Table 2. Genotype and allele distributions of *VAMP2* SNPs in both definition groups

Definition	SNP number	Sample	Genotype			p value	Allele		p value
			W/W	W/M	M/M		W	M	
Clinical response group	rs1061032	responders	18	28	13		64	54	
		nonresponders	17	25	5	0.297	59	35	0.262
	rs8067606	responders	18	27	14		63	55	
		nonresponders	15	25	7	0.434	55	39	0.488
Clinical remission group	rs1061032	remission	13	24	10		50	44	
		nonremission	22	29	8	0.434	73	45	0.395
	rs8067606	remission	13	24	10		50	44	
		nonremission	20	28	11	0.784	68	50	0.578

W = Wild-type allele; M = mutant allele.

Table 3. Haplotype distribution of *VAMP2* in both definition groups

Definition	Sample	Haplotype frequency				Global p value
		A[1]–A[2]	A–C[3]	G[4]–A	G–C	
Clinical response group	responders	0.361	0.053	0.011	0.573	
	nonresponders	0.449	0.017	0.008	0.525	
	p value	0.197	0.14	0.87	0.475	0.339
Clinical remission group	remission	0.372	0.051	0.008	0.567	
	nonremission	0.457	0.01	0.01	0.521	
	p value	0.213	0.083	0.868	0.498	0.256

[1]Minor allele of rs8067606.
[2]Minor allele of rs1061032.
[3]Major allele of rs1061032.
[4]Major allele of rs8067606.

Two limitations in this paper deserve mentioning. First, our sample size was not large enough to deny the type II error. If the relative risk is set at 1.5, a total of 150 samples would be needed to obtain over 80% statistical power. Second, we did not examine the patients' plasma concentrations of fluvoxamine. The daily fluvoxamine dosage was higher in nonremitted subjects than in those in remission, though this should be self-evident for a study design incorporating fixed-flexible dosing. However, these effects should be minimal because no correlation between plasma fluvoxamine concentration and clinical response has been reported [19]. The small but significant difference in baseline SIGH-D scores between responders and nonresponders might also have affected the results; however, the baseline SIGH-D scores could not predict clinical response in the exploratory logistic regression analysis.

In this pharmacogenetic study of fluvoxamine, our results suggest that the *VAMP2* gene is not a predictor of antidepressant efficacy in Japanese depressive patients. We must further investigate the role of the *VAMP2* gene in both the mechanisms of action of antidepressants and the pathophysiology of depression.

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〔Original Article〕

Induction of Neuroserpin Expression in Rat Frontal Cortex after Chronic Antidepressant Treatment and Electroconvulsive Treatment

Satoshi TANAKA*[1], Misa YAMADA*[2,3], Sari KITAHARA*[3], Teruhiko HIGUCHI*[4], Kazuo HONDA*[3], Kunitoshi KAMIJIMA*[1] and Mitsuhiko YAMADA*[2]

*[1] Department of Psychiatry, Showa University School of Medicine
*[2] Department of Psychogeriatrics, National Institute of Mental Health, National Center of Neurology and Psychiatry
4-1-1 Ogawahigashimachi, Kodaira, 187-8553 Japan
*[3] Department of Pharmacology, Showa University School of Pharmaceutical Sciences
*[4] Musashi Hospital, National Center of Neurology and Psychiatry

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Abstract: Using expressed sequence tag (EST) analysis, we previously identified certain molecular machinery that mediates antidepressant effects. To date, several partial cDNA fragments, termed antidepressant-related genes (ADRGs), have been isolated as ESTs from rat brain. In the present study, we identified two of the ADRGs to be rat neuroserpin. Using real-time quantitative PCR, we demonstrated increased neuroserpin mRNA expression in rat frontal cortex after chronic treatment with several classes of antidepressants, including imipramine, fluoxetine, sertraline, and venlafaxine. Electroconvulsive treatment (ECT), another therapeutic treatment for depression, also increased neuroserpin expression in rat frontal cortex. Neuroserpin is a serine protease inhibitor that is implicated in the regulation of synaptic plasticity, neuronal migration, and axogenesis in the central nervous system. In conclusion, our results support the hypothesis that neuroserpin-mediated plastic changes in frontal cortex may underlie the therapeutic action of antidepressants and ECT.

Key words: Depression, Neuronal plasticity, tPA, Microarray

Depression is one of the major psychiatric diseases. It is characterized by abnormal emotional, cognitive, autonomic, and endocrine functions. Typical antidepressants acutely inhibit monoamine reuptake, resulting in a significant increase in synaptic concentration of monoamines (noradrenaline or serotonin) (Yamada and Higuchi, 2002). With antidepressants, there is a latency period of several weeks before the onset of clinical improvement. Chronic electroconvulsive treatment (ECT) is another therapy that is widely used to alleviate depression, particularly for treating drug-resistant depression. While ECT is effective, its therapeutic mechanism remains unknown. The therapeutic benefits of ECT also have a delayed onset.

For both treatment modalities, this delay could be the result of indirect regulation of neural signal transduction systems or molecular changes in gene transcription. Indeed, antidepressants have been reported to selectively affect specific immediate early genes and transcription factors (see review by Yamada and Higuchi, 2002). Thus, it is reasonable to propose that these molecules may have an important role in the adaptive neural changes that occur following chronic antidepressant treatment. Identification of quantitative changes in gene expression that occur in the brain after chronic antidepressant treatment might yield novel molecular markers that would be useful in diagnosing and treating depression. Using expressed sequence tag (EST) analysis, we and other groups have isolated genes that are differentially expressed in rat brain after chronic antidepressant treatment (Drigues et al, 2003; Wong et al, 1996; Huang et al, 1997; Yamada et al, 1999, 2000, 2001, 2002). To date, we have cloned several partial cDNA fragments as ESTs, which we named antidepressant-related genes (ADRGs).

In the present study, we focused on ADRG116 and ADRG604, both of which were isolated independently and were found to correspond to rat neuroserpin. Neuroserpin is a neural serpin that inhibits the extracellular protease tissue-type plasminogen activator (tPA). Neuroserpin was first identified as an axonally secreted glycoprotein in neuronal cell cultures of chicken dorsal root ganglia (Osterwalder et al, 1996). It is widely expressed in the developing and adult nervous systems and is implicated in the regulation of proteases involved in synaptic plasticity, neuronal migration, and axogenesis (Hastings et al, 1997; Krueger et al, 1997; Schrimpf et al, 1997). Neuroserpin is also implicated in the regulation of emotional behavior, such as anxiety-like responses (Madani et al, 2003). Here, we provide the first report demonstrating that neuroserpin is expressed in the rat frontal cortex and that it is upregulated after chronic treatment with antidepressants and ECT.

*[2] Reprint requests should be sent to M. Yamada
山田光彦：〒187-8553 小平市小川東町 4-1-1
国立精神・神経センター精神保健研究所老人精神保健部
E-mail: mitsu@ncnp-k.go.jp

Abbreviations ADRG: antidepressant-related gene, ECT: electroconvulsive treatment, EST: expressed sequence tag, tPA: tissue-type plasminogen activator

METHODS

Experimental animals

Male Sprague-Dawley rats (age: 7–10 weeks; Sankyo Labo Service Co., Tokyo, Japan) were housed in a temperature-controlled environment with a 12-hour light/12-hour dark cycle; they had free access to food and water.

Experimental treatments

Rats were randomly assigned to either a control or treatment group. Treatments consisted of either antidepressant drugs or ECT. Six rats were used for antidepressant experiments and 4 rats were used for ECT experiments. We used the following antidepressant drugs for treatment: the tricyclic imipramine (Sigma-Aldrich, Inc., MO, USA), the selective serotonin reuptake inhibitors fluoxetine (The Lilly Laboratories, IN, USA) and sertraline (Pfizer Pharmaceuticals Inc., NY, USA), and the serotonin noradrenaline reuptake inhibitor venlafaxine (Wyeth, PA, USA). Animals in the antidepressant-treatment group received by daily intraperitoneal injection either vehicle, imipramine (10 mg/kg), fluoxetine (5 mg/kg), sertraline (10 mg/kg), or venlafaxine (10 mg/kg) for 1 day or 21 days. We determined the dose for each of the antidepressants according to the previous reports (Nibuya et al, 1996; Pei et al, 2004; Yamada et al, 2001, 2002). All drugs were dissolved in physiological saline containing 1.5% Tween-80. Rats in the ECT group were anesthetized with sevoflurane (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) before receiving a 90 mA, 0.1 sec electric shock via ear-clip electrodes. Shocks were delivered with a Ugo Basile Model 7801 Unipolar square-wave electroconvulsive stimulation pulse generator (Stoelting Co., IL, USA). Animals received either a single shock (single administration ECT group), or shocks every other day for 14 days (chronic administration ECT group). The control group was treated exactly as the ECT-treatment group, except that electric current was administered.

Animals were killed by decapitation 24 hours after the final antidepressant or ECT treatment; and the brain was quickly removed, dissected, frozen immediately in liquid nitrogen, and stored at −80°C until analysis. All studies using animals were carried out in accordance with animal protocols approved by The Institutional Animal Care and Use Committee of Showa University.

