The 5-Hydroxytryptamine₄ Receptor Agonists Prucalopride and PRX-03140 Increase Acetylcholine and Histamine Levels in the Rat Prefrontal Cortex and the Power of Stimulated Hippocampal oscillations


Received January 31, 2012; accepted March 8, 2012

ABSTRACT

5-Hydroxytryptamine (5-HT₄) receptor agonists reportedly stimulate brain acetylcholine (ACh) release, a property that might provide a new pharmacological approach for treating cognitive deficits associated with Alzheimer’s disease. The purpose of this study was to compare the binding affinities, functional activities, and effects on neuropharmacological responses associated with cognition of two highly selective 5-HT₄ receptor agonists, prucalopride and 6,7-dihydro-4-hydroxy-7-isopropyl-6-oxo-N-[3-(piperidin-1-yl)propyl]thieno[2,3-b]pyridine-5-carboxamide (PRX-03140). In vitro, prucalopride and PRX-03140 bound to native rat brain 5-HT₄ receptors with Ki values of 30 nM and 110 nM, respectively, and increased cAMP production in human embryonic kidney-293 cells expressing recombinant rat 5-HT₄ receptors. In vivo receptor occupancy studies established that prucalopride and PRX-03140 were able to penetrate the brain and bound to 5-HT₄ receptors in rat brain, achieving 50% receptor occupancy at free brain exposures of 330 nM and 130 nM, respectively. Rat microdialysis studies revealed that prucalopride maximally increased ACh and histamine levels in the prefrontal cortex at 5 and 10 mg/kg, whereas PRX-03140 significantly increased cortical histamine levels at 50 mg/kg, failing to affect ACh release at doses lower than 150 mg/kg. In combination studies, donepezil-induced increases in cortical ACh levels were potentiated by prucalopride and PRX-03140. Electrophysiological studies in rats demonstrated that both compounds increased the power of brainstem-stimulated hippocampal oscillations at 5.6 mg/kg. These findings show for the first time that the 5-HT₄ receptor agonists prucalopride and PRX-03140 can increase cortical ACh and histamine levels, augment donepezil-induced ACh increases, and increase stimulated-hippocampal power, all neuropharmacological parameters consistent with potential positive effects on cognitive processes.

Introduction

Data suggest that the cognitive deficits observed in Alzheimer’s disease (AD) are associated with the loss of cholinergic neurons and the concomitant reduction of acetylcholine (ACh) levels in two brain regions critical to cognitive and memory functions, i.e., the cortex and hippocampus (Bartus et al., 1982). The current standard of pharmacotherapy for AD uses treatment with acetylcholinesterase inhibitors (e.g., donepezil and galantamine), compounds that increase cholinergic tone throughout the brain by preventing the breakdown of ACh. At best, this nonselective increase in ACh...
levels provides moderate, short-term, symptomatic relief. Selectively targeting receptors that regulate the release of ACh and other neurotransmitters or enhance neuronal activities that are associated with cognitive and mnemonic processes might provide a new mechanistic approach to providing symptomatic relief in AD.

One promising target is the 5-HT4 receptor, a G-protein-coupled receptor that is widely distributed throughout the central nervous system (Eglen et al., 1995). Quantitative polymerase chain reaction analysis of human brain showed that the highest levels of 5-HT4 mRNA are found in the caudate and putamen, with the amygdala, temporal cortex, and hippocampus showing somewhat lower levels (Medhurst et al., 2001). In a post-mortem study of brains from patients with AD, 5-HT4 receptor levels were significantly reduced in the hippocampus and frontal cortex, by 66 and 25%, respectively (Reynolds et al., 1995).

Previous reports indicate that 5-HT4 receptor activation stimulates ACh release in rat cortex and hippocampus (Consolo et al., 1994; Mohler et al., 2007) and enhances cognition in preclinical models such as social olfactory memory, spontaneous alternation, and delayed matching (Letty et al., 1997; Terry et al., 1998; Mohler et al., 2007). 5-HT4 receptor agonists were shown to reverse muscarinic receptor antagonist-induced cognitive deficits when administered alone or in combination with acetylcholinesterase inhibitors (Fontana et al., 1996, 1997; Galeotti et al., 1998; Mohler et al., 2007; Cachard-Chastel et al., 2008). These results provide evidence linking the cognitive effects of 5-HT4 receptor agonists to the cholinergic system, and they support the potential of 5-HT4 receptor agonist action to overcome cognitive impairments associated with AD.

Histamine is a neurotransmitter that is associated with increased attention, which is an important component in many cognitive processes. Increasing brain histamine release is another potential mechanism to improve cognition in AD, because reports suggest that histaminergic neuronal activity is compromised in AD. Schneider et al. (1997) reported that the level of histidine decarboxylase, the enzyme responsible for converting histidine to histamine, was decreased approximately 50% in the frontal cortex of patients with AD. Airaksinen et al. (1991) reported that, in patients with AD, large numbers of large globular neurofibrillary tangles were found in the tuberomammillary neurons of the hypothalamus. Histaminergic neurons arise from the tuberomammillary nucleus within the hypothalamus and project throughout the brain, where they modulate the release of various neurotransmitters, including ACh. Methylphenidate and atomoxetine, two clinically effective treatments for attention-deficit/hyperactivity disorder, increase both histamine (Horner et al., 2007) and ACh (Acquas and Fibiger, 1996; Tzavara et al., 2006) levels in the rat cortex, which suggests that cortical release of these neurotransmitters might be linked to the therapeutic benefits of these agents. Therefore, the combined actions of increased ACh and histamine release could provide additional therapeutic benefits in the treatment of cognitive deficits associated with AD.

There is evidence that several putative or clinically proven cognition-enhancing compounds are capable of inducing increases in the power of brainstem-stimulated θ oscillations in the electroencephalograms (EEGs) of anesthetized rats (Kinney et al., 1999; Siok et al., 2006; McNaughton et al., 2007; Hajós et al., 2008). In several of those studies, compounds that increase either ACh release, such as donepezil, or histamine release, such as ciproxifan, were shown to increase stimulated hippocampal θ power (Kinney et al., 1999; Hajós et al., 2008). An earlier study linked increases in spontaneously occurring EEG activity in the θ frequency band of halothane-anesthetized rats to activation of 5-HT4 receptors by using zacopride, a mixed 5-HT4 receptor agonist/5-HT3 receptor antagonist (Boddeke and Kalkman, 1990).

