An Assessment of the Effects of 5-HT6 Receptor Antagonists 
in Rodent Models of Learning

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ABSTRACT

Antagonists of serotonin 6 (5-HT6) receptors have been reported to enhance cognition in animal models of learning, although this finding has not been universal. We have assessed the therapeutic potential of the specific 5-HT6 receptor antagonists, Ro 04-6790 and SB-271046, in rodent models of cognitive function. Although mice express the 5-HT6 receptor and the function of this receptor has been investigated in mice, all reports of activity with 5-HT6 receptor antagonists have utilized rat models. In the present study, receptor binding revealed that the pharmacological properties of the mouse receptor are different from the rat and human receptor: Ro 04-6790 does not bind to the mouse 5-HT6 receptor, so all in vivo testing included in the present report was conducted in rats. We replicated previous reports that 5-HT6 receptor antagonists produce a stretching syndrome previously shown to be mediated through cholinergic mechanisms, but Ro 04-6790 and SB-271046 failed to attenuate scopolamine-induced deficits in a test of contextual fear conditioning. We also failed to replicate the significant effects reported previously in both an autoshaping task and in a version of the Morris water maze. The results of our experiments are not consistent with previous reports that suggested that 5HT6 antagonists might have therapeutic potential for cognitive disorders. In our experiments, reference 5-HT6 receptor antagonists failed to demonstrate any significant effects suggestive of utility as cognition enhancing agents.
The 5-HT6 receptor was first isolated from rat striatal mRNA in 1993. It is localized almost exclusively in the CNS, including areas important for learning and memory, such as the cerebral cortex and hippocampus (Monsma, Jr. et al., 1993; Ruat et al., 1993). Polymorphisms of the 5-HT6 receptor have been associated with clinical disorders such as Alzheimer’s, bipolar affective disorder, and schizophrenia (Tsai et al., 1999a; Tsai et al., 1999b; Vogt et al., 2000), all of which are characterized by at least some degree of cognitive deficit. The suggestion that 5-HT6 receptor antagonists may have therapeutic potential as novel treatments for cognitive deficits is supported by reports that they facilitate cholinergic and glutamatergic neurotransmission. Antagonists of 5-HT6 receptors produce a behavioral syndrome of yawning/stretching/chewing, which is characteristic of cholinergic agonists (Bourson et al., 1995; Sleight et al., 1996; Sleight et al., 1998; Bentley et al., 1999), and they reduce the number of rotations produced in rats by cholinergic antagonists (Bourson et al., 1998). 5-HT6 receptor antagonists have also been shown to enhance extracellular levels of glutamate in the frontal cortex and hippocampus as revealed during microdialysis (Dawson et al., 2000; Dawson et al., 2001).

In addition, there is suggestive evidence that atypical anti-psychotics may attenuate cognitive deficits in patients with schizophrenia, perhaps through their action as 5-HT6 receptor antagonists. Atypical anti-psychotics have very high affinities for 5-HT6 receptors and block stimulation of adenylyl cyclase activity produced by serotonin (Sebben et al., 1994). They enhance extracellular levels of glutamate in the frontal cortex (Daly and Moghaddam, 1993), and chronic treatment with atypical anti-psychotics decreases 5-HT6 receptor expression in the hippocampus (Frederick and Meador-Woodruff, 1999). Most patients with schizophrenia have cognitive deficits (Meltzer and McGurk, 1999), and atypical anti-psychotics attenuate these cognitive deficits (Purdon et al., 2000). Typical anti-psychotics such as haloperidol do not mediate their effects through the 5-HT6 receptor (Bourson et al., 1995; Bourson et al., 1998; Bentley et al., 1999; Frederick and Meador-Woodruff, 1999), nor do they attenuate cognitive deficits in schizophrenia patients (Purdon et al., 2000). Taken together, these results may suggest that the cognitive effects of atypical anti-psychotics may be mediated by their action as 5-HT6 receptor antagonists.
Finally, several studies have reported that specific 5-HT6 receptor antagonists improve learning and memory in animal models. Analogues of the selective 5-HT6 receptor antagonist Ro 04-6790 attenuated scopolamine-induced deficits in a passive avoidance task (Bos et al., 2001). Ro 04-6790 also increased acquisition and consolidation in normal young rats in an operant autoshaping task, and it attenuated scopolamine-induced deficits in this task (Meneses, 2001). The selective 5-HT6 receptor antagonists Ro 04-6790, SB-271046 and SB-357134, all increased retention of a spatial mapping Morris water maze task in normal young rats (Woolley et al., 2001; Rogers and Hagan, 2001; Stean et al., 2002).

