Antidepressant-Like Pharmacological Profile of a Novel Triple Reuptake Inhibitor, (1S,2S)-3-(Methylamino)-2-(naphthalen-2-yl)-1-phenylpropan-1-ol (PRC200-SS)

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Received July 16, 2008; accepted August 7, 2008

ABSTRACT

Due to the putative involvement of dopaminergic circuits in depression, triple reuptake inhibitors are being developed as a new class of antidepressant, which is hypothesized to produce a more rapid onset and better efficacy than current antidepressants selective for serotonin or norepinephrine neurotransmission. (1S,2S)-3-(Methylamino)-2-(naphthalen-2-yl)-1-phenylpropan-1-ol (PRC200-SS), a new triple reuptake inhibitor, potently bound to the human serotonin, norepinephrine, and dopamine transporters with \( K_d \) values of 2.3, 0.63, and 18 nM, respectively. Inhibition of serotonin, norepinephrine, and dopamine uptake by PRC200-SS was also shown in cells expressing the corresponding transporter (\( K_i \) values of 2.1, 1.5, and 61 nM, respectively). In vivo, PRC200-SS dose-dependently decreased immobility in the forced-swim test in rats and in the tail-suspension test in mice, models predictive of antidepressant activity, with effects comparable with imipramine. These results in the behavioral models did not seem to result from the stimulation of locomotor activity. Consistent with the in vitro data and behavioral effects, peripheral administration of PRC200-SS (5 and 10 mg/kg i.p.) significantly increased extra-cellular levels of serotonin and norepinephrine in the medial prefrontal cortex, and of serotonin and dopamine in the core of nucleus accumbens, with reduction of levels of 3,4-dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindoleacetic acid compared with levels for saline control. Furthermore, PRC200-SS self-administration, which was used as a marker of abuse liability, was not observed with rats. Therefore, it seems that PRC200-SS may represent a novel triple reuptake inhibitor and possess antidepressant activity.

The ability of antidepressants to elevate synaptic levels of biogenic amines, such as serotonin (5-HT), norepinephrine (NE), and dopamine (DA), has long been a cornerstone of the biogenic amine hypothesis of affective illness. This hypothesis states in part that depression is a deficit of these amines in the synapse. Whereas evidence accumulated over the years supports the notion that all three of these biogenic amines need to be elevated to treat depression (Skolnick et al., 2003b), only monoamine oxidase inhibitors do so directly (Richelson, 2001). There is considerable evidence linking mesocorticolimbic dopaminergic pathways with depression, especially with the anhedonia and lack of motivation observed in many depressed patients (D'Aquila et al., 2000). Therefore, treating depressed patients with an antidepressant that elevates synaptic levels of DA, in addition to 5-HT and NE, would directly address a core feature of depression. In addition, such a drug might have a broader spectrum of activity and quicker onset of action (Skolnick et al., 2003b).

ABBREVIATIONS: 5-HT, serotonin; NE, norepinephrine; DA, dopamine; SSRI, selective serotonin reuptake inhibitor; PRC025, (1S/1R,2S/2R)-1-cyclohexyl-3-(dimethylamino)-2-(naphthalen-2-yl)propan-1-ol; PRC050, (1S/1R,2S/2R)-3-(dimethylamino)-2-(naphthalen-2-yl)-1-phenylpropan-1-ol; PRC200-SS, (1S,2S)-3-(methylamino)-2-(naphthalen-2-yl)-1-phenylpropan-1-ol; WIN 35428, (+)-2-β-carbethoxy-3-β-(4-fluorophenyl)propyline 1.5-naphthalenedisulfonate; NIMH, National Institute of Mental Health; PDSP, Psychoactive Drug Screening Program; mPFC, medial prefrontal cortex; NA, nucleus accumbens; AUG, area under the curve; ANOVA, analysis of variance; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HIAA, 5-hydroxyindoleacetic acid; DOV 21,947, (+)-1-(3,4-dichlorophenyl)-3-azabicyclo-[3.1.0]hexane hydrochloride; DOV 102,677, (1S,5R)-1-(3,4-dichlorophenyl)-3-azabicyclo-[3.1.0]hexane.
Because monoamine oxidase inhibitors can have serious pharmacodynamic interactions with other drugs and with foodstuffs that contain tyramine, relatively few patients are treated with this class of antidepressant, which can be especially effective in many patients. Thus, the most commonly prescribed antidepressants directly elevate 5-HT (e.g., citalopram), NE (e.g., desipramine), or both (e.g., venlafaxine or mirtazapine) by blocking their transporters or by blocking presynaptic receptors that regulate the release of 5-HT and NE (mirtazapine) (Richelson, 2003). Therefore, researchers have sought to find an antidepressant drug that blocks the transporters for all three key biogenic amines, 5-HT, NE, and DA, the so-called “triple reuptake” inhibitor. Other than the better efficacy as suggested with the triple reuptake inhibitor (Skolnick et al., 2003b), it is possible that the sexual dysfunction related to 5-HT transport blockade, seen very commonly with the selective serotonin reuptake inhibitors (SSRIs) (Rosen et al., 1999), would be attenuated or even eliminated due to the addition of the DA component, because DA opposes 5-HT-promoted prolactin release (Ben-Jonathan and Hnasko, 2001). In addition, due to the link of dysfunction of DA neurotransmission, triple reuptake inhibitors may be of benefit in Parkinson’s disease (Richelson, 2003) and psychostimulant withdrawal with or without depression (Paterson and Markou, 2007). If these hypotheses are proven correct, the therapeutic profile of triple reuptake inhibitors would offer clear advantages over currently available antidepressants.

