, paroxetine or the vehicle, each animal was gently placed into a plastic cylinder (22.5 cm in height, 14 cm in diameter) containing 5.0 L of water set at $25 \pm 1^\circ\text{C}$. The animal immobility time was recorded for 6 min and determined in seconds by SCANET AQ. Animals dosing and swimming test were conducted between 1:00PM and 5:00PM. To

deplete 5-HT, the mice were administered 300 mg kg⁻¹ of PCPA, an irreversible tryptophan hydroxylase inhibitor, once a day for 3 consecutive days between 7:00AM and 7:00PM. The next day, PCPA was administered in the morning, followed by the forced swimming test as described above.

Methamphetamine/chlordiazepoxide induced hyperactivity in mice

Thirty minutes after oral administration of dosing suspensions to 8-week old C57/BL6J mice, methamphetamine (10 mg kg⁻¹), a mixture of methamphetamine (10 mg kg⁻¹) and chlordiazepoxide (4 mg kg⁻¹), or saline was administered to each mouse. The animal was then placed in a plastic cage (25 x 38 x 30.5 cm), and its behavior was recorded for 90 min. After the test session, the recordings were analyzed by an Etho Vision 3.1 system (Sophia Scientific Co., Ltd., Aichi, Japan).

Spontaneous locomotion

This experiment was performed using 7-week old male Wistar rats. One hour after administration of DSR-98776 or 0.5% methylcellulose, the animals were placed in plastic cages, and their spontaneous locomotion was measured for 15 min by an automatic behavior analyzing system, SCANET AQ (Melquest Ltd., Toyama, Japan).

Data analysis

EC₅₀ values of glutamate, IC₅₀ values of DSR-98776 and MPEP in the intracellular calcium assay, and RO₅₀ value of DSR-98776 and MPEP in *ex vivo* binding assay were determined using Dx calculation (logistic curve fitting) with measured value input function in Stat Prelinica Version 1.0.3295. In the rat and mouse forced swimming test, differences in immobility time and spontaneous locomotion between test-compounds treated groups and the vehicle treated group were analyzed with one-way ANOVA followed *post hoc* parametric Dunnett's multiple comparison test. In the methamphetamine/chlordiazepoxide-induced hyperactivity model, differences in spontaneous locomotion between the test compound-saline treated group vs. vehicle-saline treated group, compound-methamphetamine treated group vs. vehicle-methamphetamine treated group, and compound-methamphetamine/chlordiazepoxide treated group vs. vehicle-methamphetamine/chlordiazepoxide treated group were analyzed with one-way ANOVA followed *post hoc* parametric Dunnett's multiple comparison test. In PCPA induced 5-HT depletion model, the effect of PCPA, DSR-98776, and paroxetine on immobility time and the interaction between PCPA and DSR-98776 or paroxetine were analyzed with two-way ANOVA. Statistical analysis in

the ANOVA and the parametric Dunnett's multiple comparison tests was performed using SAS system, and values of $p < 0.05$ (two-tailed test) were considered as statistically significant.

Results

Intracellular calcium assay

Glutamate concentration-dependently increased intracellular calcium concentration in HEK293 cells stably expressing cloned human mGlu₅ receptors with an EC₅₀ value of 311.1 ± 50.3 nM (mean ± S.E.M. n=3) (Figure 2). To confirm the inhibitory activity of DSR-98776 on mGlu₅ receptors, changes in the concentration-response curve for calcium response stimulated by glutamate in the presence of DSR-98776 at the concentration of 0, 30, 100, 300 nM, and 1 μM were evaluated. The effects of MPEP at 0, 3, 10, 30, and 100 nM on calcium concentration-response curves were also evaluated. DSR-98776 shifted glutamate concentration-response curve to the right and decreased maximal response (Figure 2A). These changes were consistent with those produced by MPEP (Figure 2B). DSR-98776 and MPEP attenuated the increase in calcium response caused by 1 μM of glutamate (a concentration that gave about 80% of maximum agonist response) with IC₅₀ values of 66.7 ± 5.9 and 3.9 ± 0.7 nM, respectively.

Radioligand binding assay

The binding site of DSR-98776 for the mGlu₅ receptor was determined using radioligands capable of binding to either the orthosteric or allosteric site of mGlu₅

receptor. MPEP (1 μ M) and DSR-98776 (10 μ M) did not inhibit the binding of [3 H]-quisqualic acid to mGlu₅ receptor orthosteric site. The relative inhibitory percents to total binding were -11.3 ± 5.1 and $-1.4 \pm 6.2\%$, respectively, mean \pm S.E.M. (n=3, Figure 3A). On the other hand, glutamate (1 mM), an intrinsic ligand for the mGlu₅ receptor, almost completely blocked the binding of [3 H]-quisqualic acid with a relative inhibitory percent to total binding of $91.9 \pm 6.9\%$, mean \pm S.E.M. (n=3). As for mGlu₅ receptor allosteric site, both MPEP (1 μ M) and DSR-98776 (10 μ M), but not glutamate (1 mM), inhibited the binding of [3 H]-MPEP to this site [relative % inhibition of 98.2 ± 1.3 (MPEP), 92.9 ± 1.0 (DSR-98776), and -19.5 ± 18.5 (glutamate), mean \pm S.E.M. n=3] (Figure 3B).

DSR-98776 effects on off-target receptors, channels, transporters and enzymes

DSR-98776 (1 and 10 μ M) showed much weaker inhibitory activity for other mGlu receptors, including mGlu₁, ₂, ₄, ₆, and ₈ receptors than for the mGlu₅ receptor (Table 1). In addition, in the 43 conducted binding assays for various receptors, channels, and transporters, DSR-98776 showed little affinity (< 50% inhibition at 10 μ M) for all the off-targets listed in Table 2. Moreover, DSR-98776 did not have any inhibitory activity on pig catechol-O-methyltransferase and human monoamine oxidase-A even at 10 μ M

(-8 and 4 %, respectively).