EST analysis

Total RNA from rat frontal cortex was extracted in Isogen reagent (Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer's instructions. Isolated total RNA was then dissolved in RNase-free water, and RNA concentration was estimated by UV spectrometry. Total RNA samples were treated with RNase-free DNase I (Nippon Gene Co., Ltd.) for 30 min at 37°C, then were purified by phenol-chloroform extraction. The first-strand cDNA was synthesized with reverse transcriptase (Invitrogen, CA, USA), 1 μM of oligo-dT primer, and 2 μg of total RNA treated with DNase I, then diluted to a final volume of 100 μl. EST analysis was then carried out in the presence of [^{33}P] dATP (Life Science Products, Inc., MA, USA) with an mRNA fingerprinting kit (Clontech, CA, USA) according to the manufacturer's instructions. Radiolabeled PCR products were then analyzed electrophoretically on denaturing 6% polyacrylamide gels. Three individual samples from each treatment group were applied side-by-side and visualized by autoradiography.

Identification of both ADRG116 and ADRG604

ADRG microarray development and fluorescence image analysis was done as described previously (Yamada et al, 2000). Briefly, we amplified each ADRG cDNA insert using vector primers and negative controls, and we spotted in duplicate ten different kinds of housekeeping genes onto glass slides using a GMS417 Arrayer (Affymetrix, Inc., CA, USA). To make the fluorescence-labeled probe for hybridization, poly-A$^+$ RNA was purified from total RNA that was pooled from three independent control or treated groups. Probes were fluorescently tagged by converting 1 μg of poly-A$^+$ RNA from the control and treated samples to cDNA in the presence of either Cy-5- or Cy-3-dUTP, respectively. Hybridization of probes to the microarray was done competitively and in duplicate. The probes were mixed and placed on an array, overlaid with a coverslip, and allowed to hybridize for 16.5 hours at 65°C. After hybridization and washing, each slide was scanned with a GMS418 Array Scanner (Affymetrix, Inc.). Gene expression levels were quantified and analyzed using ImaGene software (Bio-Discovery Ltd. Swansea, UK). Sequence analysis of both ADRG116 and ADRG604 was performed by dideoxy sequencing methods. Homology search and sequence alignment was done using the FASTA search servers at the National Center for Biotechnology Information.

Real-time quantitative PCR

Previously, two cDNAs encoding neuroserpin, rNS-1 (2917 bp) and rNS-2 (1599 bp), were reported (GenBank accession numbers AF193014 and AF193015 respectively; Hill et al, 2000). The principal difference between them was the length of the 3′ untranslated region. We have found that ADRG604 corresponds to rNS-1 (Fig. 1). Therefore, we have analyzed the changes of rNS-1 expression in mRNA level in the present study. PCR primers were designed with Primer Express Software (Applied Biosystems). The primers used for rNS-1 were 5′-CAGCACATTTTCACAGACAGAGATT-3′ and 5′-AGGGCATTGTATTATTAGTGCAGATTAA-3′ (Invitrogen) (black arrows in Fig. 1). The primers we used for amplification of the β-actin reference gene were 5′-TCGCTGACAGG-ATGCAGAAGG-3′ and 5′-GCCAGGATAGAGCCACCAAT-3′ (Invitrogen). We confirmed the presence of a single PCR product on agarose gel after electrophoresis. In addition PCR fragments were sequenced, and their identity was ascertained (data not shown).

Quantification of rNS-1 expression in rat brain was performed with real-time quantitative PCR and an ABI PRISM 7000 instrument (Applied Biosystems, CA, USA). A quantity of cDNA corresponding to 20 pg of total RNA was amplified by PCR in duplicate. The SYBR® Green PCR Core Reagents Kit (Applied Biosystems) was used for fluorescent detection of cDNA. Real-time quantitative PCR conditions were as follows: 50°C for 2 min then 95°C for 10 min for one cycle, followed by 50 cycles of 95°C for 15 s, 60°C for 1 min. We performed the real-time quantitative PCR amplification of rNS-1 and β-actin at the same time. Then we used the

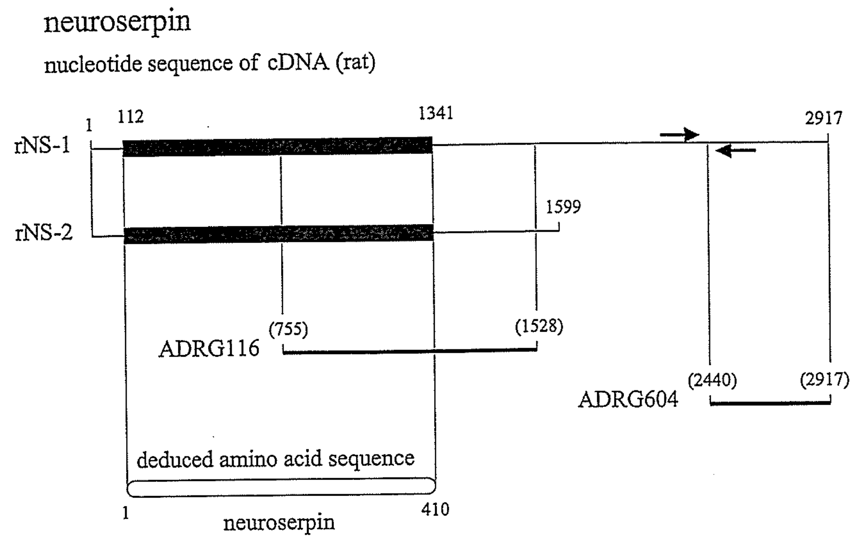

Fig. 1 Structure of the cDNAs encoding rat neuroserpin, rNS-1 and rNS-2 (GenBank accession numbers AF193014 and AF193015 respectively) and their deduced amino acid sequence. Homology analysis of ADRG116 and ADRG604 using DDBJ/EMBL/GenBank databases revealed significant matches to the rat neuroserpin gene. The sizes of the ADRG116 and ADRG604 fragments obtained from EST analysis were 774 bp and 478 bp, respectively. Black arrows represent the oligonucleotide primer pair used for real-time quantitative PCR experiments.

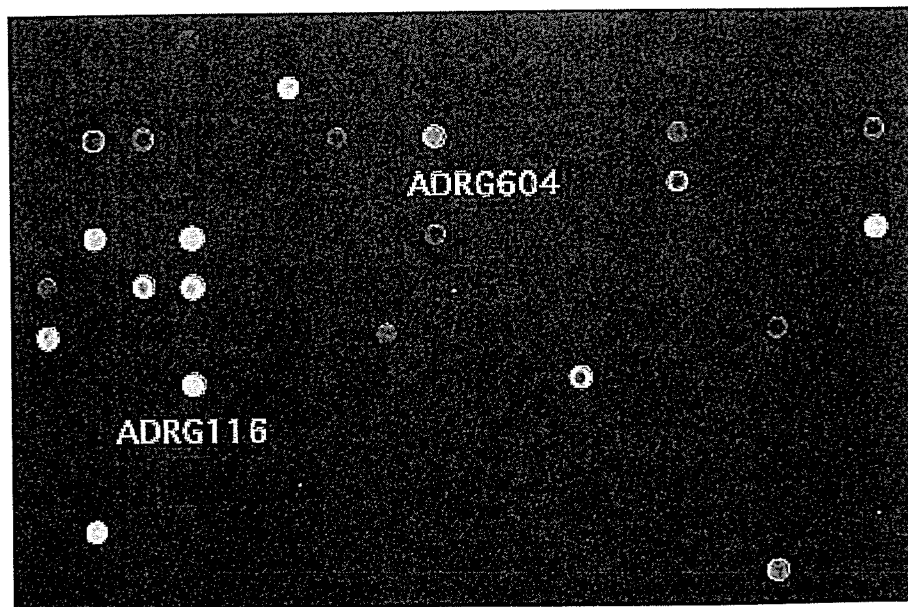

Fig. 2 Image analysis of ADRG microarray after hybridization with fluorescent probes. The pseudo-color image representing hybridization within the control sample (green) and the chronic sertraline-treatment sample (red) are overlapped. As expected, we obtained low background and consistent results in duplicate experiments. The spots within the blue rectangles represent ADRG116 and ADRG604. Interestingly, the fluorescence intensities of the spots representing ADRG116 and ADRG604 increased 3.72 times and 1.67 times, respectively, after chronic sertraline treatment compared to those of the controls.

Table 1 rNS-1 expression in rat brain after chronic antidepressant treatment as assessed by real-time quantitative PCR[a)]

Brain region	Treatment				
	Control	Imipramine	Fluoxetine	Sertraline	Venlafaxine
Frontal cortex	100±5	130±12	126±2*	149±15	137±12*
Hippocampus	100±15	81±8	102±15	74±8	82±6
Hypothalamus	100±4	104±8	120±10	118±15	94±17

a) Total RNA was extracted from rat brain after each treatment and used for real-time quantitative PCR (see Methods; $n=6$). Data represent percentages of control values (means±SEM). Differences were assessed using Student's t-test. *: $P<0.05$ was regarded as significant.

Table 2 rNS-1 expression in rat brain after ECT as assessed by real-time quantitative PCR[a)]

Brain region	Treatment		
	Control	Single administration	Chronic administration
Frontal cortex	100±6	120±16	121±8*
Hippocampus	100±10	85±8	80±4
Hypothalamus	100±12	89±5	89±7

a) Total RNA was extracted from rat brain after each treatment and used for real-time quantitative PCR (see Methods; $n=4$). Data represent percentages of control values (means ± SEM). Differences were assessed using Student's t-test. *: $P<0.05$ was regarded as significant.

