The Novel 5-Hydroxytryptamine$_{1A}$ Antagonist LY426965: Effects on Nicotine Withdrawal and Interactions with Fluoxetine

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ABSTRACT
LY426965 [((2S)-(+)1-cyclohexyl-4-[4-(2-methoxyphenyl)-1-piperazinyl][2-methyl-2-phenyl-1-butanone monohydrochloride] is a novel compound with high affinity for the cloned human 5-hydroxytryptamine (HT)$_{1A}$ receptor ($K_i = 4.66$ nM) and 20-fold or greater selectivity over other serotonin and nonserotonin receptor subtypes. Both in vitro and in vivo studies indicate that LY426965 is a full antagonist and has no partial agonist properties. LY426965 did not stimulate $[^{35}S]$guanosine-5'-O-(3-thio) triphosphate (GTP$_S$) binding to homogenates of cells expressing the cloned human 5-HT$_{1A}$ receptor in vitro but did inhibit 300 nM 5-HT-stimulated $[^{35}S]$GTP$_S$ binding with a $K_i$ value of 3.07 nM. After both p.o. and s.c. administration, LY426965 blocked the lower lip retraction, flat body posture, hypothermia, and increase in rat serum corticosterone induced by the 5-HT$_{1A}$ agonist 8-OH-DPAT (8-hydroxy-2-dipropylaminotetralin). In pigeons, LY426965 dose-dependently blocked the stimulus cue induced by 8-OH-DPAT but had no 8-OH-DPAT-like discriminative properties. LY426965 completely reversed the effects of nicotine withdrawal on the auditory startle reflex in rats. In microdialysis experiments, LY426965 administered together with fluoxetine significantly increased extracellular levels of serotonin above those achievable with fluoxetine alone. In electrophysiological studies, the administration of LY426965 produced a slight elevation of the firing rate of 5-HT neurons in the dorsal raphe nucleus of anesthetized rats and both blocked and reversed the effects of fluoxetine on 5-HT neuronal activity. These preclinical results indicate that LY426965 is a selective, full 5-HT$_{1A}$ antagonist that may have clinical use as pharmacotherapy for smoking cessation and depression and related disorders.

Selective antagonists of the serotonin$_{1A}$ [hydroxytryptamine$_{1A}$ (5-HT$_{1A}$)] receptor have been proposed to have clinical use in a variety of neuropsychiatric disorders, including anxiety, depression, smoking cessation, and Alzheimer’s disease (Rasmussen et al., 1997; see Rasmussen and Rocco, 1995, for review). Recently, 5-HT$_{1A}$ antagonists have been shown to be able to attenuate the effects of nicotine withdrawal on the auditory startle reflex (Rasmussen et al., 1997). In addition, 5-HT$_{1A}$ antagonists have been proposed for adjunctive use with selective serotonin reuptake inhibitors (SSRIs) for the treatment of depression. In clinical trials, some studies have shown significant effects of pindolol in enhancing the antidepressant effect of SSRIs, whereas other studies have failed to support this hypothesis (Artigas et al., 1994; Berman et al., 1997). One recent report concludes that pindolol can accelerate the antidepressant action of SSRIs in previously untreated patients but not in treatment-resistant patients (Perez et al., 1999). Conversely, another recent study concludes that pindolol can increase the antidepressant effect of fluoxetine in treatment-resistant patients but does not accelerate the onset of antidepressant effects (Maes et al., 1999). One limitation of these clinical studies is the compound used to test the hypothesis. Pindolol has its highest affinity for the $\beta$-adrenergic receptor and is a partial agonist at the 5-HT$_{1A}$ receptor (Chopin et al., 1994). WAY-100635 is a selective, full antagonist at 5-HT$_{1A}$ receptors and has become a standard preclinical research tool (Forster et al., 1995). However, WAY-100635 is rapidly metabolized, has a limited duration of action, and is not active after p.o. administration (our unpublished observations). Thus, the evaluation of 5-HT$_{1A}$ antagonists in human disease states awaits the development of a full and selective 5-HT$_{1A}$ antagonist that is amenable to clinical trials. Here, we describe the in vitro and in vivo pharmacology of a novel, selective 5-HT$_{1A}$

ABBREVIATIONS: 5-HT, hydroxytryptamine; DRN, dorsal raphe nucleus; SSRI, selective serotonin reuptake inhibitor; GABA, $\gamma$-aminobutyric acid; GTP$_S$, guanosine-5’-O-(3-thio)triphasphate; 8-OH-DPAT, 8-hydroxy-2-dipropylaminotetralin; DMSO, dimethyl sulfoxide.
antagonist, (2S)-(+)-1-cyclohexyl-4-[4-(2-methoxyphenyl)-1-piperazinyl]-2-methyl-2-phenyl-1-butane monohydrochloride (LY426965; Fig. 1), including its activity in tests thought to be predictive of clinical use for smoking cessation and depression and related disorders.

**Materials and Methods**

**Receptor Binding.** All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Benzodiazepine, histamine, muscarinic, dopaminergic receptor binding assays were performed according to the cited reference with minor modifications (Table 1). Frozen rat brain tissue (whole brain for benzodiazepine, histamine, and $\alpha_{1}$, $\alpha_{2}$, and $\beta$-adrenergic assays; cortex for muscarinic and GABA assays; or corpus striatum for dopamine assays) for each receptor assay was homogenized using a Polytron homogenizer PT-10 (setting 6 for 15 s $\times$ 2; Brinkmann Instruments, Westbury, NY). For the benzodiazepine, histamine, and $\alpha_{1}$, $\alpha_{2}$, and $\beta$-adrenergic assays, the tissue was homogenized in 25 volumes of 0.25 M sucrose and centrifuged at 1000 $g$ for 10 min. The supernatant was centrifuged at 40,000 $g$ for an additional 10 min, and the resulting pellet was resuspended in buffer (Table 1) and centrifuged an additional 10 min at 40,000 $g$. For muscarinic and dopamine assays, the tissue was homogenized in 25 volumes of buffer (Table 1) and centrifuged at 40,000 $g$ for 10 min. The resulting pellet was resuspended in buffer and centrifuged for an additional 10 min at 40,000 $g$. Cortex for GABA assays was prepared using freezing, thawing, and Triton X-100 extraction to remove endogenous GABA (Williams and Risley, 1979). For all assays, the final concentration of radioligand and the concentration used for each antagonist dose-response curve, LY426965 was dissolved in 25% 2-hydroxypropyl-beta-cyclodextrin (Research Biochemicals International, Natick, MA, respectively).