Venlafaxine (Fig. 1) is a 5-HT and NE reuptake inhibitor with 100-fold less affinity at the NE transporter than at the 5-HT transporter and a minimal effect on blocking the DA transporter (Tatsumi et al., 1997). In collaboration with Dr. Paul Carlier, we synthesized a series of compounds based on the structure of venlafaxine (Carlier et al., 1998). Our previous study found that two of these analogs, PRC025 and PRC050 (both racemic mixtures), are both highly potent triple reuptake inhibitors with activity in animal models used for antidepressant screening (Shaw et al., 2007). We have isolated the pure enantiomers of PRC050, and the present study focuses on PRC200-SS, which is the more active enantiomer (Fig. 1). To characterize the in vivo properties of PRC200-SS on monoamine transporters, we studied its effects on extracellular monoamine levels in the brain with the use of microdialysis in freely moving rats. The forced-swim test and tail-suspension test, animal models commonly used to evaluate potential antidepressants, were used to predict the antidepressant effect of PRC200-SS. Effects on locomotor activity were also assessed, because false positives in the antidepressant-like tests occur with compounds that stimulate this activity. Finally, because DA reuptake-blocking properties of drugs are potentially associated with abuse, we tested PRC200-SS in a drug self-administration paradigm with rats.

Materials and Methods

Animals. Male Sprague-Dawley rats (200–300 g) and male C57BL/6 mice (25–35 g) were used for these studies. Animals were housed in a temperature- and humidity-controlled facility with a 12-h light/dark cycle and free access to food and water throughout the study. Experiments were conducted during the light phase. All animal procedures were reviewed and approved by the Mayo Clinic Institutional Animal Care and Use Committee and were consistent with American Association for the Accreditation of Laboratory Animal Care guidelines.

Human Transporter Binding Assays. The equilibrium dissociation constants ($K_d$) for binding to human transporters in membranes was determined as previously described (Liang et al., 2005).

Fig. 1. Structures of PRC200-SS and other analogs in comparison to venlafaxine.
branial preparations from human embryonic kidney 293 cells expressing the 5-HT, NE, and DA transporters were determined using a previously described method in our laboratory (Tatsumi et al., 1997). In brief, cells were harvested and collected by centrifugation at 110g for 5 min at 4°C. The pellets were homogenized in the respective binding assay buffer using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and then centrifuged twice at 35,600g for 10 min at 4°C. The final pellet was suspended in assay buffer and stored at −80°C until assayed. The final protein concentration was determined by using the BCA Assay (Pierce Biotechnology, Inc., Rockford, IL). [3H]Citalopram (5 nM), [3H]miansetine (5 nM), and [3H]WIN 35428 (10 nM) (PerkinElmer Life and Analytical Sciences, Waltham, MA) were used as radioligands for human 5-HT, NE, and DA transporters, respectively. Compounds were tested in duplicate at 11 different concentrations, spanning 3 orders of magnitude. Data were analyzed by the LIGAND program (Munson and Rodbard, 1980) and presented as geometric means ± S.E.M., which was calculated as described by De Lean et al. (1982).

Neurotransmitter Transport by Intact Cells. The inhibitor constants (Ki) of PRC200-SS for blocking transport of [3H]5-HT, [3H]NE, and [3H]DA (PerkinElmer Life and Analytical Sciences) into human embryonic kidney 293 cells expressing the corresponding human transporter were determined with the use of methods modified from those described previously (Shaw et al., 2007). In brief, medium was removed from cells, which were then washed with phosphate-buffered saline. Oxygenated Krehe-HEPES buffer (pH 7.4) was then added to the flask, and the cells were gently scraped and triturated. Cells were distributed into wells of a 96-well plate. To achieve equilibrium conditions for the antagonists, aliquots of cell suspension were preincubated for 30 min with drugs (over 11 different concentrations) at 37°C. The uptake was initiated by the addition of the radiolabeled neurotransmitter to the cell suspension and was stopped after 10 min by rapid filtration of the contents of each well with the use of a Brandel cell harvester (Brandel Inc., Gaithersburg, MD). The final concentration of radiolabeled neurotransmitter in the assay was 10 nM. Data were again analyzed by the LIGAND program (Munson and Rodbard, 1980) and presented as geometric means ± S.E.M. as described above.