To investigate the potential of the 5-HT4 receptor as a target for symptomatic relief of AD, we characterized the cognitive profiles of two 5-HT4 receptor ligands, prucalopride and PRX-03140 (PRX-03140 is also known in the literature as VRX-03011; see Mohler et al., 2007) (Fig. 1). These compounds are highly selective 5-HT4 receptor agonists that were under development for the treatment of chronic constipation and cognitive deficits related to AD, respectively (Briejer et al., 2001; Mohler et al., 2007). Both compounds reportedly improve cognitive function in preclinical behavioral models. In this study, we first established the in vitro binding and functional characteristics of prucalopride and PRX-03140 at rat 5-HT4 receptors. In vivo binding and exposure studies were then performed to establish the relationship between 5-HT4 receptor occupancy and compound levels in rat brain. Subsequently, in vivo microdialysis and EEG studies were conducted to compare the effects of these compounds on neuropharmacological measures in the rat cortex and hippocampus that are implicated in the improvement of cognitive function.

**Materials and Methods**

**Animal Care.** All animal experiments were conducted in accordance with animal-use protocols approved by the Pfizer local institutional animal care and use committee and with the National Institutes of Health guidelines (Institute of Laboratory Animal Resources, 1996). Male Sprague-Dawley rats were obtained from Charles River Laboratories (Raleigh, NC) or Harlan Laboratories (Indianapolis, IN). Animals were housed on a 12-h light/dark cycle with free access to food and water and were allowed to acclimate for at least 5 days after arrival. For radioligand administration during
in vivo receptor occupancy (RO) studies, rats were purchased with preimplanted jugular vein catheters.

In Vitro Binding Studies. Binding assays were performed in 96-well format, by using rat brain membranes prepared from striatal tissue. For each 96-well plate, ~500 mg of tissue was homogenized in 50 mM Tris buffer (pH 7.4 at 4°C) containing 2.0 mM MgCl₂, by using a Brinkmann Polytron homogenizer (Thermo Fisher Scientific, Waltham, MA), and was centrifuged at 40,000 g for 10 min. The pellet was suspended in homogenization buffer and placed in a 37°C water bath for 10 min, to break down any endogenous serotonin. The membrane suspension was then centrifuged at 40,000 g for 10 min, and the resulting pellet was resuspended in assay buffer (50 mM HEPEPS, pH 7.4) containing 2 mM MgCl₂. Test compounds were prepared as 10× stock solutions in 50 mM Tris HCl buffer (pH 7.6) containing 10% dimethylsulfoxide and were assayed in duplicate at each test concentration. Incubations were initiated with the addition of tissue (225–250 μg per well) to 96-well plates containing test compounds (25 μl) and 0.5 nM [³H]GR113808 (PerkinElmer Life and Analytical Sciences, Waltham, MA), in a final volume of 250 μl. The Kᵦ value for [³H]GR113808 binding ranged from 0.4 to 1 nM, and nonspecific binding was determined in the presence of 10 μM GR125847, a 5-HTᵦ receptor antagonist. After a 30-min incubation period at 37°C, assay samples were filtered rapidly through Whatman 25-mm GF/B filters (VWR International, Bridgeport, NJ) and rinsed with ice-cold 50 mM Tris buffer (pH 7.4 at 4°C). Membrane-bound [³H]GR113808 levels were determined with liquid scintillation counting.

In Vitro Functional Activity. To prepare either fresh or frozen assay-ready cells, the BacMam expression system (Invitrogen, Carlsbad, CA) was used. DNA for each of the three isoforms of the rat 5-HT₄ receptor (4s, 4l, and 4e) was obtained from DNA 2.0 (Menlo Park, CA), subcloned into BacMam vectors, and then used to generate Bacmid DNA, which was confirmed to have the correct insert in polymerase chain reaction assays. The Bacmid was used to transfect GFP/B filters (Brandel, Gaithersburg, MD) and rinsed with ice-cold 50 mM Tris buffer (pH 7.4 at 4°C). Membrane-bound [³H]GR113808 levels were determined with liquid scintillation counting.

In Vivo Receptor Binding. An in vivo binding assay was developed by using a tritiated version of the 5-HT₄ receptor position emission tomography ligand [³H]SB207145 (Marner et al., 2009). Rats (~200 g at use, n = 4 per group) were given subcutaneously administered test compound at the time points specified. Doses ranged from 1 to 32 mg/kg for prucaloride and from 0.32 to 32 mg/kg for PRX-03140. Fifteen minutes before euthanasia, rats received a 1 ml/kg intravenous injection of [³H]SB207145 (100 μCi/ml, 20–25 μCi per rat) through a jugular vein catheter. Rats were then euthanized through live decapitation, and brains were removed. Trunk blood was reserved to obtain plasma for compound exposure analysis. The striatum (from all subjects) and cerebellum (from the vehicle control group, only to define nonspecific binding) were dissected rapidly and homogenized for approximately 3 s each, by using a Brinkmann Polytron at its highest setting (5–6), in 10 volumes (100 mg/ml) of ice-cold assay buffer (50 mM HEPEPS, 2 mM MgCl₂, pH 7.4 with NaOH). Three replicates of 200 μl of the resulting homogenates were filtered separately through 0.3% polyethyleneimine-soaked Whatman 25-mm GF/B filters (VWR International, Bridgeport, NJ), by using a 10-place filtration manifold (Hoefer Inc., Holliston, MA). The filters were then washed twice with 5 ml of assay buffer and transferred to glass scintillation vials. Ten milliliters of scintillation cocktail (Beckman Coulter, Fullerton, CA) were added to each vial, and the filters were soaked overnight before counting in a LS6500 scintillation counter (Beckman Coulter). The rest of the brain was frozen on dry ice and reserved for compound exposure analysis.