Standard Curve Method (User Bulletin, ABI PRISM 7000 Sequence Detection System) for quantification. Briefly, for rNS-1 and β-actin, an absolute standard curve was obtained by plotting the cycle of threshold following PCR amplification of serial dilutions of the control cDNA template. Data are given as percentages of control values (means ± SEM). Differences were assessed using Student's t-test. A value of $P<0.05$ was regarded as statistically significant.

RESULTS

Identification of ADRG116 and ADRG604 as rat neuroserpin

In the present study, we used an ADRG microarray for high-throughput secondary screening to identify genes commonly affected by antidepressants. Fig. 2 shows the pseudocolor image of the ADRG microarray after hybridization with samples obtained from frontal cortex of sertraline-treated rats. As expected, we obtained low background and consistent results in duplicate experiments. After normalization of the signals with both negative and positive controls, several spots of interest on the ADRG microarray showed increased or decreased fluorescence intensities after chronic sertraline treatment. Interestingly, the fluorescence intensities of the spots for both ADRG116 and ADRG604 increased 3.72 times and 1.67 times, respectively, after chronic sertraline treatment when compared to controls (Fig. 2).

Sequence and homology analysis of ADRG116 and ADRG604 (identified by EST analysis to be two independent clones) using DDBJ/EMBL/GenBank databases revealed significant matches to the rat neuroserpin gene (Hill et al, 2000). This finding strongly indicated that neuroserpin was upregulated after chronic sertraline treatment. Fig. 1 shows the structure of the cDNAs encoding rat neuroserpin and the deduced amino acid sequence. The open reading frame spanned from the 112th base to the 1341st base. The deduced amino acid sequence of neuroserpin contained 410 amino acid residues and a putative reactive site loop that binds the active site of target proteases (Osterwalder et al, 1996). The sizes of the ADRG116 and ADRG604 fragments obtained from EST analysis were 774 bp (755–1528th) and 478 bp (2440–2917th), respectively.

Real-time quantitative PCR

The differential expression of rNS-1 in both antidepressant and ECT groups was confirmed by real-time quantitative PCR. As shown in Table 1, chronic administration of imipramine, fluoxetine, sertraline, and venlafaxine increased rNS-1 expression in rat frontal cortex when compared to control samples. On the other hand, single administration of these antidepressants did not affect rNS-1 expression in this region of the brain (data not shown). Interestingly, in the hippocampus and hypothalamus, chronic treatment with these antidepressants did not significantly affect rNS-1 expression (Table 1).

The expression of rNS-1 was also significantly increased in rat frontal cortex after chronic administration of ECT (Table 2). Single administration of ECT also increased rNS-1 expression, but not significantly. Single and chronic administration of ECT did not affect rNS-1 expression in the hippocampus or hypothalamus (Table 2).

DISCUSSION

The serine protease tPA is found not only in the blood where it primarily acts as a thrombolytic enzyme, but also in the central nervous system where it promotes events

associated with synaptic plasticity. Neuroserpin, a serine protease inhibitor (serpin) that reacts preferentially with tPA, is located in regions of the brain where tPA protein is also found, indicating that neuroserpin is a selective inhibitor of tPA in the central nervous system. Growing evidence suggests that tPA participates in many physiological and pathological events in the central nervous system, and that neuroserpin plays an important role as a natural regulator of tPA activity in these processes (Hastings et al, 1997; Yepes and Lawrence, 2004).

In the adult mouse central nervous system, neuroserpin is most strongly expressed in the neocortex, the hippocampal formation, the olfactory bulb, and the amygdala (Krueger et al, 1997). During embryonic development in the mouse, it is expressed in the differentiating fields of most central nervous system regions (Krueger et al, 1997). Moreover, neuroserpin has been reported to regulate neurite outgrowth in nerve growth factor-treated PC12 cells (Parmar et al, 2002). Interestingly, neuroserpin-deficient mice display a selective reduction in locomotor activity in novel environments, an anxiety-like response in the O-maze, and a neophobic response to novel objects (Madani et al, 2003). Neuroserpin-overexpressing mice display reduced center exploration in the open-field test and neophobic responses in a novel object test. These findings suggest that neuroserpin plays some sort of role in the regulation of emotional behavior (Madani et al, 2003).

In the present study, we observed a significant increase in rNS-1 expression in rat frontal cortex after chronic treatment with antidepressants. This altered pattern of rNS-1 expression was also observed in rat frontal cortex after chronic ECT. On the other hand, a single administration of antidepressants failed to induce rNS-1 expression, suggesting that the induction of rNS-1 is due to the long-term therapeutic action of antidepressants. Clinically, a period of several weeks passes before the onset of the therapeutic effect of antidepressants.

As shown in the results, the data obtained from the microarray study was more apparent than that of real-time quantitative PCR. This would be due to the technical differences between hybridization-based analysis and PCR-based quantification. We believe that the real-time quantitative PCR is much more accurate than the microarray analysis.

Although induction of rNS-2 by antidepressants and ECT are yet to be elucidated, our data suggest that neuroserpin may be one of the common functional molecules induced after these treatments. In addition, we need to confirm the induction of neuroserpin at the protein level. We did not use ANOVA for statistical analysis due to the small number of subjects for each treatment. Therefore, we must be careful in interpreting our observations.

The frontal cortex is one of several brain regions involved in the endocrine, emotional, cognitive, and vegetative abnormalities found in depressed patients. Indeed, glucose metabolism, blood flow, and electroencephalographic activity are altered in the frontal cortex of depressed patients (Drevets et al, 1992).

It has also been suggested that the long-term actions of antidepressants may be mediated by changes in neural plasticity (Duman, 2002; McEwen and Olie, 2005; Yamada and Higuchi, 2002). Alterations in certain functional proteins (e.g., protein kinase C and GAP-43) related to neural plasticity have been reported in the brain of depressed suicide victims (Hrdina et al, 1998). Taken together, our results also support the hypothesis that neuroserpin-mediated plastic changes in the frontal cortex may contribute to the therapeutic actions of antidepressants and ECT.

In rat hypothalamus, rNS-1 expression was not affected by chronic antidepressant administration or chronic ECT. Interestingly, in rat hippocampus, rNS-1 expression seemed to have a tendency to decrease with these treatments, although not significantly. The hippocampus and hypothalamus represent other regions of the brain implicated in the pathophysiology of depression (Sheline et al, 1996; Barden, 2004). Although pharmacological actions targeting a single brain region may possibly mediate the therapeutic effects of antidepressants, pharmacological actions targeting multiple brain regions are more likely to contribute to the actual therapeutic effects of antidepressants and ECT. Studies to further characterize the neuronal circuitry of these brain regions will help to elucidate the neuroanatomical substrates of antidepressant effects. We used only one dose for each antidepressant in the present study. rNS-1 expression in hippocampus or hypothalamus could be changed with higher doses.

In this study, a single administration of ECT also induced rNS-1 expression in rat frontal cortex, although not significantly. Clinically, ECT has been considered to be a more rapid and effective treatment for major depression than are antidepressant drugs (Segman et al, 1995). Our results are consistent with the hypothesis that the rapid induction of neuroserpin in frontal cortex underlies the relatively rapid onset of the therapeutic action of ECT. Further characterization of neuroserpin as a functional protein in the central nervous system is needed to test this hypothesis.

In conclusion, we identified neuroserpin as a novel molecular target for antidepressants and ECT. Our findings offer novel insights into the actions of these treatments that may be of both basic and clinical significance.

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Original Papers

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The G196A polymorphism of the brain-derived neurotrophic factor gene and the antidepressant effect of milnacipran and fluvoxamine

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Keizo Yoshida *Department of Psychiatry, Nagoya University Graduate School of Medicine, Akita, Japan.*
Hisashi Higuchi *Omagari City Hospital, Akita, Japan.*
Mitsuhiro Kamata *Yuri Kumiai General Hospital, Akita, Japan.*
Hitoshi Takahashi *Yokote Kohsei Hospital, Akita, Japan.*
Kazuyuki Inoue *Department of Pharmaceutical Science, Akita University Hospital, Akita, Japan.*
Toshio Suzuki *Department of Pharmaceutical Science, Akita University Hospital, Akita, Japan.*
Kunihiko Itoh *Department of Clinical Pharmacology and Genetics, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan.*
Norio Ozaki *Department of Psychiatry, Nagoya University Graduate School of Medicine, Akita, Japan.*

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Abstract

Prediction of the response to different classes of antidepressants has been an important matter of concern in the field of psychopharmacology. The purpose of the present study was to investigate whether the G196A polymorphism of the brain-derived neurotrophic factor (BDNF) gene is associated with the antidepressant effect of milnacipran, a serotonin norepinephrine reuptake inhibitor, and fluvoxamine, a selective serotonin reuptake inhibitor. The subjects of our previous study of milnacipran (n=80) and fluvoxamine (n=54) were included in the present study. Severity of depression was assessed with the Montgomery Åsberg depression rating scale (MADRS). Assessments were carried out at baseline and at 1, 2, 4 and 6 weeks of treatment. Polymerase chain reaction was used to determine allelic variants. In all subjects receiving milnacipran or fluvoxamine, the *G/A* genotype of the BDNF G196A polymorphism was associated with a significantly better therapeutic effect in the MADRS scores during this study. When milnacipran and fluvoxamine-treated subjects were analysed independently, the *G/A* genotype group showed greater reduction of MADRS scores than other genotype groups, irrespective of which antidepressant was administered. These results suggest that the BDNF G196A polymorphism in part determines the antidepressant effect of both milnacipran and fluvoxamine.