In contrast to all the studies which support the therapeutic potential of 5-HT6 receptor antagonists, Russell and Dias (2002) reported that they were unable to replicate any of the therapeutic effects of 5-HT6 receptor antagonists. The objective of the studies included in the present report was to assess the therapeutic potential of 5-HT6 receptor antagonists for enhancing cognitive function in rodent models. Both rats and mice express the 5-HT6 receptor, and both species have been used to investigate the function of this receptor. Initially, we planned to assess 5-HT6 receptor antagonists in both mouse and rat models of cognitive behavior. However, although mice have been used to assess the therapeutic potential of 5-HT6 receptor antagonists (Bourson et al., 1998), all positive effects with 5-HT6 receptor antagonists have been detected in rats, and our own initial work with mice failed to detect therapeutic effects with 5-HT6 receptor antagonists (data not shown). Examination of the literature revealed that four critical residues have been identified for ligand binding to the 5-HT6 receptor, and one of these four residues is different in the mouse receptor when compared with rat and human receptor (Boess et al., 1998; Kohen et al., 2001). Therefore, the objective of our first experiment was to determine if 5-HT6 receptor antagonists bind to the mouse receptor. This study revealed that the pharmacological properties of the mouse receptor are different from the rat and human receptor, so we subsequently conducted all efficacy tests in rats. In addition, since most of the published literature demonstrating the therapeutic potential of 5-HT6 receptor antagonists was produced with Ro 04-6790 or SB-271046, we focused our efforts on attempting to replicate the initial preclinical experiments using these two standards.
MATERIALS and METHODS

**Animals.** These studies were conducted in an animal care facility certified by the American Association for Accreditation of Laboratory Animal Care, and all experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee before the studies were initiated. All rats were from Harlan Sprague-Dawley (Indianapolis, IN): male Sprague-Dawley, 285 ± 1.8g, N=297; male hooded Long-Evans, 282 ± 4.8g, N=69; and male Wistar, 360 ± 4.7g, N=160. Rats were housed in polycarbonate cages in a temperature-controlled room with a 12:12hr light:dark cycle. All rats were housed 3-4 per cage, and food and water was available *ad libitum*, except for the Wistar rats that were used in the food reward autoshaping task, which were singly housed and maintained on a restricted diet, 12-15g per day of standard rat chow, until their body weights were approximately 85% of *ad lib* fed rats, at which time behavioral testing was initiated.

**Cloning and transfection of human, rat and mouse 5-HT6 receptors.** The mouse and rat 5HT-6 receptors were obtained by PCR amplification using whole rat (adult male Sprague-Dawley) or mouse (adult male BALB/c) brain cDNA (Clontech, Palo Alto, CA) followed by TA cloning, insert excision and purification, and finally cloning into the pCDNA3.1 vector (Invitrogen, Frederick, MD). The mouse insert was amplified using 0.4µM each of the following primers: 5'-ATGGTTCCAGAGCCCGGCCCTGTCAAC-3' and 5'-TCAGTTCATGGGGGAACCAAGTGGATGCTG-3'. The rat insert was amplified using 0.4µM each of the following primers: 5'- ATGGTTCCAGAGCCAGGCCCTGTCAAC-3' and 5'-CTCCAATGGCCAGCTCTTGACCTGGTCA-3'. The completed vectors were transformed into DH5αF competent cells (Invitrogen) and a large scale prep of DNA was prepared using the Qiafilter Plasmid Maxi Kit (Qiagen, Valencia, CA) for sequence confirmation followed by transfection. The human 5-HT6 receptor was obtained from David R. Sibley (National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland). It was subcloned into pCDNA3.1 as well.

The constructs were transiently transfected into HEK 293 human embryonic kidney cells using Lipofectamine Plus (Invitrogen). Briefly, 1.5 x 10^7 cells were plated into 150mm tissue culture plates 24
hrs prior to transfection. Cells were transfected using 8µg DNA, 40µl plus reagent and 60µl lipofectamine reagent per plate according to manufacturer's instructions. Following a 3-hour transfection incubation, cells were fed fresh medium and were harvested 48 hours later. Control transfections with a fluorescent protein demonstrated an 80% transfection efficiency.

Cells were rinsed, scraped up and homogenized with a polytron. The cell homogenate was centrifuged for 30 minutes at 30,000 x g. The pellet was resuspended in 50mM Tris, pH 7.2, 1mM EDTA plus Sigma Mammalian Protease Inhibitor (St. Louis, MO). Protein concentration was determined using bicinchoninic acid (Pierce, Rockford, IL).