Microdialysis Procedures. Microdialysis was performed in freely moving, male, Sprague-Dawley rats (300–350 g), according to standard procedures. In brief, Bioanalytical Systems guide cannulas (Bioanalytical Systems, West Lafayette, IN) were implanted in the prefrontal cortex (anteroposterior, +3.2 mm from bregma; mediolateral, +0.7 mm (left) from bregma; dorsoventral, −1.3 mm from the dura), under isoflurane anesthesia, and were fixed to the skull with dental acrylic. After guide cannula implantation, animals were housed separately in isolation boxes, with free access to food and water, and were maintained on a 12-h light/dark cycle (lights on at 6:00 AM and lights off at 6:00 PM). A 4-mm Bioanalytical Systems microdialysis probe was inserted into the guide cannula on the day before testing, and artificial cerebrospinal fluid (147 mM NaCl, 1.3 mM CaCl₂, 2.7 mM KCl, 1.0 mM MgCl₂) was perfused overnight at 0.3 to 0.4 μl/min. The probe flow rate was increased to 2 μl/min at approximately 7:30 AM on the day of testing, and dialysates were collected at 15-min intervals. For ACh analysis with electrochemical detection, 100 nM neostigmine bromide was added to the artificial cerebrospinal fluid, to facilitate reliable detection of basal ACh levels.

Acetylcholine Analysis with High-Performance Liquid Chromatography. For conventional analysis, ACh levels were analyzed with high-performance liquid chromatography (HPLC) by using a modification of the Bioanalytical Systems acetylcholine-choline assay kit (MF-8910; Bioanalytical Systems). In brief, ACh was separated at a flow rate of 1 ml/min, at a temperature of 28°C, on two 10-cm analytical columns (MF-6150; Bioanalytical Systems) connected in series, by using a mobile phase containing 35 mM Na₂HPO₄, 0.1 mM EDTA, and 0.005% ProClin 150 preservative (Bioanalytical Systems), adjusted to pH 8.5 with phosphoric acid. ACh then underwent reaction in a postcolumn acetylcholinesterase/ choline oxidase immobilized-enzyme reactor (MF-6151; Bioanalytical Systems) to yield hydrogen peroxide, which was detected electrochemically with a platinum electrode maintained at a potential of +0.5 V versus Ag/AgCl. Chromatography data were collected and quantified through comparison with known standard concentrations, by using EZChrom Elite software (Agilent Technologies, Santa Clara, CA).

Acetylcholine Analysis with Liquid Chromatography-Tandem Mass Spectrometry. In combination studies with donepezil, diazysate ACh levels were determined by using liquid chromatography-tandem mass spectrometry (LC-MS/MS), in the absence of locally perfused neostigmine. Microdialysates (sample volume, 30 μl) were collected at 15-min intervals, into glass vials containing 4 μl of 10% acetic acid, by using a refrigerated fraction collector; samples were stored frozen at ~80°C for later analysis. Before analysis, deuterated acetylcholine-1,2,2-d₄, bromide (70 μl, 200 ng/ml) was
added to each sample as an internal standard. Analytes (injected sample volume, 10 μl) were separated on a Waters Atlantis Hilic column (100 × 2.1 mm; particle size, 3 μm), at a temperature of 35°C, by using a Waters Acquity Uplift perfomance liquid chromatography system (Waters, Milford, MA). Separation was achieved at a flow rate of 0.22 ml/min by using binary solvent gradient elution; solvent A consisted of 20 mM ammonium formate in 1% formic acid, pH 3.4, and solvent B was 100% acetonitrile. Each cycle began with a linear gradient from 10% to 70% solvent A over 3 min, and the composition was then held at 70% solvent A for 1.5 min before returning to 10% solvent A in 0.5 min. The effluent from the HPLC column was directed at the electrospray interface of the mass spectrometer. LC-MS/MS analyses were performed by using a Sciex API 3000 triple-quadrupole mass spectrometer equipped with a TurboIonSpray source (AB Sciex, Framingham, MA). The ion spray voltage was set at 1500 V and the source temperature at 450°C. The mass spectrometer was operated in the positive-ion electrospray mode with the following parameters: declustering potential, 25 V; focusing potential, 100 V; entrance potential, 5 V; collision cell exit potential, 22 V. Nitrogen was used for both the curtain gas and the collision gas, with ion energies of 6 and 8 eV, respectively. ACh and deuterated ACh were monitored by using multiple-reaction monitoring (MRM) mode. The MRM transitions of m/z 146.2→87.1 and 150.2→91.3 were sequentially monitored for the detection of ACh and deuterated ACh, respectively. LC-MS/MS data were collected and analyzed through comparison with known standard concentrations, by using Analyst 1.4.1 software (AB Sciex).

Histamine Analysis with HPLC. Histamine levels in microdialysates were determined by using on-line HPLC in conjunction with fluorescence detection. Histamine was separated by using a Waters Atlantis C18 HPLC column (150 × 3.0 mm; particle size, 3 μm), with a mobile phase consisting of 160 mM KH2PO4, pH 4.5, 0.45 mM octanesulfonic acid, 1% methanol, and 0.1 mM EDTA, which was delivered at 0.26 to 0.50 ml/min. The eluent line was connected with a T-piece to a reagent line, through which a 0.002% solution of octanesulfonic acid, 1% methanol, and 0.1 mM EDTA, which was Elite software (Agilent Technologies). The MRM transitions of m/z 146.2→87.1 and 150.2→91.3 were monitored by using EZChrom Elite software (Agilent Technologies).