DSR-98776 *ex vivo* inhibition of [³H]-MPEPy binding to mGlu₅ receptor

Next, DSR-98776 and MPEP occupancy of mGlu₅ receptor was evaluated by *ex vivo* inhibition of [³H]-MPEPy binding to this receptor. In the mouse forebrain, DSR-98776 and MPEP produced a dose-dependent mGlu₅ receptor occupancy with RO₅₀ (the dose causing 50% of receptor occupancy) values of 5.5 and 5.6 mg kg⁻¹, respectively (Figure 4A). In the rat cortex, DSR-98776 also produced dose-dependent receptor occupancy with an RO₅₀ value of 0.74 mg kg⁻¹. In addition, occupancy rates after daily oral administration of DSR-98776 for 4 days were almost equivalent to the occupancy rate produced by DSR-98776 after single oral administration (Figure 4B).

DSR-98776 antidepressant-like effect in the rat forced swimming test

As shown in Figure 5A, DSR-98776 significantly decreased immobility time following a 7-day consecutive administration at 1 and 3 mg kg⁻¹ compared to the vehicle [$F(3,38) = 6.52, p < 0.01$]. This compound had no effect on spontaneous locomotion in an open field throughout the treatment period [$F(3,20) = 0.65, p > 0.05$] (Figure 5B). On the other hand, animals treated with paroxetine (3 and 10 mg kg⁻¹) for 14 days reduced

immobility time compared to the animals treated with the vehicle [$F(3,33) = 3.29, p < 0.05$] (Figure 5C). Treatment with paroxetine for 7-day had no effect on rats immobility time [$F(2,15) = 0.09, p > 0.05$] (Figure 5D).

DSR-98776 antidepressant-like effect in the mouse forced swimming test

As shown in Figure 6A, DSR-98776 at 10 and 30 mg kg⁻¹ significantly decreased immobility time compared to vehicle treatment [$F(3,32) = 8.70, p < 0.01$]. Mice treated with paroxetine at 30 mg kg⁻¹ also showed significant reduction of immobility time [$F(3,32) = 5.15, p < 0.01$] (Figure 6B).

DSR-98776 antidepressant-like effect in mice with depleted 5-HT

The antidepressant-like effects of DSR-98776 and paroxetine in mice with depleted 5-HT (PCPA-treated mice) were evaluated in the forced swimming test. Two-way ANOVA revealed that DSR-98776 (30 mg kg⁻¹) significantly decreased mice immobility time [$F(1,59) = 24.11, p < 0.001$]. As shown in Figure 7A, PCPA alone didn't alter mice immobility time compared to the vehicle [$F(1,59) = 0.39, p > 0.05$], and it didn't significantly affect DSR-98776-induced decrease in mice immobility time [interaction: $F(3,59) = 0.73, p > 0.05$]. Although paroxetine also significantly reduced mice

immobility time [$F(1,60) = 7.94, p < 0.01$], unlike DSR-98776, pretreatment with PCPA inhibited paroxetine-induced decrease in mice immobility time [interaction: $F(1,60) = 4.01, p < 0.05$] (Figure 7B).

DSR-98776 anti-manic effect in methamphetamine/chlordiazepoxide-induced hyperactivity in mice

As shown in Figure 8A, DSR-98776 (30 mg kg⁻¹) significantly decreased methamphetamine/chlordiazepoxide-induced hyperactivity [$F(2,27) = 9.17, p < 0.01$], which reflects manic state. On the other hand, DSR-98776 had no significant effect on baseline locomotor activity in the vehicle-vehicle treated group and in methamphetamine alone-induced hyperactivity [baseline, $F(2,21) = 2.16, p > 0.05$; methamphetamine alone, $F(2,27) = 5.21, p < 0.05$ (Dunnet's *post hoc* test; $p > 0.05$ at 10 and 30 mg/kg vs. vehicle treated group)]. As with DSR-98776, lithium (100 mg kg⁻¹), which is clinically used as a mood stabilizer, reduced methamphetamine/chlordiazepoxide-induced hyperactivity [$F(2,13) = 7.04, p < 0.01$] without affecting baseline locomotor activity and methamphetamine alone-induced hyperactivity [baseline, $F(2,15) = 3.63, p > 0.05$; methamphetamine alone, $F(2,13) = 0.61, p > 0.05$] (Figure 8B). Carbamazepine (100 mg kg⁻¹) on the other hand, reduced

not only methamphetamine/chlordiazepoxide-induced hyperactivity [$F(2,13) = 18.46$, $p < 0.01$], but also methamphetamine alone-induced hyperactivity [baseline, $F(2,14) = 3.18$, $p > 0.05$; methamphetamine alone, $F(2,14) = 29.22$, $p < 0.01$] (Figure 8C).