5HT-6 Receptor Binding Studies. The protocol was adapted from a previous report (Boess et al., 1997). The Bmax values for the human, rat, and mouse membrane preparations were human = 18,000 fmols/mg; rat=11,400 fmols/mg; mouse =4600 fmols/mg. The membranes (2, 5 and 15 µg protein for the human, rat, and mouse receptors respectively) were added to [³H] d-lysergic acid diethylamide (LSD, 84 Ci/mmol, Amersham, Piscataway, NJ) to start the binding assay in 50 mM Tris, 2 mM MgCl₂, pH 7.4. The [³H]LSD was diluted in 0.4% bovine serum albumen, 200 µM ascorbic acid and then diluted 1:10 in the samples to give a final concentration of 2 nM. Final concentrations of compounds ranged from 10⁻⁶ to 10⁻¹⁰ M and were dissolved in dimethylsulfoxide (DMSO) with a final concentration of 1% DMSO. Non-specific binding was determined using 10 µM clozapine. The 96 well plates were shaken for 10 minutes at room temperature on a Lab Line Instruments Titer Plate Shaker (Melrose Park, IL) and then incubated for 1 hour at 37°C. The samples were filtered through Whatman GF/B membranes pretreated with 0.5% polyethyleneimine. The samples were immediately washed 5 times with 1.5 ml ice cold 20 mM Tris, pH 7.4. They were counted in a Wallac Microbeta Trilux 1450 scintillation counter (Turku, Finland).

Yawning/stretching/chewing. Rats were given vehicle injections and placed in individual, transparent chambers for one hour each day for four days before the test day, to habituate them to the observation chambers and testing procedure. On the test day, rats were placed in the observation chambers immediately after drug administration and observed continuously for yawning, stretching and chewing.
behaviors from 30-90 minutes after drug or vehicle injections (Ro 04-6790 30.0 mg/kg; SB-271046 30.0 mg/kg; physostigmine 0.1 mg/kg). Average number of yawns, stretches, and vacuous chewing movements during the one hour observation period were recorded as previously reported (Sleight et al., 1998; Bentley et al., 1999).

**Conditioned Fear.** Rats were first placed in individual sound-attenuating test chambers (Med Associates, St. Albans, VT) for a six minute conditioning session, which consisted of 2 minutes of habituation, a tone/footshock pairing (30 second 87 dB tone, 2 second 2.5 mA footshock), followed by 2 minutes with no tone or shock, another tone/footshock pairing, and a final minute of no stimuli presentation. Control rats were dosed with vehicle and placed in the fear conditioning box for 6 minutes but with no tone or shock. Rats were then removed from the test box and returned to their home cages. Percent time freezing was measured 24 hours later in a seven minute contextual memory test in the same chambers with no tone or shock. Freezing behavior was quantified with Freezview image analysis software (Actimetrics, Evanston, IL) using filter 25, and bout length of 0.75 sec. SB-271046 solutions (1.0, 10.0, and 30.0 mg/kg, p.o.) and scopolamine (1.0 mg/kg, i.p.) were co-administered 1.5 hours before the conditioning session. Ro 04-6790 (30.0 mg/kg, i.p.) and scopolamine (1.0 mg/kg, i.p.) were co-administered 30 minutes before the conditioning session. All drugs were administered before the conditioning session only.

**Autoshaping.** Ro 04-6790 was tested in an autoshaping procedure as previously reported (Meneses, 2001). On the first day of testing rats were habituated to operant chambers (Coulbourn Instruments, Allentown, PA) by filling the food trough with 50 food pellets (45 mg/pellet). As soon as all the pellets were eaten, each rat was given 10 trials. During a trial, rats were presented with a retractable lever for 8 seconds and the cage was illuminated by a house light located at the top of the cage directly above the lever. After 8 seconds the lever was retracted, the house light was extinguished and a 45mg food pellet was delivered to the food trough. If the rat pressed the lever while it was extended, it was immediately retracted, the pellet was delivered and the light was extinguished. Following pellet delivery there was a 60s intertrial interval. The following day, each rat was tested by giving them 20 trials without the initial 50-pellet habituation period. Ro 04-6790 (0, 1, 5 or 10 mg/kg, IP, 1 ml/kg, in sterile saline) was administered
immediately after training, and scopolamine (0.17 mg/kg, IP) was administered 10 minutes after the training session, as previously reported (Meneses, 2001). The dependent measure was the percentage of bar-presses during the test session. In order to overcome potential floor effects, another experiment was conducted in which rats were given additional daily test sessions, first using the same procedures as above, and then under slightly different conditions to further facilitate the rate of acquisition, in which the lever remained extended for 30 seconds, and was retracted for 10 seconds, for 50 trials each day, as previously reported (Andrews et al., 1995).