**5-HT-Stimulated [*35S*]GTP$\gamma$S Binding to Homogenates of Cells Expressing Human 5-HT$_{1A}$ Receptor.** The [*35S*]GTP$\gamma$S binding assay is based on an assay previously described (Wainscott et al., 1998) but adapted to a scintillation proximity assay (SPA) format. Incubations were performed in a total volume of 200 $\mu$l in 96-well assay plates. [*35S*]GTP$\gamma$S and GDP in assay buffer (MgCl$_2$, NaCl, EGTA in Tris-HCl, pH 7.4), 50 $\mu$l, were added to 50 $\mu$l of test compounds dissolved in water (glacial acetic acid was used to aid in solubilization of LY426965 oxalate). Wheat Germ Agglutinin (WGA) beads (Amersham Life Sciences, Inc., Arlington Heights, IL) for SPA, in assay buffer, were then added. Membrane homogenate, in assay buffer, from mouse LMTk$^+$ cells stably transfected with the human cloned 5-HT$_{1A}$ receptor was added, and the plates were covered with sealing tape (Wallac) and allowed to incubate at room temperature for 2 h. The final concentrations of MgCl$_2$, NaCl, EGTA, GDP, [*35S*]GTP$\gamma$S, and Tris were 3 mM, 120 mM, 0.2 mM, 10 $\mu$M, $\sim$0.25 nM, and 50 mM, respectively. The plates were then centrifuged at approximately 200g for 10 min at room temperature. The amount of [*35S*]GTP$\gamma$S bound to the membranes (i.e., in close proximity to the WGA SPA beads) was then determined using a Wallac MicroBeta Trilux Scintillation Counter.

**Antagonism of (2)-8-Hydroxy-2-dipropylaminotetralin (8-OH-DPAT)-Induced Lower Lip Retraction, Flat Body Posture, and Hypothermia.** 8-OH-DPAT (Research Biochemicals International; all calculations of dose based on the salt; 0.1 mg/kg) was administered s.c. 20 min before scoring to male Sprague-Dawley rats (average weight, 250 g; Harlan Sprague-Dawley, Cumberland, IN). The rats were placed into individual plastic cages with a wire floor for a 10-s observation period, and the degree of lower lip retraction and flat body posture was scored once on a scale of 0 to 3 (Wolff et al., 1997). The scorer was not blind to the treatment conditions. After the behavioral observations, the rats' core body temperature was measured by a rectal probe inserted 4.5 cm. To determine the s.c. antagonist dose-response curve, LY426965 was dissolved in 25% 2-hydroxypropyl-beta-cyclodextrin (Research Biochemicals International) and administered 35 min before the scoring. To determine the p.o. antagonist dose-response curve, LY426965 was put into solution with 5% acacia and administered 60 min before the scoring. ED$_{50}$ values were calculated for the dose-response curves using JMP software (SAS Institute, Inc., Cary, NC). To examine the duration of activity, LY426965 was administered at 10 and 20 mg/kg p.o. (in 5% acacia) at 4, 8, and 16 h before scoring. The dihydrochloride salt of LY426965 was used in these experiments.

**Antagonism of 8-OH-DPAT-Induced Increase in Rat Serum Corticosterone Concentrations.** Male Sprague-Dawley rats weighing 180 to 200 g were purchased from Harlan Sprague-Dawley, Inc. Rats were housed five per cage in a 22°C room with lights on from 7:00 AM to 7:00 PM for 1 week before experimentation. Food and water were freely available. LY426965 was injected s.c. or by gavage in a suspension of 1% carboxymethylcellulose plus 0.25% polysorbate 80 (2 ml/kg) 1 h before 8-OH-DPAT. 8-OH-DPAT (Research Biochemicals International) was dissolved in 0.01 N HCl and injected at 0.3 mg/kg s.c. Control rats received vehicle injections.

![Fig. 1. Structure of LY426965.](image-url)
The experiments were conducted in pigeon operant conditioning chambers (Med Associates, East Fairfield, VT) that were placed in light- and sound-attenuated enclosures equipped with ventilation fans and white noise generators. During each session, both the right and the left response keys were transilluminated by white stimulus lights, and a houselight was turned on in the chamber. Mixed grain could be presented through an opening centered beneath the response keys. During grain presentation, this opening was illuminated, and the right and left key lights, as well as the houselight, were extinguished.

**Antagonism of 8-OH-DPAT-Induced Discriminative Stimulus in Pigeons**. Six male white Carneau pigeons (Palmetto Pigeon Plant, Sumter, SC) were housed in individual stainless steel cages with water and crushed oyster shells continuously available, except during experimental sessions. The pigeons were maintained at approximately 85% of their free feeding body weights by postsession supplemental feedings of ProGrains for Pigeons (Purina Mills Inc., St. Louis, MO). All testing was conducted during the illuminated phase of the light/dark cycle (6:00 AM to 6:00 PM).