Keywords

antidepressant effect, genetic polymorphism, fluvoxamine, major depressive disorder, milnacipran

Introduction

Prediction of the response to different classes of antidepressants has been an important matter of concern in the field of psychopharmacology. A consistent relationship between the antidepressant effect and the plasma concentrations of selective serotonin (5-HT) reuptake inhibitors (SSRIs) has not been obtained (Burke and Preskorn, 1999), although early pharmacokinetic studies identified significant relationships between the antidepressant effect and plasma concentrations of several tricyclic antidepressants (Perry *et al.*, 1987). In terms of serotonin norepinephrine (NE) reuptake inhibitors (SNRIs), venlafaxine showed a positive association between antidepressant efficacy and plasma concentrations (Charlier *et al.*, 2002), while this relationship was not observed for milnacipran (Higuchi *et al.*, 2003).

Recent progress in pharmacogenetics has facilitated investigation of the relationship between genetic polymorphisms and the antidepressant response. Genetic polymorphisms of the 5-HT and NE transporter have been investigated intensively, because they are believed to be the primary target of SSRIs and SNRIs. As a

Corresponding author: Keizo Yoshida, Department of Psychiatry, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan.
Email: cxw01076@nifty.com

result, several interesting findings have been reported (Malhotra *et al.*, 2004; Yoshida *et al.*, 2004), but there is no consistent evidence to predict the antidepressant response. Thus, further pharmacogenetic studies of antidepressants must be performed in order to predict the antidepressant response adequately.

Recently, it has been proposed that antidepressants eventually cause critical genes to be activated or inactivated, no matter how they act on receptors and enzymes (Stahl, 2000). One of the likeliest candidate genes is brain-derived neurotrophic factor (BDNF), which belongs to a family of neurotrophic factors including neurotrophin-3/4/5 and nerve growth factor and has an important role as a potent modulator of synaptic transmission and plasticity. Substantial evidence supports that BDNF is involved not only in cognitive processes, such as memory and learning, but also in the pathophysiology of mood disorders and in the mechanism of antidepressant action, as follows. Expression of BDNF mRNA is down-regulated by either acute or repeated stressful conditions of immobilization (Smith *et al.*, 1995). An antidepressant effect in both the learned helplessness and the forced swimming tests is observed as early as 3 days after a single infusion of BDNF into the hippocampus (Shirayama *et al.*, 2002). Chronic treatment with tranylcypromine, a monoamine oxidase inhibitor, caused a significant increase in BDNF mRNA in the rat hippocampus (Russo-Neustadt *et al.*, 1999), and chronic administration of amitriptyline, a tricyclic antidepressant, significantly increased BDNF protein levels in the rat hippocampus and prefrontal cortex (Okamoto *et al.*, 2003). Thus, the BDNF gene is a plausible candidate gene for mood disorders and pharmacogenetic studies of the antidepressant response.

The G196A polymorphism in exon IIIA is located within the propeptide region of the BDNF gene. Several association studies have examined the G196A polymorphism and vulnerability for bipolar or major depressive disorders (Hong *et al.*, 2003; Nakata *et al.*, 2003). These studies have found no major role for the polymorphism in the pathophysiology of mood disorders, although Egan *et al.* (2003) reported that it influences human memory and hippocampal function. So far only one pharmacogenetic study of antidepressants and the BDNF G196A polymorphism has been carried out (Tsai *et al.*, 2003); in this study, the response to treatment with fluoxetine was evaluated for only 4 weeks and the response rate was as low as 33.6%.

In the current 6-week study, we examined the effect of the BDNF G196A polymorphism on the antidepressant effect of milnacipran, an SNRI, and fluvoxamine, an SSRI. In addition, we investigated another polymorphism of C132T in the non-coding region of exon V of the BDNF gene, which was detected and named C270T by Kunugi *et al.* (2001). Plasma concentrations of milnacipran and fluvoxamine were investigated to evaluate patients' compliance and an influence on the antidepressant effect.

Materials and methods

Subjects

The subjects in our previous studies (Yoshida *et al.*, 2002; Yoshida *et al.*, 2004) were included in the present study. The subjects were Japanese patients who fulfilled DSM-IV criteria for a diagnosis of major depressive disorder and whose scores on the Montgomery Åsberg depression rating scale (MADRS) (Montgomery and Åsberg, 1979) were 21 or higher. Patients with other axis I disorders (including dementia, substance abuse, dysthymia, panic disorder, obsessive–compulsive disorder and generalized anxiety disorder) and those with axis II disorders determined by clinical interview were excluded. Patients with a history of childhood disorders were also excluded, as were patients with severe non-psychiatric medical disorders. The patients were 20–69 years of age and had been free of psychotropic drugs at least 14 days before entry into the study. After complete description of the study to the subjects, written informed consent was obtained. This study was approved by the Ethical Committee of Akita University School of Medicine. The clinical characteristics of the patients are shown in Table 1. There was no significant difference between responders and non-responders in regard to sex, age, number of previous episodes and presence of melancholia. There was no significant difference in clinical characteristics when milnacipran and fluvoxamine-treated patients were analysed independently (data not shown). The number of previous depressive episodes was very low. Indeed, most of the patients (milnacipran: 64/80, fluvoxamine: 41/54) were in their first episode.

Table 1 Clinical characteristics of the patients in the milnacipran and fluvoxamine treatment (responders and non-responders)

	Responders (n=85)	Nonresponders (n=49)		p
Sex (male/female)	34/51	16/33	χ^2=0.72	0.40[a]
Age (yr) (± SD)	50.7±12.4	52.2±12.8	t=−0.68	0.50[b]
No. of previous episodes (± SD)	0.48±1.7	0.33±0.7	t=0.77	0.44[b]
Melancholia (+/-)	21/64	15/34	χ^2=0.55	0.46[a]

[a] Analysis performed with the use of the χ^2 test.
[b] Analysis performed with the use of the unpaired t test.

Milnacipran treatment

Milnacipran was administered twice daily (the same dose after dinner and at bedtime) for 6 weeks. The initial total daily dose was 50 mg/day, and after a week it was increased to 100 mg/day. Patients with insomnia were prescribed brotizolam, 0.25 or 0.5 mg, a benzodiazepine sedative hypnotic, at bedtime. No other psychotropic drugs were permitted during the study. Of 96 enrolled patients, ten did not complete the study; five patients because of side effects, one patient because of severe insomnia and four patients without explanation. Of the 86 patients who completed the 6-week study, six patients were excluded from the current analysis because plasma samples revealed very low milnacipran concentrations, indicative of poor compliance. Patients who completed the study included 52 women and 28 men, 49 outpatients and 31 inpatients, and ranged from 25 to 69 years of age (mean age=51.4±12.2 (±SD)).

Fluvoxamine treatment

Fluvoxamine was administered twice daily (the same dose after dinner and at bedtime) for 6 weeks. The initial total daily dose was 50 mg/day. The daily dose was increased to 100 mg/day after a week and was increased to 200 mg/day after another week. Concomitant administration of psychotropic drugs was restricted as in the milnacipran study. Of 66 enrolled patients, nine did not complete the study; four patients because of side effects and five patients without explanation. Of the 57 patients who completed the 6-week study, three patients were excluded from the current analysis because plasma samples revealed very low fluvoxamine concentrations, indicative of poor compliance. Patients who completed the study included 32 women and 22 men, 43 outpatients and 11 inpatients, and ranged from 24 to 69 years of age (mean age=51.2±13.2 (±SD)).

Data collection

Depression symptom severity was assessed with the use of the MADRS. Assessments were conducted at baseline and at 1, 2, 4 and 6 weeks after initiation of antidepressant treatment. A clinical response was defined as a 50% or greater decrease in the baseline MADRS score. Clinical remission was defined as a final MADRS score less than ten (Hawley *et al.*, 2002). Collection of blood samples was performed 12 hours after drug administration at bedtime, 4 weeks after initiation of each antidepressant treatment.

Genotyping

The BDNF G196A polymorphism was determined by a minor modification of the method of Tsai *et al.* (2003). The BDNF C132T polymorphism was determined by a minor modification of the method of Szekeres *et al.* (2003). Primers and enzymes used in this study were the same as previous studies; the conditions of the polymerase chain reaction and the chemical reagents were adjusted to our instruments.

Quantification of plasma milnacipran/fluvoxamine concentration

Plasma concentrations of milnacipran were measured with high performance liquid chromatography (HPLC). Details of the method have been described previously (Higuchi *et al.*, 2003). Plasma concentrations of fluvoxamine were measured with HPLC. Details of the method have been described previously (Ohkubo *et al.*, 2003). Genotyping and measurement of plasma concentrations were performed by laboratory personnel blind to the identity and clinical antidepressant effect of the patients. Moreover, clinicians were unaware of the genotyping results and the plasma milnacipran concentrations of each patient.

Statistical analysis

Differences in patient characteristics were analysed with the use of the unpaired t-test or chi-square test where appropriate. Differences in the MADRS scores during this study were examined with the use of two-way repeated measures analysis of variance (ANOVA), with genotype and time as factors. When significant interaction between factors was observed, contrasts were used to enable comparisons between each two of the three genotype groups. Differences in the MADRS scores at each evaluation point were examined with the one-way factorial ANOVA followed by the Fisher's PLSD test. Genotype deviation from Hardy-Weinberg equilibrium was evaluated by the chi-square test. Genotype distribution and allele frequencies were analysed with the use of the chi-square test. Plasma concentrations of milnacipran or fluvoxamine were analysed with the use of one-way factorial ANOVA in each genotype group; an unpaired t-test was then used to analyse differences between groups who were or were not responsive to milnacipran or fluvoxamine. Statistical analysis was performed using StatView version 5.0 (SAS Institute Inc., Cary, NC), except the two-way repeated measures ANOVA with contrasts was performed using SuperANOVA version 1.11 (Abacus Concepts, Inc., Berkeley, CA). Power analysis was performed with the use of G·Power (Buchner *et al.*, 1996). All tests were two-tailed; alpha was set at 0.05.