Morris water maze. Ro 04-6790 was tested in the Morris water maze as previously reported (Woolley et al., 2001). Briefly, rats were administered saline vehicle or Ro 04-6790 at the optimal dose (30 mg/kg, IP, 1 ml/kg) 30 minutes before daily acquisition training. During acquisition training rats received three, 90 second trials per day for 3 days, to swim to a hidden platform, with 20 seconds on the platform at the end of each trial. They were then given one probe trial per day, without drug, 7, 10 and 14 days after the end of acquisition training. During probe trials, the hidden platform was removed, rats were allowed to swim for 60 seconds, and swimming duration within a 10 cm annulus around the former platform location was quantified. One experiment was conducted with Sprague-Dawley rats and another experiment was conducted with Long Evans Hooded Rats.

SB-271046 was also tested in the Morris water maze as previously reported (Rogers and Hagan, 2001). Briefly, rats were dosed with vehicle or SB-271046 at the optimal dose (10 mg/kg, PO, 2 ml/kg in 1% methylcellulose) 2 hours before acquisition and probe test sessions. Rats were tested in four 60-second acquisition trials on day 1, and 6 acquisition trials per day on days 2-5. Probe trials were conducted immediately after the last acquisition trial, and again 4, 7 and 10 days after the end of acquisition training. Latency to reach the platform was recorded during acquisition trials, and the percent time in the target quadrant was the dependent measure during probe trials.

Drugs. Ro 04-6790 and SB-271046 were synthesized at Bristol-Myers Squibb (Wallingford, CT) and the structures confirmed using standard analytical methods. Scopolamine hydrobromide and physostigmine...
salicylate were purchased from Sigma-Aldrich (St. Louis, MO). All compounds were dissolved in sterile saline and administered IP at 1ml/kg, except SB-271046 in the conditioned fear and Morris water maze experiments, where it was suspended in 1% methyl cellulose and administered by oral gavage at 1-2 ml/kg.

Statistics. Data analyses were conducted with SAS™. In the text and all figures, data are presented as means ± SEMs. The yawning, stretching and chewing, conditioned fear and autoshaping data were analyzed with planned contrasts between the main control group and each treatment group, including repeated measures where appropriate. The data in the last autoshaping experiment was analyzed with a 2 x 2 ANOVA, including Ro 04-6790 and Scopolamine as factors in the analysis. In the Morris water maze experiments, differences between groups were analyzed separately for each trial during acquisition, as previously reported (Woolley et al., 2001). For probe trials with SB-271046, potential differences between groups in duration of time spent swimming in the target quadrant was analyzed separately for each probe trial, as previously reported (Rogers and Hagan, 2001). For probe trials with Ro 04-6790, performance was analyzed separately for each group and for each probe trial: duration of time in each quadrant was compared to the duration of time spent swimming in the target quadrant, as previously reported (Woolley et al., 2001).
RESULTS

5-HT6 Receptor Binding Studies. Receptor binding studies were performed using recombinant receptors from human, rat, and mouse. Clozapine and methiothepin were shown to have similar Ki’s at the 5-HT6 receptor from all 3 species. All the compounds tested, including SB-271046, Ro 04-6790 and clozapine, had potencies in the rat receptor that were similar to their potencies in the human receptors; however, a different pharmacology was observed for the mouse receptor (Fig. 1, Table 1). SB-271046 had a four-fold lower affinity at the mouse receptor than at the rat receptor, and while Ro 04-6790 had a Ki of 23.07 nM in rat receptors, it failed to inhibit LSD binding in the mouse receptor up to 1 µM (Table 1). We do not know if these changes are manifest in all mouse strains but based on these results, all subsequent in vivo experiments were conducted in rats.

Yawning/Stretching/Chewing. In an experiment with the optimal dose of SB-271046, physostigmine produced a significant increase in chewing behaviors, F(1,29)=6.27, p=0.02; and a trend towards increased yawning, F(1,29)=2.19, p=0.15. SB-271046 produced a significant increase in stretching behavior, F(1,29)=6.55, p=0.01 (Fig. 2). In an experiment examining the effects of the optimal dose of Ro 04-6790, physostigmine produced a significant increase in chewing, F(1,21)=4.44, p=0.04, and a trend towards increased yawning, F(1,21)=3.21, p=0.09; while Ro 04-6790 produced a significant increase in the number of stretches, F(1,21)=8.72, p=0.008, and vacuous chewing, F(1,21)=4.44, p=0.04 (Fig. 3).