Hippocampal θ Recordings. Details of the methods for recording brainstem-stimulated EEGs were published previously (Hajós et al., 2008). In brief, male Sprague-Dawley rats (275–325 g) were anesthetized with 1.55 g/kg urethane, followed by placement of indwelling catheters in the left femoral vein for euthanization and in the left femoral artery for blood drawing. Animals were then placed in a stereotaxic frame for the duration of the experiment and were maintained at 37°C–38°C with a thermostatically controlled heating pad. Bipolar, concentric, stainless steel recording and stimulating electrodes were manufactured by Rhodes Medical Instruments (Summerland, CA) and supplied by Kopf Instruments (Tujunga, CA). Coordinates for the hippocampal CA1 recording electrode were as follows: anteroposterior, −3.5 mm from bregma; mediolateral, +2.0 mm (left) from bregma; dorsoventral, −2.8 to −3.1 mm from dura. Coordinates for the nucleus pontis oralis-stimulating electrode were as follows: anteroposterior, −7.4 to −7.6 mm from bregma; mediolateral, +1.6 to +1.8 mm (left) from bregma; dorsoventral, −6.0 mm from dura. Differential recordings were made by using a Grass P-55 preamplifier (Grass Technologies, West Warwick, RI) with filter settings at 0.3 to 300 Hz and the 60-Hz notch filter off. All compounds were delivered subcutaneously in a volume of 1 ml/kg in 12% 2-hydroxypropyl-β-cyclodextrin in water. Experiments began 2 h after placement of the last electrode. The nucleus pontis oralis was stimulated by using a 6-s train of 0.3-ms square waves delivered at a rate of 250 pulses per s by using a Master-8 stimulator and Isoflex stimulus-isolation unit (A.M.P.I. Instruments, Jerusalem, Israel). Each 6-s stimulation period was repeated at 100-s intervals for the duration of the experiment. Current intensity was adjusted to drive hippocampal θ at a frequency between 5.9 and 6.8 Hz (range, 0.08–0.18 mA) and was then kept constant for the duration of the experiment. During this time, the EEG data were continuously monitored and saved in digital form at a sampling rate of 1000 Hz.

Determination of Compound Exposure in Rat Plasma and Brain. Plasma and brain samples were collected from in vivo binding studies or from satellite animals treated with prucalopride or PRX-03140 and were kept frozen at −20°C for later analysis with LC-MS/MS. Standard curves were prepared in respective matrices through serial dilution at concentrations of 0.49 to 2000 ng/ml for plasma and 0.49 to 2000 ng/g for brain. For plasma, a 50-μl aliquot of sample was precipitated with 300 μl of acetonitrile containing internal standard. Samples were vortex-mixed for 1 min and then centrifuged at 3000 rpm for 10 min. The supernatant (120 μl) was transferred to a 96-well plate, and an equal volume of water was added (120 μl). Frozen brain tissue was weighed, and a volume of isopropanol/water (60:40) equivalent to 4 times the mass was added before homogenization in a bead beater (BioSpec Products, Bartlesville, OK). For brain, a mixed-matrix approach was used to generate the brain standard curve, a 50-μl aliquot of blank brain homogenate matrix was added to a 50-μl aliquot of each point in the plasma curve. Likewise, 50 μl of blank plasma was added to 50 μl of each brain homogenate sample. These brain samples were then processed as described above for plasma.

LC-MS/MS analysis was performed for prucalopride and PRX-03140 by using a HPLC system consisting of triple Shimadzu LC20AD pumps (Shimadzu Scientific Instruments, Columbia, MD) with a CTC PAL autosampler (Leap Technologies, Carrboro, NC) interfaced to an API 4000 LC-MS/MS quadruopole tandem mass spectrometer (AB Sciex). Analytes were separated with a Phenomenex Synergi POLAR-RP column (30 × 2.0 mm, 4 μm, Phenomenex, Torrence, CA), through gradient elution at a flow rate of 0.4 ml/min. A 10-μl sample was injected onto the column. The mobile phase consisted of 10 mM ammonium formate in 0.1% formic acid as solvent A and acetonitrile as solvent B. The gradient was as follows: solvent B was held at 15% for 0.5 min, linearly ramped from 15% to 90% in 1.5 min, held at 90% for 0.5 min, and then ramped to 15% over 0.5 min. The mass spectrometer was operated by using positive-ion electrospray mode for both analytes in MRM mode. The ion pairs monitored were m/z 368.4/195.9 for prucalopride and m/z 378.5/144.3 for PRX-03140. All raw data were processed by using Analyst 1.4.2 software (AB Sciex).

Determination of Plasma and Brain Protein Binding. Protein binding was determined with the test compound at 1 or 2 μM in pooled rat plasma or 10% rat brain homogenerate through equilibrium dialysis in Dulbecco’s PBS (pH 7.4), as described previously (Reed-Hagen et al., 1999). The bound fraction of the test compound in undiluted brain tissue was calculated from the unbound fraction in the homogenate and the homogenate dilution factor, as described previously (Kalvass and Maurer, 2002). Prucalopride and PRX-03140 contents were determined with LC-MS/MS as described above.

Data Analysis. IC50 values for in vitro binding studies were calculated through linear regression analysis of the concentration-response data with logit transformation, and the constants for inhibition of binding (Ki) were calculated by using the Cheng-Prusoff equation. For in vitro functional assays, the maximal effect (Emax) on cAMP production for each compound was expressed as the percentage of the response observed with 10 μM 5-HT (100%). The in vitro Emax and EC50 (concentration yielding 50% of the Emax response) values were calculated with nonlinear, least-squares, curve-fitting, linear regression analysis of the concentration-response data, by using a four-parameter sigmoidal fit. For in vivo receptor binding...
studies, the percentage RO of striatal 5-HT₄ receptors at a given dose was calculated as follows: % RO = 100 × ([vehicle-treated specific binding – compound-treated specific binding]/vehicle-treated specific binding). To determine ED₅₀ and EC₅₀ values for in vivo binding studies, the dose-RO and free brain concentration-RO relationships were fitted to a four-parameter sigmoidal curve by using nonlinear, least-squares, curve-fitting with the maximal value constrained to 100% RO and the slope fixed at 1. Microdialysis time course data were expressed as the fractional change from baseline and were calculated as the ratio of the response at each time point to the average basal level (mean of last 5 or 6 samples before treatment). These data were used to calculate the overall effect of treatment as the increase in the area under the curve (AUC) over a specified time period. Statistical analysis of microdialysis data was performed by using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA). Time-course data were analyzed for statistical significance by using repeated-measures, two-way analysis of variance (ANOVA) with Bonferroni post-hoc tests. One-way ANOVA with Dunnett’s or Bonferroni post hoc tests was used to determine the statistical significance of the overall response, as measured by changes in AUC. The EEG data were quantified by using the signal from the last 5 s of each stimulation period (the first 1 s was omitted to avoid signal artifact). Signals were tapered by using a Hanning window and then were analyzed by performing a standard fast Fourier transform with a frequency resolution of approximately 0.24 Hz (Spike2 software; Cambridge Electronic Design, Cambridge, UK). From the ensuing spectrogram, the peak frequency and total power that occurred in the 4- to 9-Hz (θ) frequency range were measured. For each animal, the average frequency and total power were determined for the 0- to 30-min, 30- to 60-min, and 60- to 90-min postinjection periods by averaging the results from 18 epochs of stimulated EEG data and comparing the result with the average of the 18 epochs recorded during the 30-s preinjection period, which provided the average percent change from baseline. Group averages for each compound were then compared with the vehicle-alone group value by using a standard ANOVA. For the time course plots in Fig. 7, the power measured at each point was normalized to the average power over the entire 30-min baseline period.