Results

Minor allele frequencies for the C132T polymorphism were very low and similar to those reported by Kunugi *et al.* (2001) and Szekeres *et al.* (2003); 5.0% in the patients treated with milnacipran and 3.7% in those treated with fluvoxamine. Therefore, only the G196A polymorphism was included in the statistical analysis. The observed genotype frequencies of the G196A polymorphism were within the distribution expected according to the Hardy-Weinberg equilibrium. As the authors reported that response to fluvoxamine was associated with allelic variations of the 5-hydroxytriptamine transporter gene-linked polymorphic region (5-HTTLPR) (Yoshida *et al.*, 2002) and response to milnacipran was associated with those of the norepinephrine transporter T-182C and G1287A polymorphisms (Yoshida *et al.*, 2004), it was necessary to confirm these polymorphisms to be con-

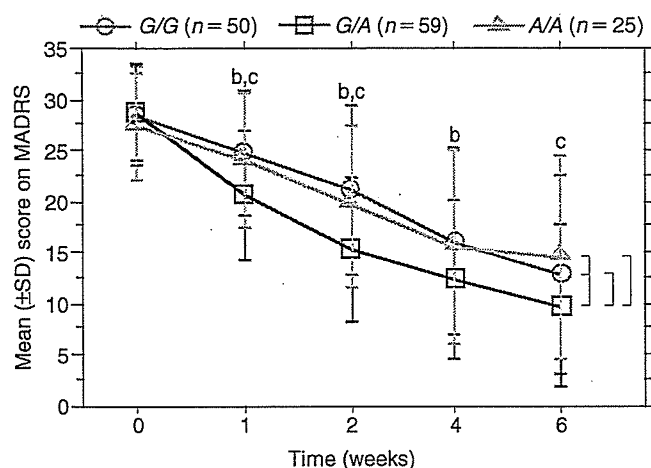

Figure 1 MADRS scores during 6 weeks of milnacipran/fluvoxamine treatment in three BDNF G196A genotype groups[a]

[a] Each point represents te mean score ± SD. Differences in the MADRS scores during this study were examined with the use of two-way repeated measures ANOVA with contrasts. Differences in the MADRS scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.
[b] Significant differences at each point between the *G/A* and *G/G* groups (p=0.0009 at week 1, p=0.0001 at week 2 and p=0.025 at week 4).
[c] Significant difference between the *G/A* and *A/A* groups (p=0.032 at week 1, p=0.019 at week 2 and 0.029 at week 6).
[d] Significant geneotype × time interaction among all three genotype groups (F=3.64, df=8, p=0.0004).
[e] Signicant genotype × time interaction between the *G/A* and *G/G* groups (F=5.21, df=4, p=0.0004)
[f] Significant genotype × time interaction between the *G/A* and *A/A* groups (F=3.99, df=4, p=0.0034).

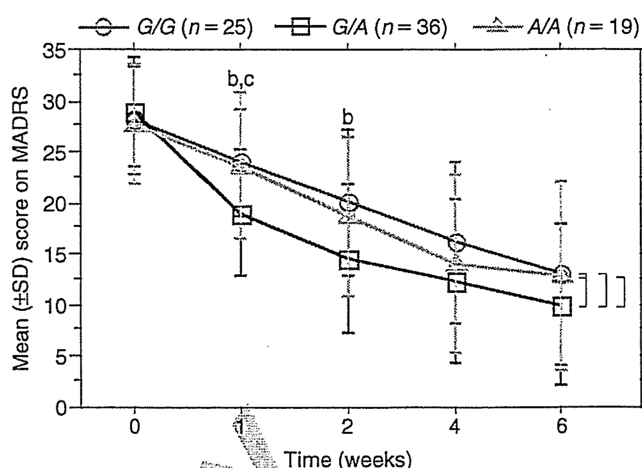

Figure 2 MADRS scores during 6 weeks of milnacipran treatment in three BDNF G196A genotype groups[a]

[a] Each point represents the mean score ± SD. Differences in the MADRS scores during this study were examined with the use of two-way repeated-measures ANOVA with contrasts. Differences in the MADRS scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.
[b] Significant difference at each point between the *G/A* and *G/G* groups (p=0.0031 at week 1 and p=0.0056 at week 2).
[c] Significant difference between the *G/A* and *A/A* groups (p=0.011 at week 1).
[d] Significant genotype × time interaction among all three genotype groups (F=2.30, df=8, p=0.021).
[e] Significant genotype × time interaction between the *G/A* and *G/G* groups (F=3.54, df=4, p=0.0077). f Significant genotype x time interaction between the *G/A* and *A/A* groups (F=2.56, df=4, p=0.039).

trolled. The genotype distribution of these genotypes was not significantly different among the G/G, G/A and A/A genotype groups of the BDNF G196A polymorphism (data not shown).

Fig. 1 shows the MADRS scores over time in relation to the BDNF G196A polymorphism for all subjects receiving fluvoxamine or milnacipran. There was no significant difference in baseline MADRS scores among each genotype group. Two-way repeated measures ANOVA including all three genotype groups indicated a significant genotype × time interaction. Contrast analysis indicated a significant genotype × time interaction between the G/A and G/G genotype groups. The MADRS score of the G/A genotype group was significantly lower than that of the G/G genotype group at 1, 2 and 4 weeks. Contrast analysis indicated a significant genotype x time interaction between the G/A and A/A groups. The MADRS score of the G/A genotype group was significantly lower than that of the A/A group at 1, 2 and 6 weeks. Contrast analysis indicated no significant genotype × time interaction between the G/G and A/A genotype groups (F=0.99, df=4, p=0.41). There was no significant difference in the MADRS score at any evaluation point between the G/G and A/A genotype groups. When milnacipran- and fluvoxamine-treated subjects were analysed independently, the G/A genotype group showed greater reduction of MADRS scores than other genotype groups, irrespective of which antidepressant was administered (Figs. 2 and 3). Mean plasma concentrations of milnacipran were 92.3±50.4 (±SD) ng/ml, 88.1±31.1 ng/ml and 91.7±36.2 ng/ml for the G/G, G/A and A/A genotype groups, respectively. There was no significant difference among the groups (F=0.99, df=2, 77, p=0.90). Mean plasma concentrations of fluvoxamine were 169.1±174.7 (±SD) ng/ml, 155.1±118.6 ng/ml and 94.8±35.3 ng/ml for the G/G, G/A and A/A genotype groups respectively. There was no significant difference among the groups (F=0.65, df=2, 51, p=0.53).

Table 2 shows the genotype distribution and allele frequencies of responders and non-responders for all subjects receiving milnacipran or fluvoxamine. The proportion of responders was higher in G/A subjects than in subjects of other genotypes, but it did not reach a significant difference. There was no significant difference in the allele frequencies between responders and non-responders. The proportion of responders was non-significantly higher in G/A subjects than in subjects of other genotypes, irrespective of which

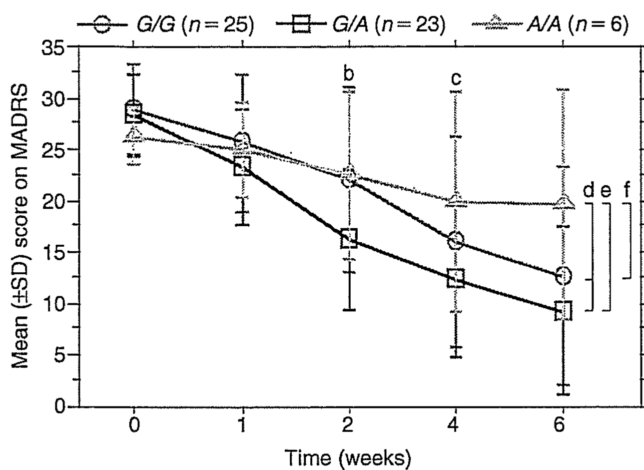

Figure 3 MADRS scores during 6 weeks of fluvoxamine treatment in three BDNF G196A genotype groups[a]

[a] Each point represents the mean score ± SD. Differences in the MADRS scores during this study were examined with the use of two-way repeated-measures ANOVA with contrasts. Differences in the MADRS scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.

[b] Significant difference at each point between the *G/A* and *G/G* groups (p=0.015 at week 2).

[c] Significant difference between the *G/A* and *A/A* groups (p=0.024 at week 6).

[d] Significant genotype × time interaction among all three genotype groups (F=2.83, df=8, p=0.0053).

[e] Significant genotype × time interaction between the *G/A* and *A/A* groups (F=4.55, df=4, p=0.0015).

[f] Significant genotype × time interaction between the *G/G* and *A/A* groups (F=2.77, df=4, p=0.029).

antidepressants were administered (Table 3 and Table 4). When remitters and non-responders were compared, there was also no significant difference in the genotype distribution (χ^2=2.53, df=2, p=0.12 for the milnacipran treatment, χ^2=3.25, df=2, p=0.20 for the fluvoxamine treatment and χ^2=4.26, df=2, p=0.12 for both treatments) and genotype frequencies (χ^2=2.53, df=1, p=0.52 for the milnacipran treatment, χ^2=0.64, df=1, p=0.64 for the fluvoxamine treatment and χ^2=4.26, df=1, p=0.63 for both treatments) (data not shown).