Receptor Binding Screen. Binding data were generously provided by the National Institute of Mental Health's (NIMH) Psychoactive Drug Screening Program (PDSP; contract no. NO1MH32004). The NIMH PDSP is directed by Dr. Bryan L. Roth (University of North Carolina at Chapel Hill, NC) and Project Officer Jamie Driscoll (NIMH, Bethesda MD). For experimental details, please refer to the PDSP Web site at http://pdsp.med.unc.edu/ and select the “Binding Assay” link.

Forced-Swim Test in Rats. The forced-swim test is widely used to screen novel compounds for potential antidepressant activity (Cryan et al., 2005). As originally described by Porosito et al. (1977), the rats were individually placed in vertical cylinders (height, 40 cm; internal diameter, 19 cm) containing water (25°C) to a level of 18 cm. Water was changed between trials, and the procedure involved a pretest and a 5-min test separated by 24 h. During the pretest, rats (adapted to the experimental room for at least 1 h) were placed in the cylinder for 15 min. After this initial exposure, the rats were dried with towels and transferred to a “drying cage” situated under a warming lamp. Fifteen minutes later, rats received intraperitoneal injections with imipramine (15 mg/kg) (Skolnick et al., 2003a) as a positive control, PRC200-SS (1, 5, or 10 mg/kg), or saline and returned to their home cages. The following day, rats were transferred to the experimental room and acclimated for at least 1 h. Rats received injections with imipramine, PRC200-SS, or saline (intrapertoneally) at the same dosages as in the pretest at 5 h and 30 min before testing and then placed in the test chambers. A time-sampling technique was used to score behavior every 5 s during the 5-min test period as described previously (Detke et al., 1995). At the end of each 5-s interval, the behavior of the rat was observed and scored based on the criteria described by Porosito et al. (1977). Scores for each behavior (mobility or immobility) were expressed as total counts per 5-min session.

Tail-Suspension Test in Mice. The tail-suspension test in mice (Steru et al., 1985) seems to be a corroboration of the forced-swim test, with possible sensitivity to a broader range of antidepressants. Pretreatment with imipramine (15 mg/kg) (Skolnick et al., 2003a) as a positive control, PRC200-SS (0.1–10 mg/kg), or saline was given 30 min before testing. Mice were then individually suspended by their tails 35 cm above the tabletop with the use of an adhesive tape placed 1 cm from the tip of the tail. Behavior was scored every 5 s throughout the 6-min test as either mobile or immobile. Mice were consid- ered immobile only when hanging passively and completely motionless. Scores for each behavior were expressed as total counts per 6-min session.

Locomotor Activity. Rats and mice were tested in a Plexiglas Opto-Varimex Minor motility chamber (Columbus Instruments, Columbus OH) to determine whether PRC200-SS affected locomotor activity. Animals were acclimated to the test chamber for 2 h and then received injections with imipramine (15 mg/kg), PRC200-SS, cocaine (positive control), or saline. Thirty minutes postinjection, activity was measured in 10-min intervals for 30 min to correspond to the time of the tail-suspension test (mice) or the forced-swim test (rats).

Microdialysis Procedure and Monoamine Assay. The effects of PRC200-SS on neurotransmitters in different parts of the brain were measured using in vivo microdialysis coupled with high-perfor- mance liquid chromatography and electrochemical detection. Each rat was cannulated in the medial prefrontal cortex (mPFC) (anterior 3.2, lateral 0.5, ventral 2.0) or nucleus accumbens (NA core) (anterior 1.6, lateral 2.4, ventral 6.9, at a 30° angle from midline) relative to bregma and skull (Paxinos and Watson, 1997). After 3 to 5 days for recovery from surgery, a microdialysis experiment was carried out on the conscious, freely moving rat. The microdialysis probe (CMA/12 with 4-mm membrane for mPFC and CMA/12 with 2-mm membrane for NA core (CMA/Microdialysis Inc., Acton, MA) was perfused at 2 μl/min with artificial cerebrospinal fluid. After at least a 2-h equilibration, dialysate samples were automatically collected every 20 min into vials containing 2 μl of perchloric acid (0.5 M) to retard oxidation of monoamines. Three baseline fractions were collected before PRC200-SS (5 or 10 mg/kg) or saline intraperitoneal injection. Monoamines and metabolites in the samples were measured on an ESA high-performance liquid chromatography coupled with a Coulochem II electrochemical detection system (ESA Inc., Chelmsford, MA) and separated on an MD-150 analytical column (3 x 150 mm, 3 μm C18; ESA Inc.) with MDTm mobile phase (ESA Inc.) at 0.6 ml/min. Potential settings for detection were E1 at −175 mV, E2 at 250 mV, and GC at 350 mV. Results were reported as percentage increase over baseline, and the area under the curve (AUC) after the injection was given as the total percentage of increase above base- line. The position of the probe was verified by visual inspection at the end of each experiment.