### Compounds
- 4-Amino-5-chloro-2,3-dihydro-N-[1-(3-methoxypropyl)-4-piperidinyl]-7-benzofurancarboxamide monohydrochloride (prucalopride), 6,7-dihydroxy-7-isopropyl-6-oxo-N-[3-piperidin-1-ylpropyl]thieno[2,3-b]pyridine-5-carboxamide (PRX-03140), (±)-2,3-dihydro-5,6-dimethoxy-2-[(1-phenylmethyl)-4-piperidinyl]methyl-1H-inden-1-one hydrochloride (donepezil), tritiated 1-methyl-1H-indole-3-carboxylic acid, [1-2-[[(methylsulfonyl)amino]ethyl]-4-piperidinyl]methyl ester ([³H]GR113080), 5-fluoro-2-methoxy-1-[2-[(methylsulfonyl)amino]ethyl]-4-piperidinyl]-1H-indole-3-methyloxycarbonylate (OR125487), and tritiated (1-methylpiperidin-4-yl)methyl-1-8-amino-7-chloro-2,3-dihydro-1,4-benzodioxine-5-carboxylate ([³H]HSB207145) were synthesized by Pfizer. For systemic administration in animal studies, all doses were corrected for salt content.

### Results

#### In Vitro Binding and Functional Studies

In vitro binding studies conducted in brain membranes prepared from striatal tissue demonstrated that prucalopride had a high binding affinity for native 5-HT₄ receptors in rat brain (Kᵢ = 30 nM). PRX-03140 was somewhat less potent, with a binding affinity that was approximately 3.5-fold weaker than that of prucalopride. In vitro functional studies in HEK-293 cells expressing various rat 5-HT₄ receptor isoforms (4l, 4s, and 4e) showed that prucalopride potently stimulated the production of cAMP (EC₅₀ = 15–26 nM) and possessed high levels of intrinsic activity (E_max = 52–77%), compared with the cAMP response observed with a full agonist (10 μM 5-HT). PRX-03140 possessed functional potencies for the various rat isoforms (EC₅₀ = 13–23 nM) that were similar to those of prucalopride but displayed lower intrinsic activity levels (E_max = 22–33%). Results of the in vitro binding and functional studies are summarized in Table 1.

#### In Vivo Receptor Occupancy Studies

Striatal tissue was selected for in vivo binding studies on the basis of preliminary experiments that indicated that this brain region had the highest receptor levels and therefore would provide the greatest window for evaluation of occupancy levels in vivo (data not shown). Prucalopride displaced striatal [³H]SB207145 binding in a dose-dependent manner over the range of 0.32 to 32 mg/kg, with an ED₅₀ of 8 mg/kg (Table 2). Corresponding total plasma and brain exposures ranged from 17 to 2595 ng/ml and from 17 to 2160 ng/g, respectively. The unbound fractions of prucalopride in plasma and brain were estimated to be 0.75 and 0.32, respectively. On the basis of these data, the EC₅₀ for [³H]SB207145 displacement by prucalopride equated to a free brain exposure of 330 nM. PRX-03140 also displaced [³H]SB207145 binding in a dose-dependent manner over the range of 0.32 to 32 mg/kg. Corresponding total plasma and brain exposures for PRX-03140 were 66 (160–500) and 130 ± 21 (86–177) nM, respectively.
ranged from 9 to 721 ng/ml and from 26 to 2850 ng/g, respectively (Table 2). The unbound fractions for PRX-03140 in plasma and brain were estimated to be 0.22 and 0.026, respectively. Although a full RO curve for PRX-03140 could not be obtained because of limitations in compound solubility, the ED$_{50}$ was estimated to be 22 mg/kg, which corresponded to a free brain EC$_{50}$ of 130 nM (Table 2).

**Effects on Cortical Acetylcholine Release.** In this set of studies, ACh levels in the rat prefrontal cortex (PFC) were determined with conventional HPLC with electrochemical detection. In the presence of 100 nM neostigmine, baseline dialysate ACh levels averaged 17 nM and ranged from 4 to 38 nM (n = 43). Vehicle administration resulted in a transient, handling-induced increase in cortical ACh levels, which returned to baseline levels within 60 min. Prucalopride was tested for its ability to increase cortical ACh levels after doses of 1, 5, and 10 mg/kg s.c. Time course data for the 5 mg/kg dose showed that the ACh levels increased 2.4-fold within 30 min after administration and gradually returned to baseline over a 3-h period (Fig. 2). Time course data for the 1 and 10 mg/kg doses showed similar profiles (data not shown). A comparison of the overall ACh responses to prucalopride treatment as changes in the AUC showed significant ($P < 0.05$) increases at 5 and 10 mg/kg (Fig. 2, inset). PRX-03140 was administered at 10, 50, and 150 mg/kg s.c. Time course data for the 150 mg/kg dose are shown in Fig. 3, which indicates a sustained increase of approximately 1.7-fold. The ACh responses to PRX-03140 at the 10 and 50 mg/kg doses were transient and similar to vehicle results (data not shown). A comparison of the overall ACh responses to PRX-03140 treatment as changes in the AUC showed a significant ($P < 0.05$) increase with the 150 mg/kg dose (Fig. 3, inset).