Rats were sacrificed by decapitation 1 h after 8-OH-DPAT injections; trunk blood was collected, and serum samples were obtained by centrifugation and stored frozen before being assayed. Rat serum corticosterone was measured by radioimmunoassay (Corticosterone 3H-Kit; ICN Biomedicals, Costa Mesa, CA). Statistical analyses were made with ANOVA using Tukey’s Honestly Significant Difference method (*P < .05) based on the mean square error. The dihydrochloride salt of LY426965 was used in these experiments.

**TABLE 2**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Radioligand</th>
<th>Buffer</th>
<th>Tissue</th>
<th>Nonspecific</th>
<th>Incubate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT1A</td>
<td>[3H]DPAT (2.0)</td>
<td>Tris + salts* 50 mM, pH 7.4</td>
<td>Transfected</td>
<td>5-HT (0.01)</td>
<td>0.5/37</td>
<td>Zgombick et al., 1991</td>
</tr>
<tr>
<td>5-HT1B</td>
<td>[3H]5-HT (5.0)</td>
<td>Tris + salts* 50 mM, pH 7.4</td>
<td>Transfected</td>
<td>5-HT (0.01)</td>
<td>0.5/37</td>
<td>Zgombick et al., 1991</td>
</tr>
<tr>
<td>5-HT1D</td>
<td>[3H]5-HT (5.0)</td>
<td>Tris + salts* 50 mM, pH 7.4</td>
<td>Transfected</td>
<td>5-HT (0.01)</td>
<td>0.5/37</td>
<td>Zgombick et al., 1991</td>
</tr>
<tr>
<td>5-HT1E</td>
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<td>Tris + salts* 50 mM, pH 7.4</td>
<td>Transfected</td>
<td>5-HT (0.01)</td>
<td>0.5/37</td>
<td>Zgombick et al., 1991</td>
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<td>5-HT1F</td>
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<td>5-HT (0.01)</td>
<td>0.5/37</td>
<td>Zgombick et al., 1991</td>
</tr>
<tr>
<td>5-HT2A</td>
<td>[3H]DOI (0.5)</td>
<td>Tris + salts* 50 mM, pH 7.4</td>
<td>Transfected</td>
<td>5-HT (0.01)</td>
<td>0.5/37</td>
<td>Zgombick et al., 1991</td>
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<tr>
<td>5-HT2B</td>
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<td>Tris + salts* 50 mM, pH 7.4</td>
<td>Transfected</td>
<td>5-HT (0.01)</td>
<td>0.5/37</td>
<td>Zgombick et al., 1991</td>
</tr>
<tr>
<td>5-HT2C</td>
<td>[3H]DOI (0.5)</td>
<td>Tris + salts* 50 mM, pH 7.4</td>
<td>Transfected</td>
<td>5-HT (0.01)</td>
<td>0.5/37</td>
<td>Zgombick et al., 1991</td>
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<td>5-HT3</td>
<td>[3H]5-HT (5.0)</td>
<td>Tris + salts* 50 mM, pH 7.4</td>
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<td>5-HT (0.01)</td>
<td>0.5/37</td>
<td>Zgombick et al., 1991</td>
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<td>5-HT5</td>
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<td>0.5/37</td>
<td>Zgombick et al., 1991</td>
</tr>
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</table>

* Salts: 10 mM MgCl2, 0.5 mM EDTA, 10 μM pargyline, and 0.1% ascorbate.

**TABLE 1**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Radioligand</th>
<th>Buffer</th>
<th>Tissue</th>
<th>Nonspecific</th>
<th>Incubate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-Adrenergic</td>
<td>[3H]Prazosin (0.2)</td>
<td>Tris-Cl 50 mM, pH 7.7</td>
<td>Whole brain</td>
<td>WB4101 (0.1)</td>
<td>1/25</td>
<td>Green grass and Bremner, 1979</td>
</tr>
<tr>
<td>α2-Adrenergic</td>
<td>[3H]Rauwolscine (0.4)</td>
<td>Tris + EDTA (1 mM) 50 mM, pH 6.9</td>
<td>Whole brain</td>
<td>Yohimbine (1.0)</td>
<td>1/23</td>
<td>Boyajian and Leslie, 1987</td>
</tr>
<tr>
<td>β-Adrenergic</td>
<td>[3H]IICP (0.05)</td>
<td>Tris-Cl 50 mM, pH 7.7</td>
<td>Whole brain</td>
<td>(−)-Propranolol (1.0)</td>
<td>1/23</td>
<td>Bylund and Snyder, 1976</td>
</tr>
<tr>
<td>Dopamine D1</td>
<td>[3H]SCH23390 (0.2)</td>
<td>Tris + salts* 50 mM, pH 7.4</td>
<td>Corpus striatum</td>
<td>SCH23390 (0.03)</td>
<td>1/23</td>
<td>Billard et al., 1984</td>
</tr>
<tr>
<td>Dopamine D2</td>
<td>[3H]Raclopride (0.8)</td>
<td>Tris + salts* 50 mM, pH 7.4</td>
<td>Corpus striatum</td>
<td>Spiperone (0.03)</td>
<td>1/23</td>
<td>Hall et al., 1988</td>
</tr>
<tr>
<td>Benzodiazepine</td>
<td>[3H]Flunitrazepam (1.0)</td>
<td>Tris-Cl 50 mM, pH 7.4</td>
<td>Whole brain</td>
<td>Clonazepam (1.0)</td>
<td>1/4</td>
<td>Brooks and Rich, 1989</td>
</tr>
<tr>
<td>Histamine H1</td>
<td>[3H]Pyrimidazam (2.0)</td>
<td>Na2HPO4 50 mM, pH 7.5</td>
<td>Whole brain</td>
<td>Promethazine (1.0)</td>
<td>0.5/23</td>
<td>Tran et al., 1978</td>
</tr>
<tr>
<td>GABAα</td>
<td>[3H]Muscimol (2.0)</td>
<td>Tris-Cl 50 mM, pH 7.4</td>
<td>Cortex</td>
<td>GABA (10.0)</td>
<td>1/4</td>
<td>Williams and Risley, 1989</td>
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<tr>
<td>Muscarinic</td>
<td>[3H]QNB (1.0)</td>
<td>Na2HPO4 50 mM, pH 7.4</td>
<td>Cortex</td>
<td>Atropine (1.0)</td>
<td>1/23</td>
<td>Yamamura and Snyder, 1974</td>
</tr>
</tbody>
</table>