Conditioned Fear. In the conditioned fear experiment with Ro 04-6790, there were significant differences between [1] the no-shock controls and the shocked, vehicle-treated controls, F(1,75)=32.52, p=0.0001, [2] the scopolamine-treated group and the vehicle group, especially during the later time points - the treatment x minute interaction between those groups was statistically significant, F(6,450)=2.18, p=0.04; [3] the no-shock controls versus the scopolamine-treated group, F(1,75)=15.20, p=0.0002; and [4] the Ro 04-6790 group versus the scopolamine treated group, F(1,75)=5.58, p=0.02 (Fig. 4A). Ro 04-6790 at 30 mg/kg reduced the amount of freezing beyond that seen in animals treated with scopolamine only, an effect which is consistent with impaired performance.
In the conditioned fear experiment with SB-271046, there were significant differences between [1] the no-shock controls and the shocked, vehicle-treated controls, F(1,126)=51.65, p=0.0001, [2] the scopolamine-treated group and the vehicle group, F(1,126)=5.18, p=0.02; [3] the no-shock controls and the scopolamine-treated group, F(1,126)=24.66, p=0.0001; and [4] the SB-271046 30mg/kg treated group and the scopolamine-treated group, F(1,126)=6.93, p=0.01 (Fig. 4B). Again, the effect of SB-271046 at 30 mg/kg was in the direction of impaired performance, relative to the group treated with scopolamine alone. Lower doses of SB-271046 did not produce significant effects, but even at 1.0 and 10.0 mg/kg, the trend was in the direction of impaired performance, relative to animals treated with scopolamine alone.

**Autoshaping.** In the first autoshaping experiment with Ro 04-6790, there were no significant increases in bar-pressing in animals with any dose of Ro 04-6790, F's(1,73)<1.27, p's > 0.26 (Fig. 5A). In the second autoshaping experiment examining the ability of Ro 04-6790 to attenuate the effects of scopolamine, dosing rats with 0.17 mg/kg scopolamine after the first session did not significantly reduce bar-pressing 24 hours later, and Ro 04-6790 did not increase bar-pressing rate on its own or in animals dosed with scopolamine, F's(1,43)< 1.0, p's > 0.40 (Fig. 5B). In the third autoshaping experiment, rats were repeatedly tested with scopolamine (0.17 mg/kg) and Ro 04-6790 administered after each session, using the dose of Ro 04-6790 (5 mg/kg) that previously produced peak effects (Meneses, 2001). In order to facilitate acquisition of bar-pressing and overcome any potential floor effects, an additional session was conducted using the Meneses procedure, and 5 more sessions with 50 trials per day, with the duration of lever extension increased from 8 seconds to 30 seconds. Rats did begin to acquire this task - bar-pressing increased over days, and a scopolamine deficit was eventually evident, the day x scopolamine interaction was statistically significant, F(6,318)=2.34, p=0.03 (Fig. 6). Ro 04-6790 (5mg/kg) did not facilitate acquisition or attenuate the scopolamine-related deficit, in fact, there was a trend for Ro 04-6790 to impair performance in both vehicle and scopolamine-treated rats, but that trend was not significant.

**Morris Water Maze.** In the test of Ro 04-6790 in both albino Sprague-Dawley and hooded Long-Evans rats, latencies to reach the target platform during acquisition trials improved over trials (Fig. 7). In the
albino Sprague-Dawley rats, there was a significant difference between the vehicle and Ro 04-6790 only on the second trial of acquisition training, in which the drug-treatment group had longer latencies than the vehicle-treated group, $F(1,28)=6.79$, $p=0.01$ (Fig. 7A). In the hooded Long-Evans rats, there was a significant difference between the vehicle and Ro 04-6790 only on the fourth trial of acquisition training, in which the drug-treatment group had longer latencies than the vehicle-treated group, $F(1,27)=6.89$, $p=0.01$ (Fig. 7B).