The plasma concentrations of milnacipran or fluvoxamine were not significantly different between responders and nonresponders, as shown in our previous studies (Yoshida *et al.*, 2002; Yoshida *et al.*, 2004).

This study of both milnacipran and fluvoxamine had a power of 0.16 to detect a small effect, 0.88 to detect a medium effect and 0.99 to detect a large effect in the genotype distribution (n=134). For the allele frequency analysis (n=268), this study had a power of 0.37 to detect a small effect, 0.99 to detect a medium effect and 0.99 to detect a large effect. In the power analysis, effect size conventions were determined according to the method of Buchner *et al.* (1996) as follows: small effect size=0.10, medium effect size=0.30 and large effect size=0.50 (alpha=0.05).

Discussion

The present study revealed that the BDNF G196A polymorphism affected the efficacy of both milnacipran and fluvoxamine. The G/A genotype of this polymorphism was associated with a significantly better therapeutic effect in the MADRS scores during this study, although the difference in final therapeutic response was not significant between the G/A and other genotype groups.

The results of this study are not well explained by the findings by Egan *et al.* (2003). Their human study showed that the *A* allele was associated with poorer episodic memory, abnormal hippocampal activation as determined by functional magnetic resonance imaging (fMRI), and lower hippocampal n-acetyl aspartate levels as assayed by MRI spectroscopy. According to their expression study, high concentrations of KCl induced detectable release of G-BDNF, whereas the activity-dependent release of A-BDNF was severely reduced and sometimes not detectable. Thus, the presence of the *G* allele is related to appropriate hippocampal function, neuronal function and activity-dependent BDNF release. In consideration of these findings, it is difficult to interpret the present results.

However, several aspects should be considered before trying to interpret our study based on the findings by Egan *et al.* (2003). First, the behavioural and mood abnormalities associated with major depressive disorder appear to result from disturbances

Table 2 Genotype distribution and allele frequencies in responders and non-responders (milnacipran/fluvoxamine treatment)[a]

	Genotype distribution[b]			Allele frequency[c]	
	G/G	*G/A*	*A/A*	*G*	*A*
Responder	29 (34.1%)	43 (50.6%)	13 (15.3%)	101 (59.4%)	69 (40.6%)
Non-responder	21 (42.9%)	16 (32.6%)	12 (24.5%)	58 (59.2%)	40 (40.8%)

[a] Analysis performed with the use of the χ^2 test.

[b] No significant difference between responders and nonresponders (χ^2=1.32, df=2, p=0.12).

[c] No significant difference between responders and nonresponders (χ^2=0.001, df=1, p=0.97).

Table 3 Genotype distribution and allele frequencies in responders and non-responders (milnacipran treatment)[a]

	Genotype distribution[b]			Allele frequency[c]	
	G/G	*G/A*	*A/A*	*G*	*A*
Responder	13 (26.0%)	26 (52.0%)	11 (22.0%)	52 (52.0%)	48 (48.0%)
Non-responder	12 (40.0%)	10 (33.3%)	8 (26.7%)	34 (56.7%)	26 (43.3%)

[a] Analysis performed with the use of the χ^2 test.
[b] No significant difference between responders and non-responders (χ^2=2.80, df=2, p=0.25).
[c] No significant difference between responders and non-responders (χ^2=0.030, df=1, p=0.57).

Table 4 Genotype distribution and allele frequencies in responders and non-responders (fluvoxamine treatment)[a]

	Genotype distribution[b]			Allele frequency[c]	
	G/G	*G/A*	*A/A*	*G*	*A*
Responder	16 (45.7%)	17 (48.6%)	2 (5.7%)	49 (70.0%)	21 (30.0%)
Non-responder	9 (47.4%)	6 (31.6%)	4 (21.0%)	24 (63.2%)	14 (36.8%)

[a] Analysis performed with the use of the χ^2 test.
[b] No significant difference between responders and nonresponders (χ^2=3.45, df=2, p=0.18).
[c] No significant difference between responders and nonresponders (χ^2=0.53, df=1, p=0.47).

mainly in the temporolimbic-frontal-caudate network (Drevets, 1999; Czeh *et al.*, 2001), although several lines of research support the notion that the hippocampus is also an important region in the pathophysiology of major depressive disorder (Campbell and Macqueen, 2004). Therefore, the functional effect of the BDNF G196A polymorphism on the temporolimbic-frontal-caudate network is necessary to understand the present results adequately. However, such information is extremely limited; to our knowledge, there have been no reports investigating this issue using functional brain imaging, such as fMRI or positron emission tomography.

Only one cognitive study (Foltynie *et al.*, 2005) investigated the effect of the BDNF G196A polymorphism on performance of planning ability in Parkinson's disease using the Tower of London (TOL) task, a test of working memory (Robbins, 1996). The TOL task is reported to increase relative regional cerebral blood flow in the dorsolateral prefrontal cortex, lateral premotor cortex, rostral anterior cingulate cortex and dorsal caudate nucleus (Dagher *et al.*, 1999). Foltynie *et al.* (2005) revealed that the *A* allele of the BDNF G196A polymorphism was associated with better performance at the TOL task. This result is inconsistent with the results by Egan *et al.* (2003), who reported that the presence of the *A* allele was associated with impaired function in the hippocampus. The exact mechanism underlying this discrepancy is unclear. The study by Foltynie *et al.* (2005) was performed in Parkinson's disease not in major depressive disorder. However, it is possible that the functional effects of the BDNF G196A polymorphism differ among areas of the brain in major depressive disorder, and this regional difference in the temporolimbic-frontal-caudate network and the hippocampus may contribute to the better antidepressant effect in patients with the *G/A* genotype.

Additionally, some other studies indicated that subjects heterozygous for the BDNF G196A polymorphism have significant differences in expression of dichotomous or quantitative phenotypes than those homozygous for either allele. Momose *et al.* (2002) reported that homozygosity of the BDNF G196A polymorphism was more frequent in patients with Parkinson's disease. This finding suggests that the *G/A* genotype is less susceptible to Parkinson's disease than other genotypes. Tsai *et al.* (2003) reported a trend to a higher percentage change of the total Hamilton Depression Rating score for heterozygote patients in comparison to homozygote patients after fluoxetine treatment for 4 weeks. Their results are consistent with those of the present study and suggest that the *G/A* genotype is related to a favourable antidepressant effect. Besides the possible regionally different effects of the BDNF G196A polymorphism on brain function, another possibility is that the polymorphism may be in linkage disequilibrium with an as yet unidentified functional polymorphism with a molecular heterotic effect (Comings and MacMurray, 2000).

One major limitation of this study is the relatively small number of subjects, especially in the fluvoxamine arm. A second limitation is the relatively small end point treatment differences. These limitations may increase the possibility of a false positive and make it difficult to conclude that the BDNF G196A polymorphism is the common genetic factor for prediction of the antidepressant effect of both milnacipran and fluvoxamine. Further

studies with a larger number of subjects are needed not only to confirm the results of this study but also to investigate the interaction of many genes, including the BDNF gene, on the mechanisms of antidepressant action.

Acknowledgements

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PHARMACOKINETICS AND DISPOSITION

Terbinafine increases the plasma concentration of paroxetine after a single oral administration of paroxetine in healthy subjects

Norio Yasui-Furukori · Manabu Saito ·
Yoshimasa Inoue · Takenori Niioka · Yasushi Sato ·
Shoko Tsuchimine · Sunao Kaneko

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Abstract
Objective Paroxetine is believed to be a substrate of CYP2D6. However, no information was available indicating drug interaction between paroxetine and inhibitors of CYP2D6. The aim of this study was to examine the effects of terbinafine, a potent inhibitor of CYP2D6, on pharmacokinetics of paroxetine.
Methods Two 6-day courses of either a daily 150-mg of terbinafine or a placebo, with at least a 4-week washout period, were conducted. Twelve volunteers took a single oral 20-mg dose of paroxetine on day 6 of both courses. Plasma concentrations of paroxetine were monitored up to 48 h after dosing.
Results Compared with the placebo, terbinafine treatment significantly increased the peak plasma concentration (C_{max}) of paroxetine, by 1.9-fold (6.4±2.4 versus 12.1±2.9 ng/ml, $p<0.001$), and the area under the plasma concentration-time curve from zero to 48 h [AUC (0–48)] of paroxetine by 2.5-fold (127±67 vs 318±102 ng/ml, $p<0.001$). Elimination half-life differed significantly (15.3±2.4 vs 22.7±8.8 h, $p<0.05$), although the magnitude of alteration (1.4-fold) was smaller than C_{max} or AUC.
Conclusion The present study demonstrated that the metabolism of paroxetine after a single oral dose was inhibited by terbinafine, suggesting that inhibition of CYP2D6 activity may lead to a change in the pharmacokinetics of paroxetine. However, further study is required to confirm this phenomenon at steady state.