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a Salts: 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, and 1 mM MgCl2.
group, the “8-OH-DPAT” key was the right key, whereas for the third pigeon, it was the left key. Thirty consecutive responses on the injection-appropriate key resulted in 4 s access to grain. Responses on the inappropriate key reset the response requirement on the injection appropriate key. After the pigeons exhibited a reliable discrimination, control data were collected from the training sessions that continued to occur on Monday, Tuesday, and Thursday. Substitution tests were conducted on Wednesday and Friday if performance on the preceding training days met the minimal criterion of 90% correct responding. On test days, responding on either key resulted in grain presentation. The test session lasted until 30 grain presentations occurred or 30 min elapsed. The percentage of responses that occurred on the 8-OH-DPAT appropriate key and the rate of responding (in responses/s) were recorded. Dose-response curves were determined by averaging the data obtained from each pigeon. A drug was considered to have fully substituted for 8-OH-DPAT if 80% or more responding occurred on the drug key. Thirty percent or less responding on the drug key indicated a lack of substitution, whereas intermediate values were considered to be partial substitution. At the start of the present experiment, all pigeons had extensive experience with this paradigm.

LY426965 Studied as an Agonist. Various doses of LY426965 were injected 20 min before the start of the test session to the pigeons trained to discriminate 0.16 mg/kg 8-OH-DPAT from saline.

LY426965 Studied as an Antagonist. Various doses of LY426965 were injected 15 min before 0.64 mg/kg 8-OH-DPAT, which was administered 20 min before the test session to the pigeons trained to discriminate 0.64 mg/kg 8-OH-DPAT from saline.

LY426965 was dissolved in 5% 2-hydroxpropyl-β-cyclodextrin and administered i.m. in a volume of 1 mg/ml. The dihydrochloride salt of LY426965 was used in these experiments (n = 3/dose).

Nicotine Withdrawal-Enhanced Auditory Startle Response. Forty male Long-Evans rats weighing 325 to 350 g were surgically implanted with s.c. osmotic minipumps (Alzet Corporation, Palo Alto, CA) that delivered 6 mg of nicotine tartrate (calculated as the base/day) for 12 days. On the 12th day, the osmotic pumps were removed, and approximately 24 h after the removal of the nicotine pumps, auditory startle testing was initiated. Startle testing was conducted across 25 trials when a 120 ± 2 dBA auditory stimulus was presented and peak amplitude (Vmax) was recorded. To evaluate the effect of LY426965, rats were orally gavaged with various doses of LY426965 (0, 0.1, 1.0, and 10 μg/kg) 1 h before startle testing. Startle responses were averaged across the 25 auditory startle trials, and the mean values for the rats in each treatment group were analyzed by ANOVA to detect differences in the magnitude of auditory startle responses when nicotine-withdrawn rats were treated with various doses of LY426965. The analysis of variance was followed by Tukey’s standardized range tests for post hoc comparisons of group means to detect significant differences (P < 0.05) between the non-nicotine-treated rats and the rats treated with nicotine to detect differences among the nicotine-treated animals administered various doses of LY426965. In a separate group of naïve animals, the effects of LY426965 on baseline startle were averaged across the 25 trials (n = 6/treatment group) at oral doses of 1, 3, and 10 mg/kg and evaluated with ANOVA followed by Tukey’s post hoc comparisons to detect effects produced by LY426965 that differed (P < 0.05) from normal startle responses. The dihydrochloride salt of LY426965 was used in these experiments.

Microdialysis. Male Sprague-Dawley rats (270–300 g; Harlan Sprague-Dawley) were housed under a reverse light period (lights off 9:00 AM to 9:00 PM). We have previously shown that fluoxetine has been reported to be 24% for serotonin with this type of probe. Basal levels were measured for at least 90 min before drug administration. The basal values were converted to percentage of basal. Mean percentages were determined for each treatment phase, and repeated tests were used to detect significant increases or decreases from basal levels. The dihydrochloride salt of LY426965 was used in these experiments.

Electrophysiology. Male Sprague-Dawley rats (300–350 g; Charles River, Portage, MI) were anesthetized with chloral hydrate (400 mg/kg i.p.); supplemental doses of anesthetic agent were administered through the lateral tail vein. The anesthetized rats were mounted in a stereotaxic apparatus. While in the stereotaxic apparatus, the rat’s body temperature was maintained at 35–37°C by placing them on a heating pad. After the rat’s skull was exposed, a cisternal drain was used to help prevent tissue swelling. Burr holes were then drilled in the rat’s skull for the placement of recording electrodes. To construct recording electrodes, single-barrel glass micropipettes (Radnoti, Starbore glass) were pulled with a Narishige PE-2 vertical puller; the resulting fine tips were broken back, and the barrels were back-filled with 2 M NaCl. Electrode impedances were 2.5 to 3.5 MΩ measured with a Winstom Electronics BL-1000 microelectrode tester. The tip of the recording electrode was lowered to the dorsal border of the dorsal raphe nucleus (DRN) and then advanced, using a micropositioning device (Burleigh 6000), in 3-μm increments.