Analyses of probe trial data revealed no evidence that Ro 04-6790 increased retention of the target location in either albino Sprague-Dawley rats or hooded Long-Evans rats (Fig. 8). Although the hooded Long-Evans rats had more of a preference for the target annulus during the first probe trial, both albino Sprague-Dawley rats and hooded Long-Evans rats spent more time swimming in the target annulus on the first probe trial, 7 days after the end of acquisition training, regardless of whether they were in the vehicle or Ro 04-6790 group. The Ro 04-6790-treated Sprague-Dawleys may not have had quite as strong a preference for the target location as the vehicle-treated rats during the first probe trial, since they failed to discriminate between the target quadrant and one of the adjacent quadrants (Fig. 8A, top left panel). In the second probe trial, 10 days after the end of acquisition training, vehicle-treated Sprague-Dawley rats had no preference for the target annulus, while the Ro 04-6790-treated rats swam in the target annulus more than in one of the adjacent quadrants, but the trend was just the opposite in hooded Long-Evans rats, where only the vehicle-treated group swam more in the target annulus than in one of the adjacent quadrants. On the third probe trial, 14 days after the end of acquisition training, there was no preference to swim in the target annulus in any of the groups. In fact, the hooded Long-Evans rats treated with Ro 04-6790 actually exhibited a preference to swim in quadrants other than where the target had been located (Fig. 8B, bottom right panel).

Hooded Long-Evans rats rapidly acquired the Morris water maze task to asymptotic levels of performance, but SB-271046 did not affect latencies to find the Morris water maze, $F$'s$(1,38)<1.4$, $p$'s$>0.25$ (Fig. 9A). Rats also exhibited evidence that they were utilizing spatial-mapping strategies, since they swam almost 50% of the time in the target quadrant during the first probe trial immediately after the
last acquisition trial (Fig. 9B). The spatial mapping strategy extinguished over repeated probe trials, as the rats spent less and less time swimming near the former target location. There was a trend for the vehicle-treated rats to spend more time swimming in the target quadrant on the third probe trial, and a trend for the SB-271046-treated rats to spend more time swimming in the target quadrant on the fourth probe trial, but there were no statistically significant differences between the vehicle-treated and the SB-271046-treated groups on any of the probe trials, F's(1,38)<1.75, p>0.20.
DISCUSSION

The results of the present experiments demonstrate, for the first time, that there are differences in the pharmacological properties of the mouse 5-HT6 receptor, relative to the human and rat receptor. Although the mouse, rat and human receptors are fairly homologous (>84% identical), four critical residues had been identified for ligand binding to the 5-HT6 receptor, and one of these four residues is different in the mouse than in the rat and human receptor (Boess et al., 1998; Kohen et al., 2001). Site mutations of the rat receptor that alter the one residue that is unique to the mouse receptor, significantly affect ligand binding (Boess et al., 1998), but those site mutations were not identical to the mouse receptor sequence, and receptor binding had not been reported previously with the mouse 5-HT6 receptor. Receptor binding studies included in the present report show that both Ro 04-6790 and SB 271046 have high affinity to rat and human receptors, but Ro 04-6790 did not bind to the mouse receptor, and SB 271046 had a lower affinity at the mouse receptor than at the rat or human receptor. These results make it clear that mice should not be used to assess the therapeutic potential of 5-HT6 receptor antagonists unless the compounds are shown to bind to the mouse receptor, and the results of previous studies assessing compounds such as Ro 04-6790 in mice need to be re-evaluated (Bourson et al., 1998).

Based on the results of the receptor binding experiments, all behavioral assessments included in the present report were conducted in rats. Our results replicated the finding that 5-HT6 receptor antagonists produce stretching, a behavioral syndrome mediated by cholinergic facilitation (Bourson et al., 1995; Sleight et al., 1996; Sleight et al., 1998; Bentley et al., 1999). We detected stretching with both Ro 04-6790 and with SB-271046. Previous studies had not seen increased stretching with SB-271046 except to accentuate the effects produced by the AChE inhibitor physostigmine [(Stretton J, unpublished data, c.f. (Reavill and Rogers, 2001) and (Routledge, abstract in British Journal of Pharmacology 127(Suppl.), 21P. 1999)]. Our tests with SB-271046 may have been more sensitive to stretching behavior because we habituated our rats to the testing procedure on numerous occasions before conducting the testing. These
habituation periods reduce activity levels during the test, which make it easier to observe stretching if it occurs.