**Combined Effects of 5-HT$_4$ Receptor Agonists and Donepezil on Acetylcholine Release.** In this set of compound combination experiments, dialysis samples were collected in the absence of locally perfused neostigmine and ACh levels were determined with LC-MS/MS analytical method, was similar to that shown in Fig. 2. The ability of donepezil (0.32 mg/kg s.c.) to increase cortical ACh levels was examined in the presence and absence of prucalopride (Fig. 4). Vehicle treatment pro-

---

**Fig. 2.** Prucalopride increases ACh levels in the rat prefrontal cortex. Time course data show the ACh responses after treatment with vehicle or prucalopride (5 mg/kg s.c.). Test compounds were dosed at time 0. Data represent mean ± S.E.M. for six or seven animals and are expressed as fractional changes from average baseline levels (baseline = 1). Inset, overall changes in cortical ACh levels expressed as increases in the AUC for the 0- to 3-h time period after prucalopride treatment with 1, 5, or 10 mg/kg s.c., compared with vehicle. *, $P < 0.05$ versus vehicle. PRU, prucalopride; Veh, vehicle.

**Fig. 3.** PRX-03140 increases ACh levels in the rat prefrontal cortex. Time course data show the ACh responses after treatment with vehicle or PRX-03140 (150 mg/kg s.c.). Test compounds were dosed at time 0. The vehicle data are the same as in Fig. 2. Data represent mean ± S.E.M. for five or six animals and are expressed as fractional changes from average baseline levels (baseline = 1). Inset, overall changes in cortical ACh responses expressed as increases in the AUC for the 0- to 3-h time period after PRX-03140 treatment with 10, 50, or 150 mg/kg s.c., compared with vehicle. *, $P < 0.05$ versus vehicle. PRX, PRX-03140; Veh, vehicle.

**Fig. 4.** Prucalopride potentiates the ACh increase caused by donepezil treatment in the rat prefrontal cortex. Time course data show the ACh levels after dosing. Data represent mean ± S.E.M. for five or six animals and are expressed as fractional changes from average baseline levels (baseline = 1). Inset, comparison of overall ACh responses to treatment with prucalopride and/or donepezil versus vehicle. Data represent changes in ACh levels expressed as increases in the AUC for the 0- to 4-h time period after dosing. *, $P < 0.05$, vehicle versus donepezil; †, $P < 0.05$, vehicle versus donepezil/prucalopride; §, $P < 0.05$, donepezil versus donepezil/prucalopride. Don, donepezil; PRU, prucalopride; Veh, vehicle.
duced a transient increase in cortical ACh levels that was similar in magnitude and duration to the response observed in studies conducted in the presence of locally perfused neostigmine (Fig. 2). Systemic administration of donepezil at 0.32 mg/kg s.c. resulted in a rapid 7.6-fold increase in ACh levels, which decreased to 1.6-fold above baseline over a 90-min period after dosing. Treatment with prucalopride alone at 10 mg/kg s.c. produced an ACh response that was similar in magnitude and duration to the maximal response seen in the presence of neostigmine. Combined treatment with donepezil and prucalopride produced rapid, prolonged, 4-h elevation of cortical ACh levels that greatly exceeded the responses to donepezil or prucalopride individually (Fig. 4). Comparison of the overall ACh responses, expressed as increases in the AUC, indicated that the response to combined treatment was significantly greater than that to donepezil alone (P < 0.05) (Fig. 4, inset). In addition, the AUC with the combined treatment was 1.9-fold greater than the sum of the AUCs for the individual treatments, which suggests a potentiating effect. The cortical ACh response to donepezil (0.32 mg/kg s.c.) was also tested in combination with a 50 mg/kg s.c. dose of PRX-03140, a dose that did not significantly affect ACh release on its own (Fig. 5). Similar to the combined effect of prucalopride and donepezil, coadministration of PRX-03140 with donepezil produced a transient increase (1.8-fold) in cortical histamine levels, the result of handling during the injection procedure, which decreased back to baseline levels within 75 min. In comparison, the time course data for prucalopride (5 mg/kg s.c.) revealed a rapid increase that peaked within 30 min after dosing and persisted over the 3-h test period. A comparison of the responses with vehicle results showed significant (P < 0.05) overall increases (AUCs) in histamine levels at the 5 and 10 mg/kg doses that were similar in magnitude, which suggests that these were maximal responses (Fig. 6, inset). PRX-03140, dosed at 50 mg/kg s.c., also caused a sustained elevation in cortical histamine levels and yielded an overall increase (P < 0.05) that was similar in magnitude to the responses seen with prucalopride. At 5 mg/kg, PRX-03140 failed to increase histamine levels, compared with vehicle treatment (Fig. 6, inset).

**Effects of Prucalopride and PRX-03140 on Hippocampal EEGs.** As reported previously (Kinney et al., 1999; Siok et al., 2006), electrical stimulation of the nucleus pontis oralis region of the brainstem reticular formation produced highly rhythmic θ (4–8-Hz) oscillations in the CA1 region of the dorsal hippocampal formation. The starting θ frequency (mean ± S.E.M.) for each group of animals before injection was as follows: vehicle, 6.14 ± 0.03 Hz; prucalopride, 6.12 ± 0.04 Hz; PRX-03140, 6.43 ± 0.04 Hz. The baseline power (mean ± S.E.M.) measured in the 4- to 8-Hz frequency range for each group was as follows: vehicle, 31,527 ± 1764 μV²; prucalopride, 30,080 ± 1116 μV²; PRX-03140, 31,545 ± 949 μV².