Operant Self-Administration of PRC200-SS in the Rat. To perform an initial assessment of potential abuse liability of PRC200-SS, operant self-administration experiments were performed. In brief, operant conditioning chambers (MED Associates, St. Albans, VT) placed in sound-attenuated outer chambers were used. First, rats were trained to press the lever in the operant chamber for sucrose pellets to receive a maximum of 20 reinforcements. Once the rats acquired the operant behavior, a surgical procedure was performed on the rats to insert cannulae in their jugular veins. After a 1-week recovery period, the rats were reintroduced to the operant chambers and infused intravenously with PRC200-SS (0.1, 0.5, or 1 mg/kg/infusion in 90-μl volume), cocaine (1 mg/kg/infusion) as posi- tive control, or saline contingent upon pressing the active lever during 1-h daily sessions. The active lever for drug infusion was the same lever used for sucrose reinforcement. The ratio of doses of cocaine-induced behavioral changes (sensitization, 10 mg/kg i.p.) (Segal and Kuczenski, 1992) versus cocaine intravenous self-admin-
istration (0.1–1 mg/kg/infusion) (Schenk et al., 1987) in rats is more than 10. Based on this rationale, PRC200-SS dosages (0.1, 0.5, and 1 mg/kg/infusion i.v.) were based on the effective doses of PRC200-SS (1, 5, and 10 mg/kg i.p.) in the forced-swim test in rats (see results below). When drug self-infusion had stabilized (using cocaine acquisition as the standard), the numbers of infusions were recorded in the final drug self-infusion session by MED Associates computer software (MED Associates). During the entire phase of the experiment, food access was restricted to 20 g/rat/day given immediately after the operant session, except during the surgery and recovery period, when there was free access to food and water.

Statistical Analysis. For the behavioral tests, statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey’s test for post hoc comparisons with the use of SigmaStat software (SPSS Inc., Chicago, IL). For the microdialysis experiments, two-way repeated measures ANOVA followed by Tukey’s test was used to compare the percentage increase over baseline between groups, time, and treatment as independent factors, and time as the repeated factor. Difference in AUC between groups was analyzed by one-way ANOVA using the same software. P < 0.05 was considered significant.

Results
Binding of PRC200-SS and PRC201 to Human Monoamine Transporters. The \( K_i \)s for binding of PRC200-SS to human 5-HT, NE, and DA transporters are given in Table 1, along with PRC201 (the \(-1R,2R\) enantiomer of PRC050) and several reference antidepressants for comparison. Hill coefficients at each binding site were close to unity (data not shown), suggesting that the binding obeyed the law of mass action. The order of potency for PRC200-SS binding to the three human transporters was NE (0.63 ± 0.05 nM) > 5-HT (2.3 ± 0.1 nM) > DA (18 ± 1 nM). PRC201 had less potency for binding to these three transporters than did PRC200-SS.

PRC200-SS Inhibition of 5-HT, NE, and DA Transport. The \( K_i \) values for PRC200-SS at inhibiting uptake of \([3H]5HT\), \([3H]5HT\), and \([3H]DA\) into cells expressing the respective human recombinant transporters were 1.5, 0.6, and 61 ± 4 nM, respectively (Table 2). These data were consistent with its binding affinity at the transporters and showed a similar rank order of potency.

PRC200-SS and PRC201 Receptor Binding Screen. PRC200-SS had weak (\( K_i > 1000 \) nM) or no binding activity at serotonin 5-HT\(_{1a}\), 5-HT\(_{1b}\), 5-HT\(_{1d}\), 5-HT\(_{2a}\), 5-HT\(_{2c}\), 5-HT\(_{3}\), and 5-HT\(_{5a}\), 5-HT\(_{6}\), and 5-HT\(_{7}\); \( \alpha_{1a}\)-, \( \alpha_{1b}\)-, \( \alpha_{2}\)-, \( \alpha_{2a}\)-, \( \alpha_{2b}\)-, \( \alpha_{2c}\)-, \( \beta_{1}\)-, \( \beta_{2}\)-, \( \beta_{3}\)-adrenoceptors; \( \delta\)-, \( \kappa\)-, and \( \mu\)-opioid; histamine H\(_{1}\), H\(_{2}\), H\(_{3}\), and H\(_{4}\); and muscarinic M\(_{1}\), M\(_{2}\), M\(_{3}\), M\(_{4}\), and M\(_{5}\). The \( K_i \) for PRC200-SS at the 5-HT\(_{2b}\) receptor was 430 nM, and the \( K_i \) for PRC200-SS at the \( \sigma_{1}\) receptor was 660 nM.