Keywords Paroxetine · Terbinafine · CYP2D6 · Interaction

N. Yasui-Furukori (✉) · M. Saito · Y. Sato · S. Tsuchimine ·
S. Kaneko
Department of Neuropsychiatry,
Hirosaki University School of Medicine,
Hirosaki 036-8562, Japan
e-mail: yasufuru@cc.hirosaki-u.ac.jp

Y. Inoue
MP-Technopharma Corporation,
Fukuoka, Japan

T. Niioka
Department of Pharmacy, Hirosaki University Hospital,
Hirosaki, Japan

Introduction

Paroxetine is one of the selective serotonin transporter inhibitors (SSRI) and is widely used in the treatment of mental disorders, including depression, panic disorders, and obsessive compulsive disorder [1, 2]. Paroxetine is almost completely absorbed following oral administration. However, the drug undergoes extensive first pass metabolism [3, 4]. As a result, less than 50% of a single dose of paroxetine reaches the general circulation. Paroxetine is eliminated by metabolism involving oxidation, demethylation, and conjugation [5].

An early in vivo study using healthy volunteers demonstrated a cosegregation between paroxetine and sparteine metabolism, and that steady-state plasma concentration of paroxetine in poor metabolizers (PM) was higher than extensive metabolizers (EM) [6, 7]. Moreover, an in vitro study demonstrated that quinidine and quinine, both of which are potent inhibitors, inhibit paroxetine demethylation in human liver microsomes from EM, but did not inhibit that from PM [8]. Based on these findings, it is evident that paroxetine is primarily metabolized by the cytochrome CYP2D6. However, there are few in vivo data,

indicating that paroxetine concentration is influenced by CYP2D6 modulators despite the fact that numerous in vivo and in vitro studies used paroxetine as a potent inhibitor of CYP2D6 [9–12].

Terbinafine is an orally active allylamine antifungal agent with a primarily fungicidal action in vitro [13, 14]. Clinical trials have demonstrated that orally administered terbinafine is effective in the treatment of dermatophyte infection of the skin [15]. Several in vitro studies demonstrated that terbinafine has a potent inhibitory effect on CYP2D6 activity [16–18]. Several in vivo studies indicated that terbinafine inhibited dextromethorphan [19] and desipramine [20], both of which are substrates of CYP2D6, suggesting an inhibitory effect of terbinafine on CYP2D6 in vivo. Moreover, several case reports demonstrated two cases who suffered from desipramine toxicity [21] and nortriptyline toxicity [22] induced by terbinafine. Terbinafine increased CYP2D6-mediated amitriptyline and nortriptyline concentration for at least 6 months [23], suggesting terbinafine inhibited CYP2D6-mediated metabolism of these antidepressants. Therefore, it is more likely that terbinafine affects the disposition of paroxetine. To our knowledge, however, there is no information about drug–drug interaction between terbinafine and paroxetine. The aim of this study was to confirm the effects of terbinafine on the pharmacokinetics of paroxetine.

Methods

Subjects

Twelve healthy Japanese volunteers (nine males, three females) were enrolled in this study. Their mean±SD of age (range) was 24.8±2.5 (20–35) years and mean body weight was 58.3±8.5 (46–75) kg. The Ethics Committee of Hirosaki University School of Medicine approved this study protocol, and written informed consent had been obtained from each participant before any examinations.

Study design

A randomized crossover study design was conducted at intervals of 4 weeks. One capsule containing either 125 mg of terbinafine or a matched placebo with 240 ml of tap water was given once daily at 0800 hours for 6 days. Compliance of thetest drug was confirmed by pill-count. No other medications were taken during the study periods. No meal was allowed until 4 h after dosing (1300 hours). The use of alcohol, tea, coffee and cola was forbidden during the test days.

Sample collections

Blood samplings (10 ml each) for determination of paroxetine were taken into heparinized tubes just before and 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 36 and 48 h after the administration of paroxetine. Plasma was separated immediately and kept at −30°C until analysis. At the same time as the blood samplings, blood pressure and heart rate were measured. Any adverse events were reported by each subject.

Assay

Plasma concentrations of paroxetine were measured using a high-performance liquid chromatography method developed in our laboratory. In brief, extraction procedure was as follows: to 2,000 μl of plasma sample was added 500 μl of 0.5 M NaOH, 100 μl of internal standard solution (trifluperidol 200 μg/ml) and 100 μl of methanol. Thereafter, the tubes were vortex-mixed for 10 s and 5μml of n-heptane–chloroform (70:30, v/v) was added as extraction solvent. After 10 min of shaking, the mixture was centrifuged at 2,500 *g* for 10 min at 4°C, and the organic phase was evaporated in vacuo at 40°C to dryness (TAITEC VC-960, Shimadzu, Kyoto, Japan). The residue was dissolved in 500 μl of mobile phase, then 400 μl were injected onto the HPLC system. The HPLC system consisted of Shimadzu LC-10AT high-pressure pumps, a Shimadzu CTO-10AVP column oven and a Shimadzu Work station CLASS-VP chromatography integrator (Kyoto, Japan), a Shimadzu SPD-10AVP (Kyoto, Japan) and a Shimadzu SIL-10ADVP (500-μl injection volume) (Tokyo, Japan) and a column (STR-ODS II C18 150×4.6, 3 μm) (Tokyo, Japan). The mobile phase was phosphate buffer (0.02 M, pH=4.6), acetonitrile and perchloric acid (60%) (57.25: 42.5: 0.25, v/v/v). The lowest limit of detection and quantification were 0.5 and 1.0 ng/ml, respectively, and the values of the intra-assay and inter-assay coefficient of variation were less than 10 % at all the concentrations (1.0–150 ng/ml) of calibration curves for paroxetine.

CYP2D6 genotypes

For the determination of *CYP2D6* genotype, DNA was isolated from peripheral leukocytes by a guanidium isothiocyanate method. Genotypings of CYP2D6 were performed using AmpliChip CYP450 Test DNA chip (Roche Diagnostics). The AmpliChip CYP450 Test provides materials for genotyping two cytochrome genes, encompassing 31 known mutations in the CYP2D6 gene, including duplication and gene deletion (CYP2D6*2, *3, *4, *5, *6, *7, *8, *9, *10AB, *11, *14A, *15, *17, *19, *20, *25, *26, *29, *30, *31, *35, *36, *40, *41, *1XN,

*2XN, *4XN, *10XN, *17XN, *5XN, *41XN). The alleles without activity were *3, *4, *5, *6, *7, *8, *11, *14A, *15, *19, *20, *40. The alleles which lead to the decreased enzyme activity were CYP2D6*9, *10, *17, *29, *36, *41.

Date analyses of pharmacokinetics

The peak concentration (C_{max}) and concentration peak time (t_{max}) were obtained directly from the original data. The area under the plasma concentration-time curve [AUC (0–48)] was calculated with use of the lin-lin trapezoidal rule. AUC from zero to infinity [AUC(0–∞)] and elimination half-life were determined by non-compartment model with WinNonlin Professional software (Pharsight, Cary, N.C., USA). The terminal elimination rate constant (k_e) was determined by log-linear regression of the final data points (4). The apparent elimination half-life of the log-linear phase ($t_{1/2}$) was calculated as follows: $0.693/k_e$.

Statistical analysis

Data are shown as means±SD in tables and figures. Paired *t*-test was used for the comparison of the plasma drug concentrations between two phases, i.e., placebo and terbinafine. The comparison of $t_{max}$was performed using the Wilcoxon signed-sample test. A *p*value of 0.05 or less was regarded as significant. Geometric mean ratios to corresponding values in placebo phase with 95% confidence intervals were used for detection of significant differences. When the 95% confidence interval did not cross 1.0, the result was also regarded as significant. When the calculated 90% confidence intervals with logarithmic transformation of pharmacokinetic data (C_{max}, AUC(0–48) and total AUC) fell within, on average, 80–125% for the ratio of the paroxetine plus terbinafine to paroxetine only, we regarded the paroxetine plus terbinafine as bioequivalent to paroxetine only. SPSS 13.0J for Windows (SPSS Japan, Tokyo) was used for these statistical analyses.

Results

The subjects had the following CYP2D6 genotypes: *wt/wt* (4 subjects), **10/wt* (6), **10/*10* (1) and **5/*10* (1), respectively. No subjects regarded as poor metabolizers were included. These patients were divided into three groups according to the number of mutated alleles: no mutated allele in 4, one mutated allele in 6 and two mutated alleles in 2 subjects.

Appetite loss (n=4, n=6), abdominal disturbance (n=4, n=7), diarrhea (n=1, n=1), asthenia (n=5, n=7) and sleepiness (n=3, n=4) were observed in control and terbinafine phases, respectively. All of these side effects were mild to moderate and recovered at longest within 2 days after paroxetine doses.

Plasma drug concentration-time curves during both placebo and terbinafine treatments are shown in Fig. 1. Their pharmacokinetic parameters are summarized in Table 1, and individual data are shown in Table 2. There were three subjects whose plasma concentrations of paroxetine 48 h after paroxetine dosing in control phase were under the detectable quantification (1.0 ng/ml). Although we were not able to calculate accurate averages in 12 subjects, we calculated the average at 48 h in Fig. 1, using the half of limit of quantification (0.5 ng/ml) in the three subjects. Extrapolated AUC of paroxetine was 9.3% for control and 24.9% for terbinafine, respectively.

The C_{max} of paroxetine during terbinafine treatment was higher than the corresponding value during placebo by 1.87-fold [95% confidence interval (95% CI), 1.49, 2.57-fold]. The AUC (0–48) of paroxetine during trebinafine treatment was higher than placebo by 2.53-fold (1.85, 4.58-fold). The total AUC of paroxetine during trebinafine treatment was higher than placebo by 2.88-fold (1.99, 5.41-fold). Elimination $t_{1/2}$ of paroxetine during trebinafine was significantly longer than that during placebo [1.35-fold (1.14, 1.70-fold)]. No change was found in t_{max} [1.14-fold (0.99, 1.36-fold)].