TABLE 3
Receptor binding affinities of LY426965

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Kᵢ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT₁A</td>
<td>4.66 ± 0.63 (4)</td>
</tr>
<tr>
<td>5-HT₁B</td>
<td>847 ± 158 (3)</td>
</tr>
<tr>
<td>5-HT₁D</td>
<td>139 ± 21 (2)</td>
</tr>
<tr>
<td>5-HT₁E</td>
<td>&gt;4,500 (3)</td>
</tr>
<tr>
<td>5-HT₁F</td>
<td>1385 ± 426 (3)</td>
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<tr>
<td>5-HT₂A</td>
<td>1164 ± 319 (5)</td>
</tr>
<tr>
<td>5-HT₂B</td>
<td>97.3 ± 32.2 (5)</td>
</tr>
<tr>
<td>5-HT₂C</td>
<td>&gt;3,000 (5)</td>
</tr>
<tr>
<td>5-HT₃A</td>
<td>1210 ± 305 (3)</td>
</tr>
<tr>
<td>5-HT₅A</td>
<td>2376 ± 652 (4)</td>
</tr>
<tr>
<td>5-HT₇A</td>
<td>296 ± 5 (2)</td>
</tr>
<tr>
<td>α₁-Adrenergic</td>
<td>727 ± 55 (4)</td>
</tr>
<tr>
<td>α₂-Adrenergic</td>
<td>&gt;100,000 (2)</td>
</tr>
<tr>
<td>β-Adrenergic</td>
<td>&gt;10,000 (2)</td>
</tr>
<tr>
<td>Dopamine D₁</td>
<td>&gt;10,000 (2)</td>
</tr>
<tr>
<td>Dopamine D₂</td>
<td>263 ± 19 (4)</td>
</tr>
<tr>
<td>Histamine-1</td>
<td>4880 ± 161 (3)</td>
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<tr>
<td>GABAₐ</td>
<td>&gt;10,000 (2)</td>
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<td>Benzodiazepine</td>
<td>&gt;10,000 (2)</td>
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<tr>
<td>Muscarinic</td>
<td>&gt;100,000 (2)</td>
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</tbody>
</table>
through the nucleus. Cells were considered to be serotonergic if they possessed the following characteristics: a long action potential duration (>2.5 ms), a biphase or triphasic waveform with an initial positive phase, and a slow and very regular firing pattern with a rate of 0.5 to 3.0 Hz.

For single-unit recordings, compounds were administered via the lateral tail vein with a 2-min interdose interval. The percentage change from baseline firing rate produced by each dose was calculated by determining the mean firing rate during the final 1-min period of the 2-min interdose interval. ED50 values were calculated for each cell with JMP software (version 3.1.6; SAS Institute, Inc.).

In a separate group of animals, a population survey of the DRN was conducted. In these animals, the electrode was lowered through five tracks (separated by 0.2 mm; each electrode track was completed over an approximately 20-min period) in the DRN, and the number of spontaneously active serotonin neurons and their firing rates were examined. Results were analyzed with a two-way ANOVA with paired comparisons between treatment groups (JMP software, version 3.1.6; SAS Institute, Inc.).

Fluoxetine HCl was dissolved in distilled water, and LY426965 was dissolved in 4% 2-hydroxpropyl-β-cyclodextrin (pH 5.5). The dihydrochloride salt of LY426965 was used in these experiments.

The effect was examined of cumulative i.v. administration of 8-OH-DPAT (5-HT1A agonist), LY333068 (5-HT1A partial agonist), and LY426965 on the single-unit activity of serotonergic neurons in the DRN. The effect was examined of pretreatment with LY426965 or vehicle before the acute administration of fluoxetine on the single-unit activity of serotonergic neurons in the DRN. Animals were pretreated with either 0.5 or 1.0 mg/kg LY426965 i.v. or vehicle 5 min before the cumulative i.v. administration of fluoxetine.

The number of spontaneously active neurons was examined in either untreated controls or animals pretreated with LY426965 (0.3 mg/kg s.c.) or vehicle 15 min before the administration of fluoxetine (10 mg/kg i.p.) or vehicle. In treated animals, recordings were made 60 min after the administration of fluoxetine or its vehicle.

The ability of LY426965 to reverse the effects of fluoxetine on serotonin neurons was examined. After a 5-min baseline recording, fluoxetine was administered at a dose to achieve about a 50% decrease in neuronal activity (0.5-2.0 mg/kg i.v.). When the maximal effect of fluoxetine was achieved, LY426965 or vehicle was administered i.v. in increasing cumulative doses at 2-min intervals.

Results

Receptor Binding. LY426965 has high affinity for the cloned human 5-HT1A receptor (Ki = 4.66 nM). The receptor for which LY426965 has the next highest affinity is the 5-HT2B receptor. The Ki of LY426965 for the 5-HT2B receptor is 97 nM, which is more than 20-fold higher than its affinity for 5-HT1A receptors. For all other serotonin and nonserotonin receptor subtypes examined, LY426965 has greater than 20-fold selectivity (Table 3).

5-HT-Stimulated [35S]GTPγS Binding to Homogenates of Cells Expressing Human 5-HT1A Receptor. By itself, LY426965 did not stimulate [35S]GTPγS binding to homogenates of cells expressing the human cloned 5-HT1A receptor (n = 3). The EC50 value for 5-HT in this assay was 39.5 ± 3.5 nM (n = 9). When measured as an antagonist, LY426965 inhibited 300 nM 5-HT-stimulated [35S]GTPγS binding (Ki = 3.07 ± 0.13 nM, n = 3). The minimum, -0.23 ± 0.24% relative to the response produced by 10 μM 5-HT, was not statistically different from 0 (baseline, t test; Fig. 2).