After vehicle injection, each animal exhibited a decrease in θ power over the course of the 90-min experiment, with a significant overall decrease from baseline values that averaged 14.4 ± 1.3% during the final 60- to 90-min time period. In contrast, treatment with 5.6 mg/kg prucalopride or 5.6 mg/kg PRX-03140 caused θ power to trend upward from baseline levels during the first 0 to 30 min after injection. This increase persisted for the entire length of the experiment and averaged 11.0 ± 2.4 and 11.9 ± 4.2% during the 60- to 90-min period for prucalopride and PRX-03140, respectively (Fig. 7). The increase seen after injection of either compound was not significantly different from vehicle results.
We have demonstrated for the first time that systemic administration of the 5-HT4 receptor agonists prucalopride and PRX-03140 enhances neuropharmacological measures that are thought to be associated with improvements in cognitive function. These measures include increased release of cortical ACh and histamine and increases in the power of hippocampal \( \theta \) oscillations. The results also indicate that both compounds seem to augment the increase in cortical ACh levels produced by systemic treatment with the acetylcholinesterase inhibitor donepezil. Our findings with two highly selective and brain-penetrant 5-HT4 receptor ligands confirm and extend previous findings on ACh release and \( \theta \) oscillations, which relied on central administration, used nonselective activation of 5-HT4 receptors with serotonin reuptake inhibitors, or depended on behavioral activity (Boddeke and Kalkman, 1990; Consolo et al., 1994; Yamaguchi et al., 1997; Matsumoto et al., 2001; Mohler et al., 2007). The ability of prucalopride and PRX-03140 to increase histamine levels and to augment ACh release in the presence of donepezil has not been reported previously and provides new insight into the regulation of neurotransmitter release by 5-HT4 receptor agonists.

Multiple 5-HT4 receptor isoforms arise through alternative splicing, and localization studies showed that isoforms 4a, 4b, and 4e are found in regions of the human brain associated with memory and cognition (Dumuis et al., 1988; Blondel et al., 1998; Brattelid et al., 2004). Multiple 5-HT4 receptor isoforms also exist in rats, including the 4s and 4l isoforms, which are homologs of the human 4a and 4b isoforms, respectively, and the 4e isoform (Gerald et al., 1995; Claeyesen et al., 1999). Therefore, neuropharmacological measures in rats might translate to humans.

In vitro rat binding and functional data were comparable to published values for human receptors. The rat binding \( K_i \) for prucalopride was 30 nM, a value in good agreement with reported values of 25 and 35 nM for the human 4a and 4b receptor isoforms, respectively (Krobert et al., 2005). PRX-03140 was reported to bind to human 4a and 4e receptor isoforms with \( K_i \) values of 31 and 17 nM, respectively, and showed a similar affinity (\( K_i = 36 \) nM) for native guinea pig brain 5-HT4 receptors (Mohler et al., 2007). In the current study, PRX-03140 bound to native rat 5-HT4 receptors with a \( K_i \) value of 110 nM, which is approximately 3.5-fold less potent than the values reported by Mohler et al. (2007).

In vitro functional testing demonstrated that prucalopride was a potent agonist at the rat 4s, 4l, and 4e isoforms, with \( E_{C50} \) values ranging from 15 to 26 nM. These values were similar to those reported for various human receptor isoforms (Krobert et al., 2005). The \( E_{C50} \) values for PRX-03140 were comparable to those for prucalopride and those reported for the human 4a and 4b isoforms (Mohler et al., 2007). Although the two compounds displayed similar functional potencies for the various rat isoforms, differences in their intrinsic activities were detected. Prucalopride had moderate intrinsic activity levels, ranging from 52 to 77%, values that are somewhat lower than those reported for human receptors (78–100%) (Krobert et al., 2005). In contrast, PRX-03140 possessed considerably lower intrinsic activities, with values ranging from 22 to 33%. These values are lower than those reported for the human 4a, 4b, and 4e isoforms (30–61%) (Mohler et al., 2007).

In vivo receptor binding studies provide a direct means of establishing a dose-RO relationship in animals (Grimwood and Hartig, 2009). In this report, in vivo RO studies established that prucalopride and PRX-03140 were able to penetrate the brain and bound to striatal 5-HT4 receptors. For
PRX-03140, the free brain level required to achieve 50% RO in vivo closely matched its in vitro binding $K_i$, whereas prucalopride values displayed an 11-fold separation. This difference might be attributable to the accuracy of determining RO levels in vivo or the assay conditions used to establish binding potency in vitro. Using alternative assay conditions, Shen et al. (2011) reported a binding $K_i$ of 20 nM for PRX-03140, which is 5.5-fold lower than the 110 nM value reported here. Receptor state might also have an influence. 5-HT4 receptors exist in both high- and low-affinity agonist states, which can be revealed by using agonist and antagonist radioligands (Mikami et al., 2008). Prucalopride binds to the high-affinity agonist state (Bonaventure et al., 2000), whereas the interaction of PRX-03140 at high- and low-affinity sites is unknown. A difference in the compounds' interactions with high- and low-affinity states might contribute to the discrepancy, because the in vitro assay conditions and antagonist radioligand used in this study would not discriminate between the high- and low-affinity states.

Prucalopride and PRX-03140 increased cortical ACh levels after systemic administration, confirming a previous finding showing that central administration of 5-HT4 receptor agonists increases brain ACh levels (Consolo et al., 1994). In vivo occupancy data generated in the current study indicated that prucalopride produced a maximal ACh increase at $<40\%$ RO, whereas PRX-03140 elicited a response at $>85\%$ RO. These observations agree with literature data regarding the levels of target occupancy required for efficacy of G-protein-coupled receptor ligands (Grimwood and Hartig, 2009). PRX-03140 was reported to increase hippocampal ACh levels in rats at doses (1 and 5 mg/kg) equating to low RO; however, this increase occurred only during a behavioral task, and no increase was detected in resting animals (Mohler et al., 2007). These data suggest that low-intrinsic activity 5-HT4 receptor agonists require high RO levels to increase ACh levels under resting conditions but possess the capacity to modulate ACh release in an “activated” system (e.g., behaving animals) at low occupancy levels (Mohler et al., 2007). Receptor reserve can influence the levels of intrinsic activity and RO required to elicit an agonist response. This should be considered in the interpretation of our data, because the level of 5-HT4 reserve receptors was not established in vivo. The interplay between free drug concentrations, intrinsic activity, receptor state, and RO levels required to elicit a maximal response is complex. Similar studies with other 5-HT4 receptor agonists with differing intrinsic activities might improve the interpretation and usefulness of this type of data.