Discussion

Most of the reported mGlu₅ receptor negative allosteric modulators have a typical di-substituted alkyne structure as the central scaffold (Gasparini et al., 1999; Kubas et al., 2013). However, I succeeded in obtaining a selective mGlu₅ receptor negative allosteric modulator, DSR-98776, without this core structure. My purpose in this study was to characterize this compound *in vitro* profile and to elucidate its *in vivo* therapeutic benefits in common rodent models of depression and mania. As shown in Figure 2, DSR-98776 shifted glutamate concentration-response curve to the right and decreased its maximal response in the intracellular calcium assay with cells expressing the human mGlu₅ receptor. In addition, I found that DSR-98776 did not affect the binding of [³H]-quisqualic acid to the orthosteric site of mGlu₅ receptor, but completely suppressed the binding of [³H]-MPEP to the allosteric site of mGlu₅ receptor. These findings indicate that DSR-98776 acts as a mGlu₅ receptor noncompetitive antagonist that occupies the same allosteric site of mGlu₅ receptor as MPEP, despite the two compounds structural differences. On the other hand, the IC₅₀ value of DSR-98776 for glutamate induced calcium efflux was 66.7 ± 5.9 nM, which was about 17 times weaker than that of MPEP. Porter et al. (2005) have also reported another mGlu₅ receptor negative allosteric modulator, fenobam, that has weaker functional potency than MPEP

and MTEP. In addition, a recent report has shown that Novartis' Mavoglurant (AFQ-056) exhibits an IC_{50} value of 71.6 nM in intracellular calcium detection assay (Kubas et al., 2013). These results indicate that the inhibitory activity of DSR-98776 on the mGlu₅ receptor is comparable to that of representative mGlu₅ receptor negative allosteric modulators that show promising efficacy in *in vivo* models. Furthermore, my off-target radiobinding assay together with functional and enzyme assays confirmed that DSR-98776 is a highly selective mGlu₅ receptor negative allosteric modulator.

As mGlu₅ receptor inhibitors are believed to have beneficial effects on various depressive states (Pałucha et al., 2005; Pałucha and Pilc, 2007; Pilc et al., 2002; Swanson et al., 2005; Witkin et al., 2007), I evaluated the effects of DSR-98776 in the rat and mouse forced swimming test. DSR-98776 significantly reduced rats immobility time without affecting spontaneous locomotion. Rat forced swimming test is widely used as a behavioral screening tool for assessment of the benefits of all major classes of antidepressant, including tricyclic antidepressants, monoamine oxidase inhibitors, atypical antidepressants, and electroconvulsive shock (Borsini and Meli, 1988). A delayed onset of the effect of clinical treatments can be mimicked in this test, because low dose of antidepressants produce antidepressant-like efficacy only after chronic treatment (Detke et al., 1997; Trivedi et al., 2006). In this study, DSR-98776 decreased

rats immobility time after 7 days treatment, while treatment with paroxetine, a representative SSRI, required 2 weeks for comparable efficacy. These results indicate that DSR-98776 would have earlier clinical onset than SSRIs in the treatment of depressive states. However, it would be interesting to evaluate the fast onset action of this compound in representative chronic models, such as chronic mild stress model, to support my results in the rat forced swimming test. In contrast to the significant dose-dependent efficacy of DSR-98776 in the forced swimming test, Pałucha et al. (2005) have shown that MTEP does not influence rats immobility time in the Porsolt's forced swimming test, but significantly alters mice immobility time in the tail suspension test. In this study, DSR-98776 was daily administered for 7 consecutive days compared to 3 times treatment with MTEP in Pałucha et al. report. In addition, it is believed that the dose of MTEP used in that report is beyond the effective dose range of MTEP (Pałucha-Poniewiera et al., 2014). Thus, I consider that appropriate dose range coupled with appropriate administration regimen are necessary for assessing drug efficacy in the rat forced swimming test.

Next, I confirmed the efficacy of DSR-98776 in the mouse forced swimming test and investigated whether this efficacy is achieved via serotonergic mechanisms using PCPA, which is frequently used to generate SSRI-resistant depression models with depleted

central 5-HT (Koe and Weissman, 1966; Pałucha-Poniewiera et al., 2014). In naïve mice, DSR-98776 and paroxetine significantly and dose-dependently reduced mice immobility time, suggesting an antidepressant-like effect. On the other hand, DSR-98776 showed significant antidepressant-like efficacy in both of PCPA- and saline-treated mice, while pretreatment with PCPA blocked the efficacy of paroxetine. These results indicate that DSR-98776 shows antidepressant-like efficacy via 5-HT independent mechanisms. In contrast to these results, Pałucha-Poniewiera et al. (2014) have shown the involvement of serotonergic system activation in the antidepressant-like effect of MTEP. Furthermore, Stachowicz et al. (2007) showed that MTEP dose-dependently increases 5-HT release in PFC in rat microdialysis study. It is difficult to show a reasonable explanation for these discrepancies, because MTEP is reported to be a selective for mGlu₅ receptor (Cosford et al., 2003). However, Cosford et al. (2003) showed that MTEP had weak monoamine oxidase-A inhibitory activity, which would contribute to inhibition of serotonin metabolisms. These findings suggest that a part of the antidepressant efficacy of MTEP is based on enhancement of 5-HT neurotransmission. Taken together, these results suggest that DSR-98776 would have fast acting antidepressant efficacy based on 5-HT independent mechanisms.

Inhibition of mGlu₅ receptors is expected to be beneficial not only as

antidepressant-like effect, but also as mood stabilizing effect. In addition to the fact that glutamine/glutamate ratio is significantly high in anterior cingulate cortex and parieto-occipital cortex in bipolar disorder (Öngür et al., 2008), it is reported that lithium, which is frequently used as mood stabilizer for patient with mania, robustly protects neurons against excitotoxicity, probably by reducing glutamatergic overstimulation (Nonaka et al., 1998). Other clinically effective drugs, such as antiepileptics are known to inhibit glutamate activity (Krystal et al., 2002). From this evidence, I considered that DSR-98776, which can modulate glutamatergic neurotransmission by inhibiting mGlu₅ receptor, would show a mood stabilizing effect. The antimanic effect of DSR-98776 was evaluated in methamphetamine/chlordiazepoxide-induced hyperactivity in mice. In this model, mice are treated with a mixture of a psychostimulant, including methamphetamine and D-amphetamine plus chlordiazepoxide. To distinguish antipsychotic drugs from mood stabilizers, a separate group of animals are treated with methamphetamine alone. Thus, test-drugs mood stabilizing efficacy can be detected by their efficacy against hyperactivity induced by a mixture of methamphetamine plus chlordiazepoxide and their lack of efficacy against hyperactivity induced by methamphetamine alone (Arban et al., 2005; Okada et al., 1990). In the present study, I also examined the effects of