In contrast to the stretching behavior, which is consistent with facilitation of cholinergic neurotransmission, none of our efforts to assess the therapeutic potential of 5-HT6 receptor antagonists on measures of cognitive function detected any positive effects. For example, the results of our experiment with Ro 04-6790 in the conditioned fear task were not consistent with the positive effects reported with analogs of Ro 04-6790 in the passive avoidance task (Bos et al., 2001). In another experiment, we were unable to detect significant, positive effects with SB-271046 in the conditioned fear test. Even after closely replicating the methods used previously with Ro 04-6790 and SB-271046 in an autoshaping task and in the Morris water maze (Woolley et al., 2001; Rogers and Hagan, 2001; Meneses, 2001), we failed to replicate any of the positive results reported in those studies. For example, we did not see evidence of improved acquisition or retention with Ro 04-6790 in the autoshaping task. Meneses reported bar-pressing rates of 10% for the vehicle-treated control group on the test day, and in our experiments, vehicle-treated controls pressed the bar on 6-7% of the 20 trials, which is within the expected range of Meneses’ experiments, but it is so low that there are potential floor effects which may reduce the sensitivity of this test. However, even with repeated testing, no significant effects were detected for Ro 04-6790 in this task, either in normal rats or in rats with scopolamine-induced deficits. In addition, even if increased bar-pressing rates had been detected in this task, additional studies would need to be conducted to rule out potential non-specific effects such as increased activity levels and/or disinhibition or impulsivity.

Likewise, we saw no evidence of increased retention with Ro 04-6790 in the Morris water maze using the same procedures reported previously (Woolley et al., 2001). The animals reached asymptotic levels during acquisition, they showed evidence of spatial mapping during retention trials, and performance declined with repeated probe trials, but there was no evidence that Ro 04-6790 improved acquisition or retention. If anything, Ro 04-6790 actually impaired performance during acquisition trials. It is not clear what the critical difference is between our experiments and the previous experiment that reported positive
results. However, it is interesting to note that in the previous Morris water maze experiment, the group treated with Ro 04-6790 performed significantly better than the vehicle-treated controls during acquisition trials, but this difference seemed to be due to the fact that the vehicle-treated controls suddenly performed worse than expected during the last 3 acquisition trials. Whatever the cause, if the difference in performance between the vehicle-treated controls and the group dosed with Ro 04-6790 is due to uncharacteristically poor performance among the vehicle-treated controls, this should not be interpreted as evidence that the drug improved performance.

Finally, we were also unable to replicate the positive effects reported for SB-271046 in the Morris water maze, although we replicated as precisely as possible the methods used previously (Rogers and Hagan, 2001). Our results suggest that cognition enhancing effects of Ro 04-6790 and SB-271046 are not reliable. In addition, we agree with previous criticisms suggesting that the effects previously reported were not necessarily evidence of therapeutic potential (Russell and Dias, 2002). For example, the effect in both previous Morris water maze studies may have been attributed to perseveration, rather than due to increased retention (Russell and Dias, 2002). In other words, even if we had replicated the results of the previous studies showing prolonged searching for the previous target location, it would not be appropriate to conclude that this effect was evidence of improved cognitive function, it could also be attributed to perseveration or impaired cognitive function, and additional experiments would have to be run to rule out that possibility.

Consistent with the results of the present experiments, other investigators have also reported difficulty in replicating the positive effects of Ro 04-6790 and SB-271046 in the Morris water maze (Russell and Dias, 2002), and in other models of cognitive function. Chronic ICV administration of antisense oligonucleotides to the 5-HT6 receptor did not affect performance in a conditioned fear task (Yoshioka et al., 1998), and 5-HT6 receptor knockouts had no effect in a novel object recognition test (reported in Martin et al., 1998). Not only are the therapeutic effects of 5-HT6 receptor antagonists in preclinical models of cognitive function in question, but studies that support the therapeutic potential of 5-HT6 receptor antagonists for cognitive deficits is complicated by at least one report that fails to support it. For example, while some
studies have suggested that 5-HT6 receptor antagonists enhance cholinergic and glutamatergic transmission, one study failed to detect an increase in hippocampal extracellular acetylcholine levels after administration of Ro 04-6790 (Shirazi-Southall et al., 2002), and another study failed to detect increases in glutamate release from frontal cortex after systemic or direct application of SB-271046 (Russell and Dias, 2002). The fact that hippocampal extracellular acetylcholine levels were elevated after administration of clozapine but not Ro 04-6790 (Shirazi-Southall et al., 2002) also suggests that atypical anti-psychotics might not enhance cognitive function in patients with schizophrenia through their action on 5-HT6 receptors. Recent studies also suggest that there are no differences in 5-HT6 receptor binding or receptor densities in schizophrenic patients (East et al., 2002), and find neither an association between 5-HT6 receptor polymorphisms and susceptibility to schizophrenia (Shinkai et al., 1999; Ohmori et al., 2001), nor an association between 5-HT6 receptor polymorphisms and response to clozapine in schizophrenic patients (Masellis et al., 2001). Another study reported that there were no significant differences in genotypic or allelic distribution of 5-HT6 receptors among AD patients and controls, which suggests that these polymorphisms probably do not represent major genetic risk factors for Alzheimer’s disease (Thome et al., 2001; Orlacchio et al., 2002).