PRC201 had weak (\( K_i > 1000 \) nM) or no binding activity at serotonin 5-HT\(_{1a}\), 5-HT\(_{1b}\), 5-HT\(_{1d}\), 5-HT\(_{2a}\), 5-HT\(_{2c}\), 5-HT\(_{3}\), 5-HT\(_{5a}\), 5-HT\(_{6}\), and 5-HT\(_{7}\); \( \alpha_{1a}\)-, \( \alpha_{1b}\)-, \( \alpha_{2}\)-, \( \alpha_{2a}\)-, \( \alpha_{2b}\)-, \( \alpha_{2c}\)-adrenoceptors; \( \beta_{1}\)-, \( \beta_{2}\)-, \( \beta_{3}\)-adrenoceptors; dopamine D\(_{1}\), D\(_{2}\), D\(_{3}\), and D\(_{4}\); \( \delta\)-, \( \kappa\)-, and \( \mu\)-opioid; histamine H\(_{1}\), H\(_{2}\), H\(_{3}\), and H\(_{4}\); and muscarinic M\(_{1}\), M\(_{2}\), M\(_{3}\), M\(_{4}\), and M\(_{5}\). The \( K_i \) for PRC201 was as follows: 5-HT\(_{2a}\) receptor, 280 nM; 5-HT\(_{2b}\), 580 nM; and \( \alpha_{2c}\)-adrenoceptors, 690 nM.

Effect of PRC200-SS in the Forced-Swim Test in Rats. Peripheral administration of PRC200-SS resulted in a decrease in immobility and an increase in mobility in the forced-swim test (Fig. 2). PRC200-SS at 10 mg/kg produced similar effects compared with that at the dosage of 5 mg/kg. Similar efficacy was shown for PRC200-SS at 1 mg/kg as with the positive control, imipramine, which was tested at the much higher dosage of 15 mg/kg.

Effect of PRC200-SS in the Tail-Suspension Test in Mice. PRC200-SS dose-dependently decreased immobility and consequently increased mobility of mice in the tail-suspension test (Fig. 3). No significant effect was shown at the lowest dose tested (0.1 mg/kg). The effects of PRC200-SS at 0.5 mg/kg were comparable with the effects seen with the reference antidepressant, imipramine, which was tested at a dose of 15 mg/kg.

Effect of PRC200-SS on Locomotor Activity. The results of the forced-swim test and tail-suspension tests can be invalid, if a compound causes a persistent increase in locomotor activity. To address this potential confound, locomotor activity was measured 30 min postinjection for 30 min, and the dosages of PRC200-SS were selected to correspond to the ranges tested in the forced-swim test (rats) or the tail-suspension test (mice). PRC200-SS produced no significant locomotor activity in rats at 1 and 10 mg/kg and in mice at either dose tested, with effects similar to those of imipramine (15 mg/kg) (Fig. 4). Although PRC200-SS at 5 mg/kg significantly stimulated locomotor activity in rats compared with the saline control, the stimulatory effect was significantly less in

<table>
<thead>
<tr>
<th>Compound</th>
<th>( K_i ) (nM)</th>
<th>hSERT</th>
<th>hNET</th>
<th>hDAT</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRC200-SS</td>
<td>2.3 ± 0.1</td>
<td>0.63 ± 0.05</td>
<td>18 ± 1</td>
<td></td>
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</tr>
<tr>
<td>PRC201</td>
<td>210 ± 20</td>
<td>42 ± 5</td>
<td>200 ± 30</td>
<td></td>
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</tr>
<tr>
<td>PRC050 (racemic)</td>
<td>6.0 ± 0.3</td>
<td>0.40 ± 0.05</td>
<td>120 ± 10</td>
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</tr>
<tr>
<td>Desipramine</td>
<td>17.6 ± 0.7</td>
<td>0.83 ± 0.05</td>
<td>3190 ± 40</td>
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<td></td>
</tr>
<tr>
<td>DOV 102,677</td>
<td>740 ± 140</td>
<td>1030 ± 76</td>
<td>222 ± 43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOV 21,947</td>
<td>99 ± 16</td>
<td>262 ± 41</td>
<td>213 ± 56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imipramine</td>
<td>1.4 ± 0.03</td>
<td>37 ± 2</td>
<td>8500 ± 100</td>
<td></td>
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</tr>
<tr>
<td>Nomifensine</td>
<td>1010 ± 30</td>
<td>15.6 ± 0.4</td>
<td>56 ± 3</td>
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<tr>
<td>Paroxetine</td>
<td>0.13 ± 0.01</td>
<td>40 ± 2</td>
<td>490 ± 20</td>
<td></td>
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</tr>
<tr>
<td>Sertraline</td>
<td>0.29 ± 0.01</td>
<td>420 ± 20</td>
<td>25 ± 2</td>
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<tr>
<td>Venlafaxine</td>
<td>9.0 ± 0.3</td>
<td>1060 ± 40</td>
<td>9300 ± 50</td>
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</tbody>
</table>

hSERT, human serotonin transporter; hNET, norepinephrine transporter; hDAT, dopamine transporter.
comparison to that induced by a low dose of cocaine. Otherwise, at 1 and 10 mg/kg, doses that were effective in the forced-swim test in rats, PRC200-SS had no significant stimulatory effect on activity.