lithium and carbamazepine as control drugs. Lithium is able to selectively reduce the hyperactivity induced by a mixture of methamphetamine plus chlordiazepoxide, while carbamazepine reduces not only the hyperactivity induced by a mixture of methamphetamine plus chlordiazepoxide, but also the hyperactivity induced by methamphetamine alone. On the other hand, DSR-98776, like lithium, significantly reduced the hyperactivity induced by a mixture of methamphetamine plus chlordiazepoxide at the dose that had no effect on basal activity and hyperactivity induced by methamphetamine alone. Arban et al. (2005) showed that carbamazepine and lamotrigine diminish the hyperactivity induced by methamphetamine alone and discussed that this effect on sodium channels would prevent the dopamine mediated modulation by D-amphetamine. In terms of the effect on dopamine neurotransmission, it is reasonable that DSR-98776 has no effect on the hyperactivity induced by methamphetamine alone, because this compound does not have binding affinity for dopamine related targets. Furthermore, it has been reported that lithium attenuates calcium release after desensitization of Group I mGlu receptors, including mGlu₅ receptor (Sourial-Bassillious et al., 2009). This finding indicates that DSR-98776 has a mood stabilizing effect based on non-dopaminergic mechanisms.

In conclusion, my results show that DSR-98776, a novel compound without typical

di-substituted alkyne, has a highly selective mGlu₅ receptor negative allosteric modulating effect and potent antidepressant-like efficacy. Furthermore, DSR-98776 has antimanic-like efficacy as indicated by the results in a rodent mania model. These findings indicate that DSR-98776 has great potential in the treatment of a broad range of mood disorders.

Figures and Tables

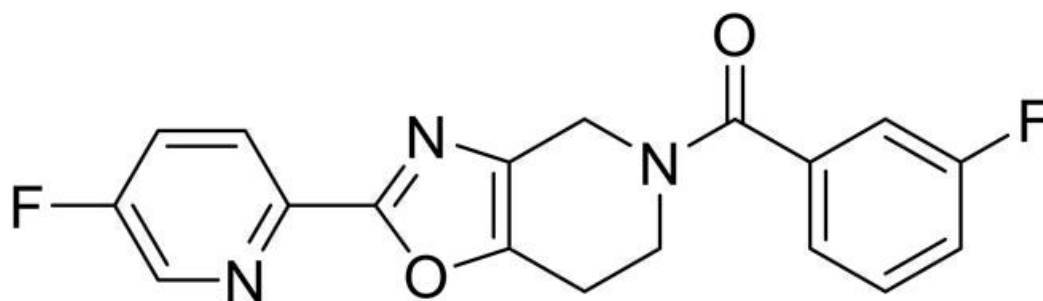


Figure 1

Chemical structure of DSR-98776

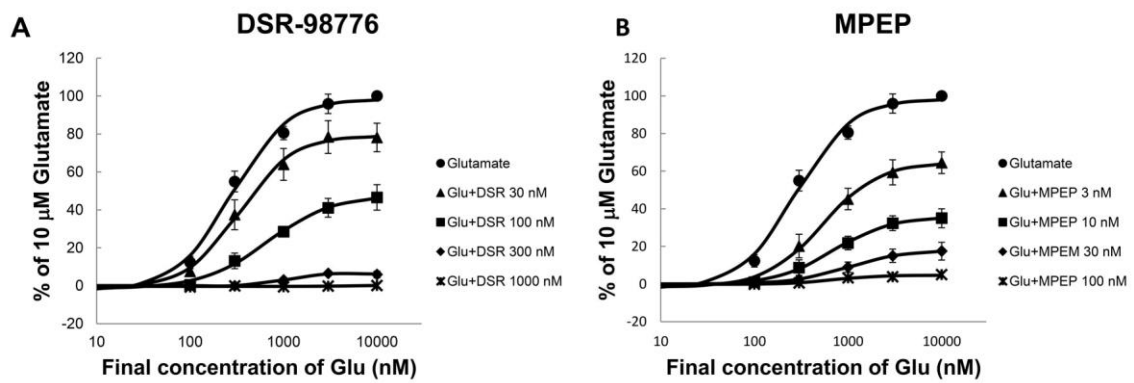


Figure 2

DSR-98776 and MPEP inhibition of calcium efflux in HEK293 cells expressing the human mGlu₅ receptor. (A, B) Effects of DSR-98776 and MPEP on concentration–response curve of glutamate for human mGlu₅ receptor. Data symbols represent the maximum fluorescent signal obtained at each concentration as mean \pm S.E.M (n=3). Each single experiment was performed in quadruplicate.