Antagonism of 8-OH-DPAT-Induced Lower Lip Retraction, Flat Body Posture, and Hypothermia. After s.c. administration, LY426965 antagonized the effects of 8-OH-DPAT on lower lip retraction, flat body posture, and hypothermia (ED50 = 0.54, 0.54, and 0.62 mg/kg s.c., respectively; n = 3 or 4/group; Fig. 3). After p.o. administration, LY426965 also antagonized the effects of 8-OH-DPAT on lower lip retraction, flat body posture, and hypothermia (ED50 = 3.0, 2.0, and 2.4 mg/kg p.o., respectively; n = 3 or 4/group; Fig. 3). The average p.o.-to-s.c. ratio for these assays was 4.40. LY426965 administered orally at 20 mg/kg was fully effective in blocking the effect of 8-OH-DPAT on lower lip retraction, flat body posture, and hypothermia for at least 8 h (n = 4/group; Fig. 4).

Antagonism of 8-OH-DPAT-Induced Increase in Rat Serum Corticosterone Concentrations. Figure 5 (top) shows that an s.c. 15-min pretreatment with LY426965 dose-dependently blocked the increase in rat serum corticosterone concentrations elicited by 8-OH-DPAT (ED50 = 8.74 mg/kg, p.o.; Fig. 5).
s.c., n = 5/group). LY426965 alone at a 10 mg/kg s.c. dose had no effect on basal levels of corticosterone, suggesting no 5-HT1A receptor agonist activity at this dose (n = 5/group; Fig. 5). Figure 5 (bottom) also shows that a 1-h pretreatment with LY426965 by gavage dose-dependently antagonized the 8-OH-DPAT-induced increase in corticosterone concentrations (ED50 = 9.19 mg/kg, p.o.; n = 5/group). Two hours after a 30 mg/kg p.o. dose of LY426965 alone, no effect was observed on basal levels of corticosterone, suggesting no 5-HT1A receptor agonist activity at this dose (Fig. 5; n = 5/group).

Antagonism of 8-OH-DPAT-Induced Discriminative Stimulus in Pigeons. After the injection of either 0.16 or 0.64 mg/kg 8-OH-DPAT under training conditions, virtually all of the pigeons’ responses were on the 8-OH-DPAT-related key, whereas after vehicle injection, very few of the responses were on this key. LY426965 did not mimic the stimulus cue induced by the low training dose (0.16 mg/kg) of 8-OH-DPAT and did not decrease response rates below those found under training conditions (n = 3/dose; Fig. 6).

LY426965 antagonized the stimulus cue induced by 0.64 mg/kg 8-OH-DPAT in a dose-related manner (Fig. 6). LY426965 also antagonized the rate decreasing effects of the 0.64 mg/kg dose of 8-OH-DPAT (Fig. 6; n = 3/dose).

Nicotine Withdrawal-Enhanced Auditory Startle Response. As depicted in Fig. 7, rats implanted with nicotine-filled minipumps displayed significant elevations in the mag-
magnitude of startle responses to auditory stimuli across the 3 days immediately after the removal of the nicotine pumps. The p.o. administration of various doses of LY426965 effectively blocked the elevation in startle responding seen in nicotine withdrawn rats ($ED_{50} = 0.1 \text{ mg/kg; } n = 8/\text{group}$).

A separate study examining the effects of LY426965 on auditory responding was conducted in a group of naïve animals. As Fig. 7 (bottom) indicates, the threshold dose for altering auditory startle responding was 10 mg/kg p.o. ($n = 8/\text{group}$).

**Microdialysis.** The administration of 10 mg/kg s.c. ($n = 4$) and 3 ($n = 3 \text{ or } 4$) and 10 ($n = 6 \text{ or } 7$) mg/kg p.o. LY426965, after the administration of fluoxetine (10 mg/kg i.p.), significantly elevated 5-HT levels in the hypothalamus above those reached by fluoxetine alone (Fig. 8).

When administered before fluoxetine, LY426965 (30 mg/kg p.o.) significantly increased 5-HT levels to 205% of baseline levels (Fig. 10). Subsequent administration of fluoxetine (10 mg/kg i.p.) increased 5-HT levels to an average of 256% (10 mg/kg p.o. LY426965) and 469% (30 mg/kg p.o.) of baseline levels ($n = 5 \text{ or } 6/\text{group}$) (Fig. 10). The overall average amount of 5-HT for all groups was 0.39 ± 0.09 pmol/20 μl.

**Electrophysiology.** The acute administration of LY426965 produced a slight, nonsignificant elevation of the firing rate of serotonin neurons (Fig. 11). In contrast, administration of the 5-HT$_{1A}$ agonist 8-OH-DPAT and the 5-HT$_{1A}$ partial agonist LY333068 both produced complete inhibition of activity ($n = 6 \text{--8/\text{group}}$).

The acute administration of moderate doses of fluoxetine (0.5–2.0 mg/kg i.v.) was able to produce a sustained, approx-
approximately 50% inhibition of serotonergic neuronal activity that was not reversed by vehicle administration (data not shown). The administration of LY426965 was able to completely reverse the inhibition of firing rate produced by fluoxetine (Fig. 12; $ED_{100} = 1.1 \pm 0.2$ mg/kg, $n = 4$).

The acute administration of higher doses of fluoxetine (4–12 mg/kg i.v.) produced a complete inhibition of serotonin neuronal activity. Pretreatment with 0.5 and 1.0 mg/kg i.v. LY426965 greatly attenuated the effects of fluoxetine (Fig. 13; $n = 3–10$/data point). Pretreatment with 0.5 mg/kg LY426965 i.v. produced an increase in the ED50 for fluoxetine from 1.08 to 10.49 mg/kg. Pretreatment with 1.0 mg/kg i.v. LY426965 blocked the inhibitory effect of fluoxetine to such a degree that an ED50 value for fluoxetine was unable to be generated.