**In Vivo Effects of PRC200-SS on Monoamines and Their Metabolites in Rat Brain.** As shown in Figs. 5 and 6, PRC200-SS (5 or 10 mg/kg i.p.) significantly increased extracellular levels of NE and 5-HT in the mPFC, and DA and 5-HT in the core of the NA, with reduction of levels of 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindole acetic acid (5-HIAA) compared with the saline control. Consistent with the behavioral results in the forced-swim test, PRC200-SS at 5 mg/kg showed comparative effects as 10 mg/kg did in mPFC. PRC200-SS caused a persistent increase in NE levels in the mPFC throughout the microdialysis period (AUC$_{0–240}$ min $\approx 674\%\cdot h$ at 5 mg/kg, AUC$_{0–240}$ min $\approx 805\%\cdot h$ at 10 mg/kg), with the peak value occurring between 180 and 220 min (Fig. 5, A and F). The enhancement of 5-HT levels in mPFC reached the highest value at 40 min postinjection and declined to the saline level at 200 min (Fig. 5B). The effect of PRC200-SS at a dose of 10 mg/kg on 5-HT levels was also found in the NA core, but with 2-fold lower peak values and 4-fold lower AUC$_{0–240}$ min compared with the respective values with the dose of 10 mg/kg in the mPFC (Fig. 6, B and F). The maximal elevation of DA levels (172%) was observed in the NA core at 40 min after administration at 10 mg/kg, and the AUC$_{0–240}$ min was approximately 300% $\cdot$ h (Fig. 6, A and F). Extracellular DOPAC, HVA, and 5-HIAA levels in both brain regions retained stable reductions throughout the study (Figs. 5, C–E, and 6, C–E). The effects on 5-HIAA were similar between these two regions at a dose of 10 mg/kg (AUC$_{0–240}$ min $\approx 140\%\cdot h$), whereas lesser decreases of DA metabolites in mPFC were observed compared with that in the NA core (Figs. 5F and 6F). There was no difference between PRC200-SS and control in DA levels in the mPFC (data not shown).

**Operant Self-Administration of PRC200-SS in the Rat.** On day 7, cocaine self-infusion had stabilized. As shown in Fig. 7, in the final drug self-infusion session, rats significantly self-administered cocaine at 1 mg/kg/infusion compared with the saline control. PRC200-SS was not self-infused at any of the dosages tested. The average number of infusions was 4.1 ± 0.8 in the PRC200-SS group at 0.1

**Table 2**

Inhibitor constants ($K_i$) for reuptake inhibition of serotonin, norepinephrine, and dopamine: PRC200-SS and reference antidepressants

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ for Uptake</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>[$^3$H]Serotonin</td>
<td>[$^3$H]Norepinephrine</td>
</tr>
<tr>
<td>PRC200-SS</td>
<td>2.1 ± 0.6</td>
<td>1.5 ± 0.9</td>
</tr>
<tr>
<td>DOV 102,677</td>
<td>1.23 ± 2.2</td>
<td>1.28 ± 3.3</td>
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<tr>
<td>DOV 21,947</td>
<td>1.0 ± 0.1</td>
<td>0.61 ± 0.07</td>
</tr>
<tr>
<td>Desipramine</td>
<td>4.1 ± 3</td>
<td>4.2 ± 1</td>
</tr>
<tr>
<td>Imipramine</td>
<td>2.7 ± 0.4</td>
<td>3.3 ± 2</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>3.4 ± 0.4</td>
<td>2.20 ± 40</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>3.9 ± 3</td>
<td>2.10 ± 20</td>
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Fig. 2. Effect of PRC200-SS on the amount of mobility and immobility in rats during the forced-swim test. Rats received injections intraperitoneally with PRC200-SS (1, 5, or 10 mg/kg), imipramine (15 mg/kg, as positive control), or saline 24 h, 5 h, and 30 min before the test. Behavior was observed every 5 s during the 5-min test period and scored as mobile or immobile. Results are expressed as the mean number of counts over the 5-min test period (±S.E.M.) ($n = 4–7$ rats). *, $P < 0.05$ versus saline treatment.
mg/kg/infusion, 4.8 ± 0.9 in PRC200-SS at 0.5 mg/kg/infusion, 4.1 ± 0.8 in PRC200-SS 1 at mg/kg/infusion, and 3.9 ± 0.8 in saline control from day 1 to 7, respectively. When animals in the PRC200-SS infusion group were reversed to sucrose pellets instead of drug infusion, lever pressing returned to maximum as observed during the operant training period (data not shown), indicating that animals had not lost the capacity to lever press for the reinforcer.