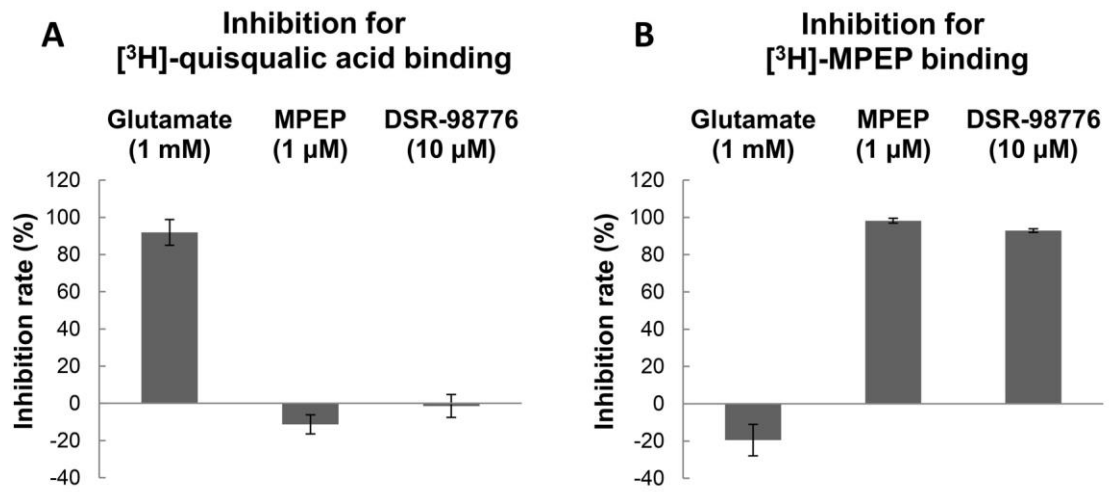


Figure 3

Inhibition of labeled compounds binding to the orthosteric or allosteric site of mGlu5 receptor. (A) Glutamate, MPEP and DSR-98776 inhibition of [3H]-quisqualic acid binding to the orthosteric site of mGlu5 receptor. Each bar represents the mean \pm S.E.M (n=3). (B) Test-compounds inhibition of [3H]-MPEP binding to the allosteric site of mGlu5 receptor. Each bar represents the mean \pm S.E.M (n=3). Each single experiment was performed in duplicate.

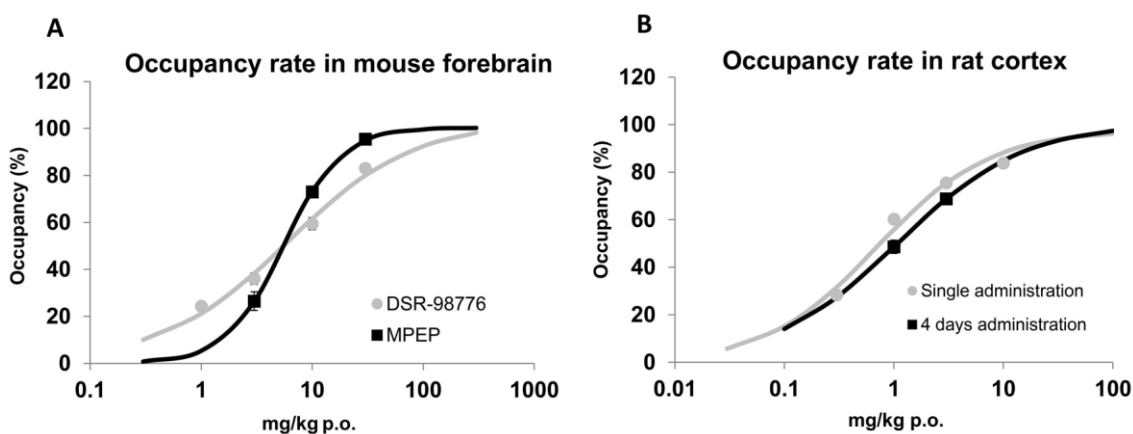


Figure 4

Ex vivo mGlu₅ occupancy by DSR-98776 and MPEP. (A) Effect of 1 h pretreatment with DSR-98776 or MPEP on *ex vivo* binding of [³H]-MPEPy in the mouse forebrain. (B) Effect of single or 4 days administration of DSR-98776 on *ex vivo* binding of [³H]-MPEPy in the rat frontal cortex. In both cases, occupancy rate was expressed as percentage of receptor labelling in the corresponding brain areas (forebrain for mice or cortex for rats) of the vehicle-treated animals. Each symbol represents the mean ± S.E.M (n=4 for mice or n=6 for rats).