Acute administration of fluoxetine (with vehicle pretreatment) produced a significant decrease in both the number of spontaneously active serotonin cells per track and their firing rates. Pretreatment with 0.3 mg/kg LY426965 s.c. completely blocked the effects of fluoxetine and produced no significant effects on its own (Fig. 14; $n = 5–11$/group).

Discussion

LY426965 has high affinity for the cloned human 5-HT1A receptor ($K_i = 4.66$ nM) and 20-fold or greater selectivity over other serotonin and nonserotonin receptor subtypes (Table 2). In an in vitro assay of 5-HT1A receptor activation, LY426965 exhibited no measurable agonist activity and was a full antagonist. Thus, LY426965 did not stimulate $[^{35}S]GTP\gamma S$ binding to homogenates of cells expressing the cloned human 5-HT1A receptor. In addition, LY426965 inhibited 300 nM 5-HT-stimulated $[^{35}S]GTP\gamma S$ binding ($IC_{50} = 26.4 \pm 1.2$ nM, $K_i = 2.76 \pm 0.12$ nM; $n = 3$).

In vivo, LY426965 also acted as a full 5-HT1A antagonist and displayed no agonist activity. Administration of the 5-HT1A agonist 8-OH-DPAT leads to the appearance of several behaviors (including lower lip retraction and flat body posture), hypothermia, and an elevation of serum corticosterone (Wolff et al., 1997). LY426965 blocked the lower lip retraction, flat body posture, and hypothermia induced by 8-OH-DPAT (0.1 mg/kg s.c.) in rats after both s.c. ($ED_{50} = 0.54$, 0.54, and 0.62 mg/kg, respectively) and p.o. ($ED_{50} = 3.0$, 2.0, and 2.4 mg/kg, respectively) administration (Fig. 4). The $ED_{50}$ p.o./$ED_{50}$ s.c. ratios for the lower lip retraction (5.5), flat body posture (3.7), and hypothermia (3.9) assays indicated good bioavailability in the rat for LY426965. The administration of an oral dose two times the $ED_{100}$ for the blockade of 8-OH-DPAT-induced effects on lower lip retraction, flat body posture, and body temperature (20 mg/kg) completely prevented the effects of 8-OH-DPAT for up to 8 h but less than 16 h. LY426965 also blocked the increase in rat serum corticosterone concentrations elicited by 8-OH-DPAT (0.3 mg/kg s.c.) after both s.c. ($ED_{50} = 8.74$ mg/kg) and p.o. ($ED_{50} = 9.19$ mg/kg) administration. The administration of LY426965 alone (10 mg/kg s.c. and 30 mg/kg p.o.) had no effect on basal levels of corticosterone, suggesting no 5-HT1A receptor agonist activity at these doses (Fig. 5). These results indicate that although it is less potent than WAY-100635 as a 5-HT1A antagonist (Forster et al., 1995), LY426965 has a much longer duration of action than WAY-100635 and, unlike WAY-100635, is active after p.o. administration.

8-OH-DPAT induces a discriminative stimulus cue that is very specific for activation of the 5-HT1A receptor. The use of a relatively low (0.16 mg/kg s.c.) training dose of 8-OH-DPAT
facilitates the detection of the agonist/partial agonist properties of novel compounds, whereas the use of a relatively high dose of 8-OH-DPAT facilitates the detection of the antagonist properties of novel compounds (Wolff and Leander, 1997). In agreement with the other assays, LY426965 acted as a full antagonist with no agonist properties in the drug discrimination assay. Thus, LY426965 did not substitute for the low-dose stimulus cue (0.16 mg/kg) of 8-OH-DPAT and did not alter response rates from rates observed during vehicle sessions (Fig. 6). LY426965 also dose-dependently antagonized the stimulus produced by the high dose of 8-OH-DPAT (0.64 mg/kg), and LY426965 antagonized the rate-decreasing effects of the 0.64 mg/kg dose of 8-OH-DPAT (Fig. 6).

Withdrawal from the chronic administration of nicotine has previously been shown to enhance the auditory startle reflex in rats (Helton et al., 1993). A variety of compounds are effective in attenuating this nicotine withdrawal-enhanced startle response, including 5-HT_{1A} antagonists (Rasmussen et al., 1996, 1997; Helton et al., 1997). LY426965 was able to completely block the enhancement of the startle response caused by nicotine withdrawal at doses that have no affect on baseline startle (Fig. 7). Because cessation of the chronic use of nicotine or tobacco in human results in withdrawal symptoms (including anxiety, irritability, difficulty concentrating, and restlessness) and withdrawal symptoms have been shown to play an important role in relapse (Hughes and Hatsukami, 1986), these results indicate that LY426965 may be able to relieve some nicotine withdrawal symptoms in humans and may represent a novel pharmacotherapy for smoking cessation.

It is interesting to note that the doses of LY426965 that were effective in attenuating the nicotine withdrawal-enhanced startle response were much lower than those needed to block the effects of the 5-HT_{1A} agonist 8-OH-DPAT. Sev-
eral possibilities may explain these results. First, for the blockade of the effects of 8-OH-DPAT, the ED$_{50}$ values for LY426965 were influenced by the concentration of the agonist used. However, the nicotine withdrawal assay is not “agonist driven”. Thus, lower doses may be required. Second, the process of chronic exposure to high levels of nicotine followed by abrupt cessation may lead to alterations in the properties of a number of receptors, including the 5-HT$_{1A}$ receptor. Indeed, recent studies indicate that 5-HT$_{1A}$ receptors are more sensitive to agonists during nicotine withdrawal (Rasmussen and Czachura, 1997). Thus, lower doses of antagonist may be needed to achieve a significant blockade of the receptor. Third, activity at a receptor other than the 5-HT$_{1A}$ receptor may contribute to the blockade of the nicotine withdrawal response by LY426965. Although it is diffi-