Discussion

Although PRC200-SS is an analog of venlafaxine (Fig. 1), a 5-HT and NE reuptake inhibitor, our novel compound is clearly a triple reuptake inhibitor. Thus, PRC200-SS bound to all three human transporters with high affinity and a rank order of potency of NE > 5-HT > DA. A similar rank order of potency was found for blockade of reuptake by intact cells expressing the human transporters. As seen in Table 1, PRC200-SS had a higher affinity for the human 5-HT, NE, and DA transporters than many currently available antidepressants (e.g., desipramine and venlafaxine), the discontinued antidepressant nomifensine, and some other new triple reuptake inhibitors in clinical trials (the DOV compounds).

The optimal potency ratio for inhibiting these three monoamine transporters is unknown, but there is a wide variation in potency ratios among clinically active dual reuptake inhibitors (Briley and Moret, 1997). Most likely, the ideal rank order would be N > D > S, considering the balance among the three transporters and the potential adverse effects associated with the transporter blockade (Liang and Richelson, 2008). As seen in Table 1, PRC200-SS (1S,2S-isomer of racemic PRC050) is much more potent than PRC201 (1R,2R-isomer of racemic PRC050) for all three monoamine transporters, a result that indicates the highly stereoselective nature of the binding to these monoamine transporters. In addition, PRC050 (Shaw et al., 2007), the racemic compound containing both PRC200-SS and PRC201, was less potent in binding to 5-HT and DA transporters compared with PRC200-SS. Taken together, both the stereoselectivity and structural specificity should be considered in the development of future triple reuptake inhibitors.

PRC200-SS and PRC201 were also screened for binding at various receptors, and, like venlafaxine (Cusack et al., 1994), these compounds had no or very weak effects on 5-HT, NE, DA, histamine, and muscarinic receptors, in particular. This suggests that certain adverse effects related to blockade of certain neurotransmitter receptors (Richelson, 2003) may not be seen with this compound.

The antidepressant-like activity of PRC200-SS was evaluated using the forced-swim test in rats and the tail-suspension test in mice. These tests are highly predictive of clinically effective antidepressants (Borsini and Meli, 1988). PRC200-SS was active in both of these models, and the effects did not seem to be due to motor stimulation, because no significant increase in motor activity was observed at doses active in these two behavioral tests. Drugs that increase locomotor activity such as psychomotor stimulants can produce false positives in the forced-swim and tail-suspension tests (Cryan et al., 2005). Although PRC200-SS at a dose of 5 mg/kg i.p. showed a significant increase in the locomotor activity in rats compared with the saline group, the magnitude of the locomotor stimulation was comparable with that observed with a previously reported triple reuptake inhibitor, DOV 21,947, which was tested under similar experimental conditions (Skolnick et al., 2003a) and was much less than that observed with a low dose of cocaine (4 mg/kg). It is interesting to note that at the higher dosage of 10 mg/kg, PRC200-SS did not show higher locomotor activity compared with the saline or cocaine group. Therefore, it is not likely that the increase in locomotor activity observed at 5 mg/kg PRC200-SS in rats would invalidate the results of the forced-swim test where a lower dose (1 mg/kg) and a higher dose (10 mg/kg) were also active in this test.

The in vivo microdialysis studies provide functional support for the inhibition of all three transporters by PRC200-SS.
at behaviorally relevant doses. Nonetheless, the magnitude of monoamine increases after PRC200-SS did not fit our prediction that was based on the affinity for the human monoamine transporters. There are several possible explanations for this result. These include species differences in affinities of PRC200-SS for the different transporters, the regional density differences of monoamine transporters, the kinetics of increase in the neurotransmitters (Popik et al., 2006), and interactions with presynaptic receptors that modulate neurotransmitter release (Garris et al., 2003). For example, immunohistochemical and neuroanatomical studies suggest that the shell of NA receives a prominent noradrenergic innervation and has most of the NE transporters, which are largely absent from the core (McKittrick and Abercrombie, 2007). These anatomical differences can explain why we did not observe NE changes in the NA core after administration of PRC200-SS. In addition, no changes of DA after injection of PRC200-SS were found in mPFC, which is known to lack DA transporters (Sesack et al., 1998). Although NE transporters in noradrenergic terminals may play a significant role in the clearance of extracellular DA in the mPFC (Yamamoto and Novotney, 1998), the present results suggest that NE transporter blockade by PRC200-SS is not sufficient to increase levels of extracellular DA, as measured by in vivo microdialysis.