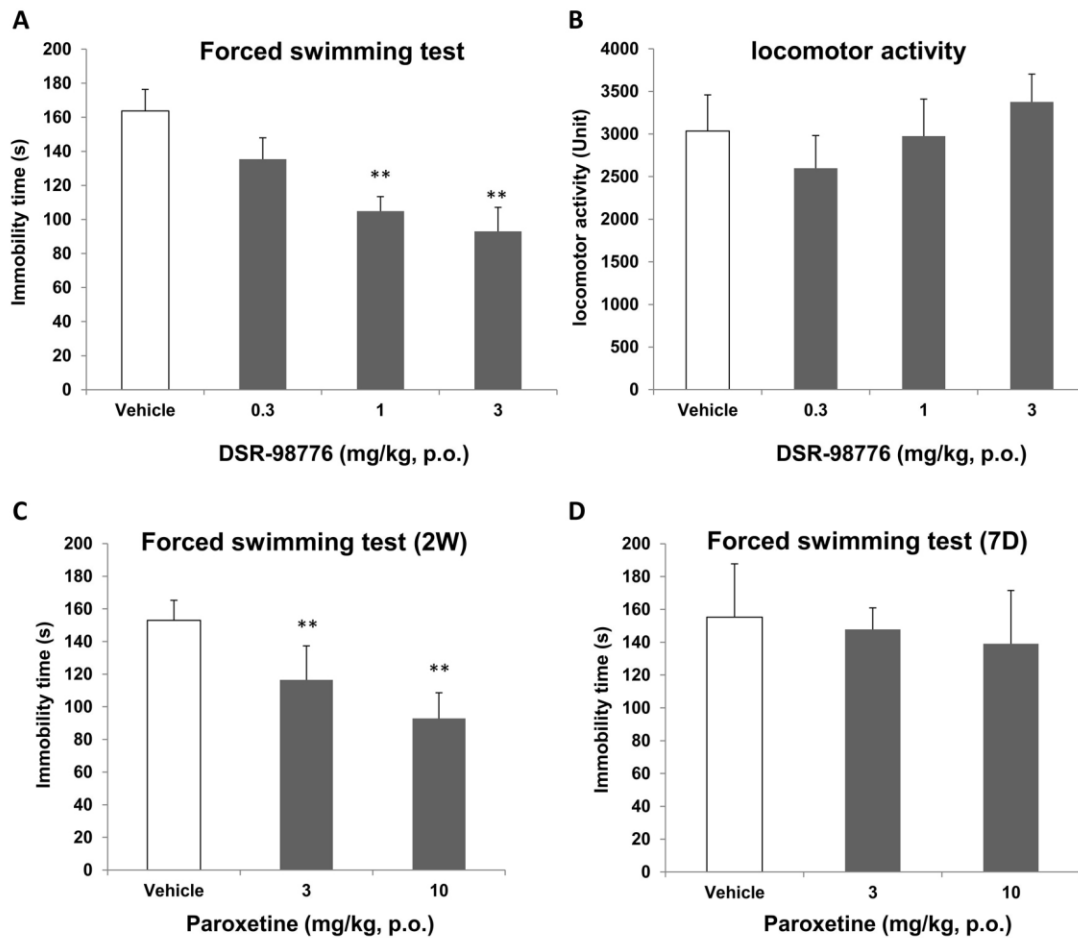


Figure 5

(A, C and D) Effects of DSR-98776 and paroxetine on immobility time in the forced swimming test in rats. Each bar represents the mean \pm S.E.M of immobility time during a 6 min test session (n=6 (for D) or 12 (for A and C) per each group). (B) Effect of DSR-98776 on locomotion. Each bar represents the mean \pm S.E.M of locomotion during a 15 min period, starting 1 h after administration of DSR-98776 (n=6 per each group). ** $p < 0.01$, compared with the vehicle-treated group using parametric Dunnett's multiple comparison test, following significant results of one-way ANOVA.

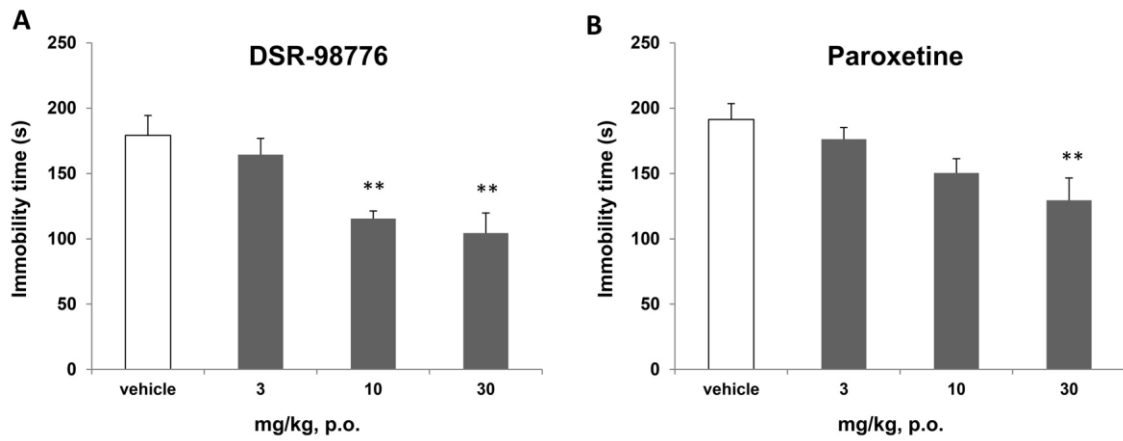


Figure 6

Effects of DSR-98776 (A) and paroxetine (B) on immobility time in the forced swimming test in mice. Each bar represents the mean \pm S.E.M of immobility time during a 6 min test session (n=9 per each group). ** $p < 0.01$, compared with the vehicle-treated group using parametric Dunnett's multiple comparison test, following significant results of one-way ANOVA.

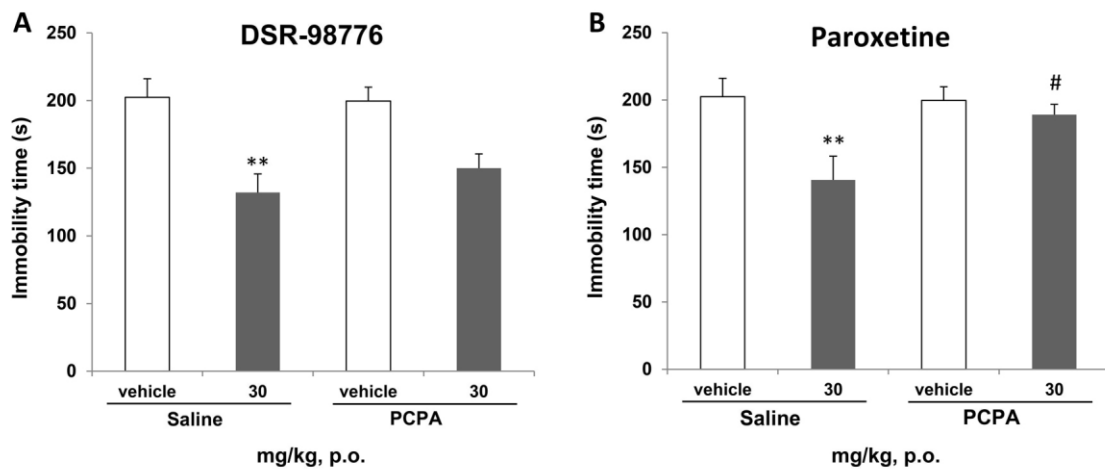


Figure 7

Effects of DSR-98776 (A) and paroxetine (B) on immobility time in the forced swimming test in PCPA treated mice. Values are expressed as the mean \pm S.E.M and were analyzed by two-way ANOVA. $**p < 0.01$ for the main effects of DSR-98776 and paroxetine *vs.* the control group; $\#p < 0.05$ for the interaction between paroxetine and PCPA treatment.