Bioequivalence analyses showed that 90% confidence interval of log-transformed C_{max}, AUC (0–48) and total AUC between two treatments were 130–148%, 116–127 and 118–128%, respectively.

There was significant correlation between number of mutated alleles for CYP2D6 and Cmax (r_s=0.772, p<0.01), AUC(0–48) (r_s=0.724, p<0.05) and total AUC (r_s=0.724, p<0.05). Significant correlations were found between

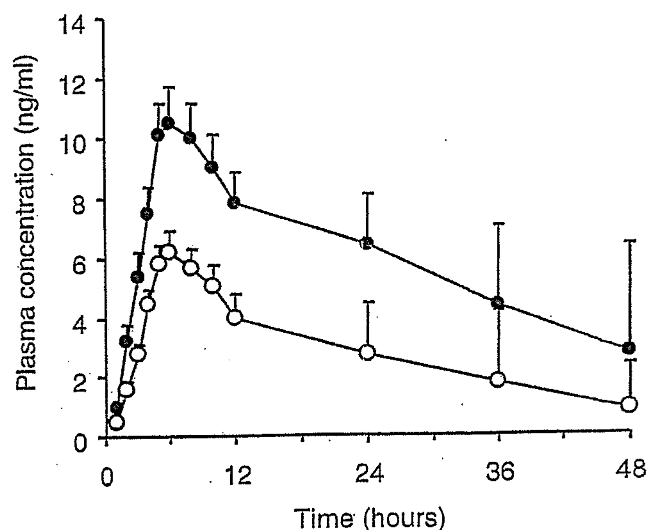

Fig. 1 Mean plasma concentration-time curves of paroxetine after a single oral 20 mg dose of paroxetine. *Open circles* indicate control and *solid circles* indicate terbinafine. *Error bars* indicate standard error

Table 1 Effects of terbinafinee treatment on paroxetine pharmacokinetic parameters after a single oral 20-mg dose of paroxetine in 12 healthy volunteers

Parameters	Control	Terbinafine	Ratio to control
C_{max}(ng/ml)	6.5±2.4	12.1±2.9***	1.92 (1.71, 2.22)
t_{max}(h)	5.0 (4.0–10.0)	6.0 (5.0–8.0)	-
AUC (0–48) (ng.h/ml)	127±67	318±102***	2.64 (2.07, 3.64)
AUC (0–∞) (ng.h/ml)	150±78	443±172***	3.03 (2.46, 4.08)
Vd/F (l)	3,599±1,515	1,577±528***	0.47 (0.36, 0.76)
CL/F (l/h)	167±76	58±44***	0.35 (0.25, 064)
Elimination half-life (h)	15.3±2.3	22.7±8.8*	1.39 (1.13, 1.86)

Data are shown as mean±SD for pharmacokinetic parameters and geometric mean
(95% confidence interval) for ratio to control
Data for tmax are shown as median and range
C_{max}, peak concentration; t_{max}, time to peak concentration in plasma; AUC (0–48), are under plasma concentration-time curve from 0 to 48 h; AUC (0–∞), AUC from 0 to infinity; Vd/F, apparent volume of distribution; CL/F, apparent total clearance
*$p<0.05$, ***$p<0.001$

number of mutated alleles for CYP2D6 and ratio of terbinafine phase to placebo for Cmax ($r_s=-0.628$, $p<0.05$), AUC(0–48) and ($r_s=-0.774$, $p<0.01$), but not total AUC ($r_s=-0.477$, ns). Elimination half-life was not correlated with number of mutated alleles for CYP2D6 ($r_s=0.211$, ns).

There was no relationship between total AUC and the observed side effects after single dose of paroxetine. No difference was found in different sequence (placeco–terbinafine versus terbinafine–placebo).

Discussion

The results of this study showed a significant increase in plasma concentration of paroxetine (C_{max} and AUC) during terbinafine treatment. Additionally, the paroxetine pharmacokinetics were not regarded as bioequivalent according to EMEA and FDA guidance. These findings imply that terbinafine increases the bioavailability of paroxetine or inhibits the metabolism of paroxetine. Our result was in accordance with the previous studies of dextrometrophan [19] and desipramine [20]. Paroxetine is almost completely absorbed following oral administration. However, because paroxetine undergoes extensive first pass metabolism, less than 50% of a single dose of paroxetine reaches the general circulation, suggesting that the first pass effect of paroxetine is more than 50% [4]. Therefore, terbinafine might inhibit the first pass effect of paroxetine in this study.

Furthermore, prolongation of elimination of half-life was observed during terbinafine coadministration. This suggests that terbinafine inhibits metabolism of paroxetine. Previous studies confirmed the decrease in their metabolites, 2-hydroxydesipramine [20] and dextrophan [19], during terbinafine as well as the increase in the substrates of CYP2D6, suggesting that terbinafine inhibits biotransformation from despramine to 2-hydroxydesipramine and from dextrometrophan to dextrophan. Unfortunately, we were unable to measure the metabolites of paroxetine because of undetectable levels in both phases.

Numbers of mutated alleles for CYP2D6 correlated well with Cmax and AUC, but not with elimination half-life in this study. This finding suggests that CYP2D6 activity is associated with the first pass effect of paroxetine. Furthermore, we found a significant relationship between CYP2D6 genotype and the magnitude of this interaction. In subjects having lower CYP2D6 activity, the inhibitory effect of

Table 2 Characteristics of healthy subjects and their individual data

No.	Age	Gender	Weight	CYP2D6	C_{max}(ng/ml)		AUC (0–∞) (ng.h/ml)		Elimination half-life (h)	
					Control	Terbinafine	Control	Terbinafine	Control	Terbinafine
1	29	Male	75	*1/*10	4.7	10.9	88	448	16.2	38.4
2	25	Male	63	*2/*10	6.2	14.2	123	547	12.4	29.6
3	21	Male	64	*1/*1	3.2	6.8	66	99	11.7	8.8
4	21	Female	47	*1/*2	4.3	11.6	72	419	17.0	15.8
5	26	Female	49	*2/*5	6.4	10.2	129	350	13.8	16.6
6	25	Male	57	*1/*1	4.8	10.2	78	350	13.3	16.6
7	20	Male	52	*5/*10	12.3	16.2	304	830	16.1	30.5
8	24	Female	46	*10/*10	9.3	16.9	286	574	16.2	22.9
9	22	Male	54	*1/*10	6.4	10.9	166	406	15.6	18.5
10	24	Male	62	*2/*10	6.9	9.9	141	409	14.1	33.0
11	25	Male	64	*1/*1	6.3	14.9	161	385	18.9	15.7
12	35	Male	66	*1/*10	6.9	12.4	185	510	19.0	25.9

C_{max}, peak concentration; AUC (0–∞), AUC from 0 to infinity;

terbinafine is expected to have been smaller. This confirms that these interactions resulted from CYP2D6 inhibition.

An earlier in vivo study demonstrated that the interphenotype difference in metabolism was less prominent at steady state than after a single dose [7]. There is ongoing controversy as to whether or not the steady-state plasma concentration of paroxetine is different between CYP2D6 genotypes. A recent study indicated no relationship between CYP2D6 genotypes and steady-state plasma concentration of paroxetine [24], although there were significant differences between the CYP2D6 genotypes [25, 26]. Therefore, the magnitude of our interaction might be reduced after repeated doses. Thus, further studies are required to confirm our finding even at steady state.

Severely depressed patients tend to suffer from dermatophytosis in skin, hair and nails due to the difficulty in self-care. On the other hand, systemic fungal infections remain a major clinical problem in immunocompromised patients and such patients tend to have negative thinking [27]. From a clinical point of view, it is more likely that terbinafine is added to depressed patients treated with paroxetine. Thus, interaction between paroxetine and terbinafine should be kept in mind by physicians. Although there is no information about drug interaction between repeated doses of these drugs, dosage of paroxetine should be adjusted based on our result showing the increased exposure of paroxetine during terbinafine.

Limitations of this study included sampling time and washout period. We had a 4-week washout period in this study. Extrapolated AUC of paroxetine was 9.3% for control and 24.9% for terbinafine. If we took blood samples for a longer period (e.g., 72 h), more accurate parameters of paroxetine might have been calculated. In addition, a case report suggested that the effect of terbinafine on CYP2D6-mediated amitriptyline and nortriptyline concentration continues for at least 6 months [23]. Madani et al. [20] suggest that the inhibitory effect of terbinafine on CYP2D6 measured by desipramine continues for 4 weeks, while Abdel-Rahman et al. [19] suggest that CYP2D6 inhibition of terbinafine measured by dextrometrophan metabolic ratios in most subjects return to baseline at 4 weeks after discontinuation of terbinafine. Although half dose (125 mg) and shorter duration (7 days) than previous studies by Madani et al. (250 mg for 21 days) and Abdel-Rahman et al. (250 mg for 14 days) were used in our study and no difference was found in different sequences (placeco–terbinafine versus terbinafine–placebo), the inhibitory effect of terbinafine on CYP2D6 might remain until the control phase.

In conclusion, the present study showed that terbinafine increased paroxetine exposure, probably because of an increase in bioavailability through CYP2D6 inhibition. A change in regulation of CYP2D6 may lead to significant alteration of paroxetine pharmacokinetics.

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