The cognition-enhancing properties of acetylcholinesterase inhibitors, such as donepezil and galanthamine, are reportedly potentiated by 5-HT4 receptor agonists (Mohler et al., 2007; Cachard-Chastel et al., 2008). Therefore, we examined the effects of prucalopride and PRX-03140 on cortical ACh release in combination with donepezil. Combining a maximally effective prucalopride dose with donepezil increased cortical ACh levels to a value that exceeded a simple additive response, which suggests potentiation. The combined actions of PRX-03140 and donepezil were tested under different conditions, whereby the combination of an ineffective PRX-03140 dose with donepezil produced moderate potentiation of ACh release. The ability of PRX-03140 to potentiate ACh release at a subeffective dose in the presence of increased cholinergic tone (i.e., donepezil treatment) supports the premise that 5-HT4 receptor agonists can modulate ACh release in pharmacologically activated neurons, and this provides a neuropharmacological explanation for the cognitive improvements observed in behavioral studies after combination treatment with 5-HT4 receptor agonists and acetylcholinesterase inhibitors.

Prucalopride and PRX-03140 also increased histamine levels in the rat PFC. Histamine is a neurotransmitter that is associated with memory and attentional processes, and its effects on these processes might be mediated in part through cross-talk with the cholinergic system (Philippu and Prast, 2001; Blandina et al., 2004). For example, histaminergic afferents to cholinergic cell bodies in the basal forebrain help to increase ACh release at cholinergic terminals in the PFC and hippocampus (Cechci et al., 2001; Bacciotti et al., 2002). Methylphenidate and atomoxetine, compounds that are used clinically to treat attention-deficit/hyperactivity disorder, were shown previously to increase ACh and histamine levels in the rat cortex (Tzavara et al., 2006; Horner et al., 2007). Taken together, these findings suggest that activation of the cholinergic and histaminergic systems is a desirable response for cognitive enhancement.

Hippocampal theta rhythm is linked to cognitive, memory-related, and attentional processes in both animals and humans (Kahana, 2006; McNaughton et al., 2007). ACh is thought to play a major role in the regulation of hippocampal theta rhythms (Vertes and Kocsis, 1997), inasmuch as administration of acetylcholinesterase inhibitors, such as donepezil, increases theta rhythm in preclinical models (Kinney et al., 1999). Because 5-HT4 receptor agonists elevate brain ACh levels, increased theta oscillation might reflect their cognitive effects in preclinical animal models. The effects of prucalopride and PRX-03140 on stimulated theta activity in the rat CA1 hippocampus were assessed. Our results demonstrated that both compounds increased theta power after 5.6 mg/kg doses. These increases were of similar magnitude as the 30% increase in theta power reported for the histamine H3 receptor antagonist ciproxifan (Hajós et al., 2008). Donepezil treatment was reported to increase theta power over a dose range of 0.1 to 3 mg/kg, with increases ranging from 50 to 350% (Kinney et al., 1999). Currently, it is not known how the magnitude of the increase observed in preclinical measures of theta power is related to the clinical efficacy of donepezil. The increase in theta power observed with prucalopride occurred at a dose that also increased cortical ACh release in the presence or absence of donepezil. In contrast, the theta response observed with PRX-03140 occurred at a dose that was approximately 30- or 10-fold lower than the doses shown to increase cortical ACh levels when given alone or in combination with donepezil, respectively. However, the theta response did occur at a dose similar to that at which cognitive improvements and increased hippocampal ACh release in behaving animals were reported (Mohler et al., 2007). One possible explanation is that the observed effect on theta activity with low doses of PRX-03140 is the consequence of electrical stimulation, which might mimic an active cholinergic state that is modulated by 5-HT4 receptor agonists at low RO levels.

The utility of 5-HT4 receptor agonists as symptomatic treatment agents for cognitive deficits associated with AD would be dependent on the presence of the receptors. Given the reported reductions in 5-HT4 receptor levels in AD brains (Reynolds et al., 1995) and the progressive nature of the
disease, it might be hypothesized that any cognitive benefits derived from this compound class would be most prevalent in the earlier stages of the disease.

5-HT_4 receptor agonists might challenge cognitive deficits associated with psychiatric disorders such as schizophrenia (Roth et al., 2004). ACh plays a critical role in cognitive processing, and its actions are mediated in part through muscarinic M1 receptors. One study reported that, in 26% of patients with schizophrenia, muscarinic M1 receptor levels were reduced by 74% in the PFC (Scarr et al., 2009). Because 5-HT_4 receptor levels are reportedly unchanged in schizophrenia (Dean et al., 1999), 5-HT_4 receptor agonists might help to restore M1 receptor-mediated neurotransmission by increasing cortical ACh levels.

In summary, we showed that two highly selective 5-HT_4 receptor agonists demonstrate potent binding and functional activity at rat 5-HT_4 receptors. In vivo RO studies demonstrated that both compounds are able to penetrate the brain and to bind to 5-HT_4 receptors in the rat striatum. Prucalopride and PRX-03140 exhibited similar neuropharmacological properties, in that both compounds increased ACh and histamine levels in the prefrontal cortex and the power of hippocampal theta oscillations. The ability of these compounds to potentiate ACh release elicited by donepezil might provide a neurochemical explanation for the cognitive improvements observed when ineffective doses of 5-HT_4 receptor agonists and acetylcholinesterase inhibitors are combined in preclinical behavioral models. These data confirm previous work suggesting that 5-HT_4 receptor agonists might have potential as a new approach to symptomatic improvement of cognitive deficits in AD.

Acknowledgments

We thank Dr. Hans Rollema for helpful discussions during the writing of this manuscript and Deborah Smith for technical assistance with in vivo occupancy studies.

Authorship Contributions

Participated in research design: Johnson, Drummond, Grimwood, Sawant-Basak, McDowell, Fisher, Stutzman-Engwall, Hajas, and Siok.

Conducted experiments: Johnson, Drummond, Miller, Tseng, Vanase-Frawley, Rubitski, Nelson, Horner, Gorczyca, and Siok.

Contributed new reagents or analytic tools: Stutzman-Engwall and Nelson.


Wrote or contributed to the writing of the manuscript: Johnson, Drummond, Grimwood, Sawant-Basak, Miller, Tseng, McDowell, Vanase-Frawley, Fisher, Stutzman-Engwall, Horner, Gorczyca, Hajas, and Siok.

References


Address correspondence to: David E. Johnson, Neuroscience Research Unit, Pfizer Global Research and Development, Eastern Point Rd., Groton, CT 06340. E-mail: david.e.johnson@pfizer.com