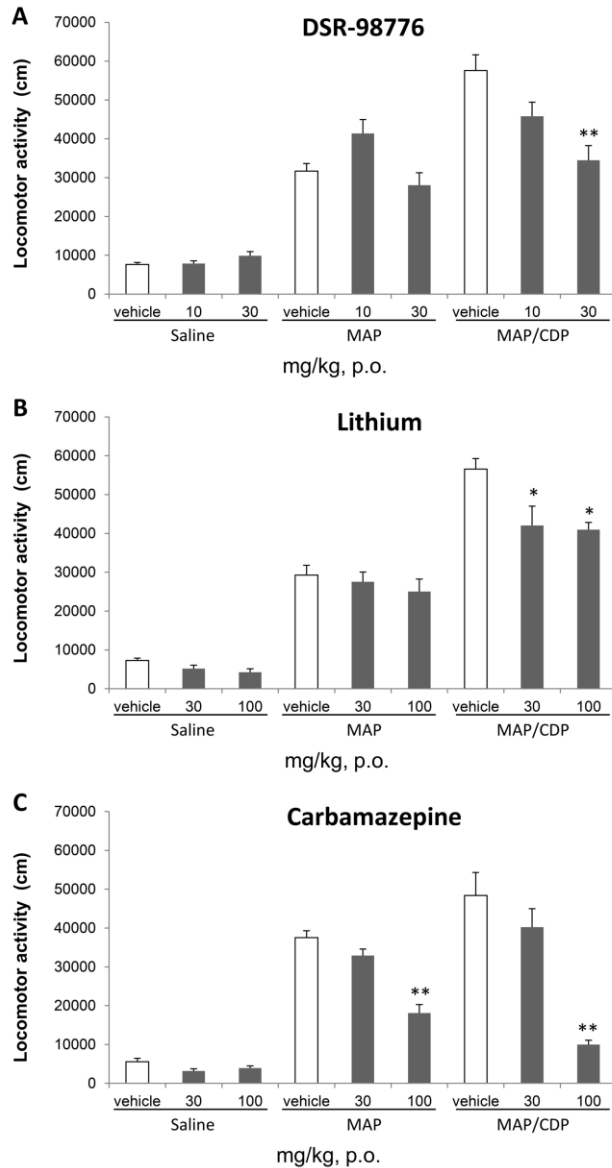


Figure 8

Effects of DSR-98776 (A), lithium (B) and carbamazepine (C) on basal activity, methamphetamine (MAP)- or MAP/chlordiazepoxide (CDP)-induced hyperactivity in mice. Each bar represents the mean \pm S.E.M of locomotion during a 90 min period, starting 1 h after administration of each test-compound (n=5-10). * $p < 0.05$, ** $p < 0.01$, compared with the vehicle-treated group using parametric Dunnett's multiple comparison test, following significant results of one-way ANOVA.

Table 1

DSR-98776 (1 and 10 μM) inhibition of calcium efflux by stimulation of various mGlu receptors

Target	Conc. (μM)	% inhibition
mGlu ₁	1	4.2
	10	-2.3
mGlu ₂	1	-4.7
	10	-14.0
mGlu ₄	1	-25.7
	10	28.0
mGlu ₆	1	-15.4
	10	-20.8
mGlu ₈	1	3.8
	10	6.6

Table 2

DSR-98776 (10 μ M) inhibition of radioligand binding to various receptors, channels and transporters

Target	Radioligand	Conc. (nM)	% inhibition	Target	Radioligand	Conc. (nM)	% inhibition
rat adrenergic α_1	[3 H] Prazosin	0.25	-9	hum muscarinic M ₁	[3 H] N-Methylscopolamine	0.80	5
rat adrenergic α_2	[3 H] Rauwolscine	0.70	1	hum nicitnic acetylcholine	[125 I] Epibatidine	0.10	-3
rat adrenergic β	[3 H] Dihydroalprenolol	0.25	1	rat opiate	[3 H] Naloxone	1.0	20
rat calcium channel L-type	[3 H] Diltiazem	2.0	4	rat potassium Channel [K _A]	[125 I] α -Dendrotoxin	0.010	-1
rat calcium channel L-type	[3 H] Nitrendipine	0.10	-6	hum potassium Channel [K _{ATP}]	[3 H] Glyburide	5.0	12
rat calcium channel L-type	[3 H] D-888	0.40	2	rat potassium Channel [SK _{CA}]	[125 I] Apamin	0.0050	1
rat calcium channel N-type	[125 I] ω -Conotoxin	0.010	0	hum serotonin 5-HT _{1A}	[3 H] 8-OH-DPAT	1.5	2
hum dopamine D ₁	[3 H] SCH-23390	1.4	10	hum serotonin 5-HT _{1B}	[3 H] GR125743	1.0	9
hum dopamine D _{2L}	[3 H] Spiperone	0.16	4	hum serotonin 5-HT _{2A}	[3 H] Ketanserin	0.50	0
hum dopamine D ₃	[3 H] Spiperone	0.70	2	hum serotonin 5-HT _{2B}	[3 H] LSD	1.2	-8
rat GABA _A	[3 H] TBOB	3.0	1	hum serotonin 5-HT _{2C}	[3 H] Mesulergine	1.0	-1
rat GABA _A	[3 H] Flunitrazepam	1.0	10	hum serotonin 5-HT ₃	[3 H] GR-65630	0.69	8
rat GABA _A	[3 H] Muscimol	1.0	49	gp serotonin 5-HT ₄	[3 H] GR-113808	0.70	5
rat GABA _B	[3 H] CGP-54626	0.60	9	hum serotonin 5-HT ₆	[3 H] LSD	1.5	0
rat glutamate, AMPA	[3 H] AMPA	5.0	-16	hum serotonin 5-HT ₇	[3 H] LSD	5.5	7
rat glutamate, kainate	[3 H] Kainic acid	5.0	4	rat choline transporter	[3 H] Hemicholinium-3	0.75	5
rat glutamate, NMDA	[3 H] CGP-39653	2.0	12	hum DAT	[125 I] RTI-55	0.15	7
rat glutamate, NMDA	[3 H] TCP	4.0	-2	rat GABA transporter	[3 H] GABA	6.0	-7
rat glycine	[3 H] Strychnine	10	-19	rat glycine transporter	[3 H] Glycine	0.050	7
hum histamine H ₁	[3 H] Pyrilamine	1.2	0	hum NET	[125 I] RTI-55	0.20	1
hum histamine H ₂	[125 I] Aminopotentidine	0.10	-14	hum SERT	[3 H] Paroxetine	0.40	10
hum imidazoline I ₂	[3 H] Idazoxan	2.0	-19				