PRC200-SS caused less of an increase in 5-HT levels in the mPFC and NA core, and its effects were shorter lasting in comparison with its effects on NE and DA in these areas, respectively. These results probably relate to activation of terminal 5-HT_{1A} autoreceptors that feed back to inhibit 5-HT release, reducing the magnitude (and possibly the duration) of the increase in extracellular 5-HT levels. SSRI administration to rodents is known to cause similar, small increases in extracellular 5-HT levels as found with PRC200-SS (Hjorth, 1993; Invernizzi et al., 1996), and this attenuated 5-HT response is readily enhanced with specific antagonists of 5-HT_{1A} autoreceptors (Hjorth, 1993; Gartside et al., 1999). Nonetheless, the ability of PRC200-SS to elevate NE and 5-HT levels in mPFC, and DA and 5-HT in the NA core over time, may be importantly related to its onset of therapeutic activity.

The slow onset and long-lasting reduction in extracellular monoamine metabolites associated with the increase in extracellular NE, DA, and 5-HT after PRC200-SS administration was also observed with another triple reuptake inhibitor, DOV 102,677 (Popik et al., 2006), the SSRI fluoxetine (Clark et al., 1996), and other monoamine uptake blockers (Gardner et al., 2006), suggesting that it might be a common property of monoamine transporter inhibitors. These results are totally consistent with the action of transporter-blocking drugs and with our understanding of the metabolism of catecholamines and 5-HT by intracellular enzymes. Upon reuptake into the nerve ending, these biogenic amines can be metabolized by monoamine oxidase and other intracellular enzymes or be repackaged into synaptic vesicles for future release (Axelrod, 1971). With blockade of this reuptake, there is less neurotransmitter available for the formation of metabolites, which leak into the extracellular space. In this regard, it has been shown that extracellular DOPAC and HVA levels are dependent on both cytoplasmic neurotransmitter levels and monoamine oxidase activity (Hurd and Ungerstedt, 1989).

A concern with drugs that block DA transporters is their potential reinforcing effects and abuse liability. For instance, although cocaine binds to DA, 5-HT, and NE transporters (Tatsumi et al., 1997), the binding to the DA transporter is thought to correlate best with potential for cocaine self-administration (Kuhar et al., 1991). In addition, many studies have verified that the mesocorticolimbic DA system is implicated in addiction by psychostimulants (Di Chiara, 2000).
Fig. 5. Effects of PRC200-SS on extracellular NE (A), 5-HT (B), DOPAC (C), HVA (D), and 5-HIAA (E) levels in mPFC. PRC200-SS (5 or 10 mg/kg i.p.) or saline (intraperitoneal) was given at zero time. Results are expressed as percentage increase above baseline (average of three untreated points), and the AUC (F) after the injection was given as the total percentage of increase above baseline, shown as mean ± S.E.M. (n = 5 rats). *P < 0.05 versus saline group.
Fig. 6. Effects of PRC200-SS on extracellular DA (A), 5-HT (B), DOPAC (C), HVA (D), and 5-HIAA (E) levels in the nucleus accumbens core. PRC200-SS (10 mg/kg i.p.) or saline (intraperitoneal) was given at zero time. Results are expressed as percentage increase above baseline (average of three untreated points), and the area under the curve (AUC) (F) after the injection was given as the total percentage of increase above baseline, shown as mean ± S.E.M. (n = 4 rats). *, P < 0.05 versus saline group.
Thus, triple reuptake inhibitors will probably receive extra scrutiny regarding their abuse liability. However, blockade of the DA transporter does not necessarily lead to drug abuse. It has been demonstrated that a fast rate of DA transporter blockade, rather than high affinity, may be more relevant to addictive potential (Volkow et al., 2005). Moreover, increased serotonergic activity is associated with decreased reinforcing potency and efficacy among drugs that increase monoaminergic neurotransmission (Wee et al., 2005; Howell et al., 2007). Although an increase in DA levels via blockade of DA transporter by PRC200-SS was observed in the NA core, an important region of the brain in drug addiction (Cadoni et al., 2000), PRC200-SS was not self-administered in the rat at the effective behavioral dosage. It should be noted that because we were limited by the amount of compound that was available to us for these studies, the self-administration experiments were not carried out for an extended period of time and at higher dosages. However, at a time in which cocaine self-administration had been strongly acquired, PRC200-SS was not self-administered at any of the dosages tested. Considering the average number of infusions during the whole session, the total infused dosage of PRC200-SS was approximately four times the single intravenous dose. Based on these initial results, it seems that PRC200-SS does not possess the same abuse liability as cocaine, although future studies will more rigorously test this idea.

Based on the results of this preclinical study, PRC200-SS may possess antidepressant activity and represents a new triple reuptake inhibitor with potential for clinical use. Whether this compound would have a more rapid onset of therapeutic action or greater efficacy than currently prescribed antidepressants needs further investigation. However, the antidepressant-like properties of this compound and other triple reuptake inhibitors certainly merit study in the clinic.

References


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