![Fig. 9. The administration of LY426965 (30 mg/kg p.o.) alone significantly increased 5-HT levels over baseline. The subsequent administration of fluoxetine (10 mg/kg i.p.) increased 5-HT levels to an average of 256% (10 mg/kg p.o. LY426965) and 469% (30 mg/kg p.o.) over baseline levels. Filled symbols indicate significantly different from basal levels (P < .05), +, significantly different from 10 mg/kg group (P < .05).](image)

![Fig. 10. Unlike the agonist (±)8-OH-DPAT and partial agonist LY333068, cumulative doses of LY426965 did not inhibit the activity of serotonergic neurons in the DRN (n = 6–8/group).](image)

![Fig. 11. The administration of LY426965 reversed the effects of fluoxetine (0.5 mg/kg i.v.) on the activity of serotonergic neurons in the DRN.](image)
cult to disprove this last possibility, it seems unlikely because LY426965 has low affinity for other receptor subtypes known to modulate the nicotine withdrawal-enhanced startle response (i.e., cholecystokinin-B metabotropic glutamate group 2/3, α2-adrenergic, and benzodiazepine) and 20-fold or greater selectivity versus more than 40 other serotonin and nonserotonin receptor subtypes examined.

We also examined the interactions of LY426965 with fluoxetine in microdialysis experiments. Previous studies have demonstrated that negative feedback, mediated via 5-HT1A somatodendritic autoreceptors, limits the 5-HT output at nerve terminals after the administration of an SSRI (Hjorth, 1993). Thus, the blockade of 5-HT1A somatodendritic autoreceptors with 5-HT1A antagonists potentiates the elevation of nerve terminal 5-HT output induced by SSRIs (Dreshfield et al., 1996; Sharp et al., 1997). This negative feedback on 5-HT cells, and subsequent limitation of 5-HT terminal output, has been hypothesized to play a role in the delayed therapeutic onset of SSRIs (Blier and de Montigney, 1983). After several weeks of treatment with an SSRI, the 5-HT1A autoreceptor desensitizes. This desensitization releases the 5-HT cells from negative feedback and allows a greater release of 5-HT and full therapeutic effects. LY426965 (3 and 10 mg/kg p.o.; 10 mg/kg s.c.), when administered with fluoxetine, significantly increased extracellular levels of serotonin above those achievable with fluoxetine alone (Fig. 8). Interestingly, the administration of 30 mg/kg LY426965 p.o. alone caused a significant increase in extracellular 5-HT (Fig. 9). This is consistent with electrophysiological evidence that 5-HT1A receptor antagonists increase neuronal activity in the unanesthetized animal (Fornal et al., 1996). Thus, these results indicate that LY426965 may have antidepressant effects by itself through increases in 5-HT levels. Furthermore, when used as adjunctive treatment, LY426965 can rapidly enhance the 5-HT terminal output and thus may accelerate the onset of therapeutic effects of fluoxetine and other SSRIs.

Fig. 12. Pretreatment with LY426965 (0.5 and 1.0 mg/kg i.v.) attenuated the effect of fluoxetine on the activity of serotonergic neurons in the DRN (n = 3–11/data point). ○, vehicle pretreatment; □, 0.5 mg/kg i.v. LY426965 pretreatment; ▼, 1.0 mg/kg i.v. LY426965 pretreatment.

Fig. 13. Pretreatment with LY426965 (3.0 mg/kg s.c.) blocks the effect of fluoxetine but has no effect alone on the number of spontaneously active serotonergic cells per track in the DRN (n = 5–11/group). ***, significantly different from vehicle + vehicle (P < .001).
Electrophysiological experiments confirmed that LY426965 is not a 5-HT$_{1A}$ partial agonist, in that the administration of LY426965 did not inhibit the activity of serotonergic neurons in the DRN. The systemic administration of 5-HT$_{1A}$ antagonists inhibits the activity of 5-HT neurons in the DRN (Sprouse and Aghajanian, 1987). The administration of 5-HT$_{1A}$ partial agonists also inhibits the activity of 5-HT neurons in the DRN, due, at least in part, to the high receptor reserve of 5-HT$_{1A}$ receptors (VanderMaelen et al., 1986). As has been shown previously, administration of the 5-HT$_{1A}$ agonist 8-OH-DPAT inhibited the activity of 5-HT neurons in the DRN (Fig. 10). LY333068 is a compound that blocks the effects of 8-OH-DPAT in behavioral experiments but has 21% intrinsic activity at the 5-HT$_{1A}$ receptor in vitro (Rocco et al., 1997). As can be seen in Fig. 10, the administration of LY333068 also completely inhibits the activity of 5-HT neurons in the DRN. However, the administration of LY426965, which has 0% intrinsic activity in in vitro experiments (Fig. 2), does not inhibit the activity of 5-HT neurons at any dose examined.

The results from the electrophysiological experiments also support the hypothesis that LY426965 may be useful in accelerating the onset of therapeutic effects of SSRIs, as Czachura and Rasmussen (2000), the administration of fluoxetine produced a long-lasting inhibition of 5-HT unit activity in the DRN. As has been shown previously (Czachura and Rasmussen, 2000), the administration of fluoxetine produced a long-lasting inhibition of 5-HT unit activity in the DRN. The subsequent administration of LY426965, when administered with fluoxetine, significantly increased extracellular levels of serotonin in the DRN. As has been shown previously (Czachura and Rasmussen, 2000), the administration of fluoxetine produced a long-lasting inhibition of 5-HT unit activity in the DRN. However, the administration of LY426965, which has 0% intrinsic activity in in vitro experiments (Fig. 2), does not inhibit the activity of 5-HT neurons at any dose examined.

These preclinical results indicate that LY426965 may have clinical use as a pharmacotherapy for smoking cessation and related disorders.

**References**


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