Summary and Conclusion

This study characterized the *in vitro* pharmacological profiles of two structurally novel antidepressant candidates, i.e. DSP-1053, a 5-HT reuptake inhibitor with 5-HT_{1A} receptor partial agonistic activity, and DSR-98776, a selective mGlu₅ receptor negative allosteric modulator, and demonstrated the superior therapeutic benefits of these compounds compared to conventional SSRIs in rodent mood disorder models.

First, both compounds showed earlier therapeutic onset than conventional SSRIs. Although activation of 5-HT_{1A} receptor is reported to fasten SSRIs therapeutic onset, I was able to confirm this hypothesis by directly comparing the efficacy of DSP-1053 to that of paroxetine, a representative SSRI, in the rat forced swimming test and olfactory bulbectomy test, both of which are suitable paradigms for investigating antidepressants onset. On the other hand, previous reports have shown that mGlu₅ receptor negative allosteric modulators lack this feature. Therefore, this report shows for the first time that a selective mGlu₅ receptor negative allosteric modulator has faster therapeutic onset than conventional SSRIs. These results indicate that addition of 5-HT_{1A} partial agonistic activity or mGlu₅ receptor inhibitory activity to 5-HT reuptake inhibition improves antidepressants therapeutic onset. Because delayed therapeutic onset is one of the main drawbacks of current antidepressants, these results are important for the development of

new medications for major depressive disorder.

Using DSP-1053, we demonstrated in this study that partial activation of the 5-HT_{1A} receptor contributes to a fast adaptation to the feeling of nausea and therefore reduction in emetic episodes. Since patients adherence to antidepressants is directly related to the frequency and intensity of side effects, this finding would be useful in alleviating antidepressants gastrointestinal side effects and reducing treatment incompleteness rate.

This study also showed that the mGlu₅ receptor negative allosteric modulator DSR-98776 is effective not only for the depressive state but also for the manic state of mood disorder. There remain significant unmet medical needs for the treatment of mania, because mania symptoms significantly affect patient's life and can be controlled by mood stabilizers and atypical antipsychotics, both of which have severe side effects.

Although the precise mechanism of the antimanic effect of mGlu₅ receptor inhibition is unknown, improved monoaminergic transmission and channel blocking, which are the main mechanisms of action of current antidepressants, are not likely to be involved in the downstream efficacy of DSR-98776 based on the *in vitro* pharmacological profiling.

Therefore, my findings indicate that modulation of glutamatergic neurotransmission, including inhibition of the mGlu₅ receptor, is a promising therapeutic option for patients with bipolar disorder.

In conclusion, I have shown in this study that the newly synthesized antidepressant candidate DSP-1053 and DSR-98776 show superior therapeutic benefits than available antidepressant. Since all current antidepressants, including tricyclic antidepressants, SSRIs, SNRIs and monoamine oxidase inhibitors, act by enhancing 5-HT and norepinephrine concentration in the synaptic cleft, a broad number of receptors, including both target and off-target receptors are simultaneously stimulated. Therefore, drugs with reduced side effects and improved efficacy by working to specific receptors are expected to be promising approach for future drug development. In addition, focusing on neurotransmitters other than the monoamines is obviously a promising approach for the discovery of new antidepressant. A number of effective neurotransmitters, including several neuropeptides and steroid hormones have been reported. Among them, glutamatergic neurotransmission is considered to be the most promising target due to the positive results of a number of clinical and non-clinical studies. This study demonstrated that both the 5-HT_{1A} receptor and mGlu₅ receptor are promising targets to compensate for the shortcomings of current antidepressants. In the future, not only further non-clinical evidences but also clinical evidences are expected to be piled up to make this research achievement helpful for patients who are unsatisfied with today's armamentarium.

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List of Theses

I have published these research archives in following theses.

- 1) Kato T, Matsumoto Y, Yamamoto M, Matsumoto K, Baba S, Nakamichi K, Matsuda H, Nishimuta H, Yabuuchi K (2015). DSP-1053, a novel serotonin reuptake inhibitor with 5-HT_{1A} partial agonistic activity, displays fast antidepressant effect with minimal undesirable effects in juvenile rats. *Pharmacol Res Perspect* 3: e00142.

- 2) Kato T, Takata M, Kitaichi M, Kassai M, Inoue M, Ishikawa C, Hirose W, Yoshida K, Shimizu I (2015). DSR-98776, a novel selective mGlu₅ receptor negative allosteric modulator with potent antidepressant and antimanic activity. *Eur J Pharmacol* 757: 11-20.

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