Several previous studies have reported linear dose-response curves with more and more robust, positive effects of Ro 04-6790 up to the highest dose tested, 30 mg/kg (Sleight et al., 1998; Bentley et al., 1999; Woolley et al., 2001). Ro 04-6790 also attenuated scopolamine-induced rotations with a maximal effect at 30 mg/kg (Bourson et al., 1998), therefore, we tested Ro 04-6790 at this optimal dose of 30 mg/kg during observations for stretching, in the conditioned fear task, and in the Morris water maze. Likewise, several studies have reported that consistent, robust effects were obtained with SB-271046 at 10 mg/kg (Routledge et al., 2000; Dawson et al., 2000; Dawson et al., 2001), including tests for retention in the Morris water maze (Rogers and Hagan, 2001), so we tested SB-271046 at this optimal dose in the Morris water maze. Large sample sizes were tested with these optimal doses, and when we were unable to detect significant effects with Sprague-Dawley rats in the Morris water maze, we repeated the study with Long-Evans rats. The autoshaping task was also conducted with a very large sample size and testing was continued until we could be sure that the lack of significance was not due to a floor effect. All these
decisions were made in an attempt to maximize the probability of either detecting a beneficial effect on
cognitive function, or of replicating the results of previously published studies that reported statistically
significant effects with 5-HT6 receptor antagonists.

Despite our best efforts to detect significant therapeutic effects on measures of cognitive function, it is
impossible to prove that a treatment is inactive or that it has no therapeutic efficacy, and no matter how
many studies are conducted, it is always possible that additional studies might still uncover some
potential efficacy. For example, it is possible that the use of different doses or different dosing times, or
the use of an experimental design that was not confounded with extinction effects, may have detected
therapeutic effects of 5-HT6 receptor antagonists on measures of cognitive function. We would
emphasize that our efforts to demonstrate efficacy and/or to replicate previous positive results were fairly
extensive, and that we made every effort to maximize our chances of detecting therapeutic effects.

However, we do not conclude from the present experiments that 5-HT6 receptor antagonists have no
therapeutic efficacy. Instead, the negative results of the present experiments simply raise questions about
the reliability and validity of the therapeutic potential of 5-HT6 receptor antagonists. For results to be
accepted as valid, they must be reliable, and we would simply argue that the therapeutic potential of 5-
HT6 receptor antagonists cannot be accepted as valid until they can be shown to produce therapeutic
effects reliably.

The results of the present experiments suggest that 5-HT6 receptor antagonists may not have therapeutic
potential for cognitive disorders. Alternatively, there may be some differences between the studies, which
are critical for producing and detecting positive and potentially therapeutic effects. It is clearly impossible
to prove that a treatment has no therapeutic potential, and replicating the procedures used in previous
reports does not constitute an exhaustive assessment of the therapeutic potential of this target. However,
knowledge about the reliability and robustness of the results in preclinical studies would help to more
accurately assess the therapeutic potential of novel compounds and the predictive validity of the models.
If a treatment such as 5-HT6 receptor antagonism ultimately does or does not have efficacy in the clinic,
determining the differences between the preclinical studies which produced positive and negative results may allow us to determine which tests and approaches appear to have greater predictive validity.
Acknowledgements: The authors gratefully acknowledge Lynn Balanda for performing the cell transfections, and Dr. Cen Xu for help with the pharmacology.
References


Daly DA and Moghaddam B (1993) Actions of clozapine and haloperidol on the extracellular levels of excitatory amino acids in the prefrontal cortex and striatum of conscious rats. *Neurosci.Lett.* **152**:61-64.


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Figure Legends

Fig. 1: Ro 04-6790, SB-271046, and clozapine inhibit $[^3]$H]LSD binding to HEK 293 cell membranes expressing the human, rat or mouse receptors.

Fig. 2: Yawning/stretching/chewing from 30-90 minutes after vehicle, physostigmine (0.1 mg/kg) or SB-271046 (30 mg/kg), n's=10-11.

Fig. 3: Yawning/stretching/chewing from 30-90 minutes after vehicle, physostigmine (0.1 mg/kg) or Ro 04-6790 (30 mg/kg), n's=8.

Fig. 4: Percent of time spent freezing in 7-minute conditioned fear test. SCOP = scopolamine; No-Shock Vehicle Controls = control rats not given any shock during conditioning session. [A] Ro 04-6790 (30 mg/kg) did not attenuate scopolamine-induced deficits, n's=19-20. [B] SB-271046 did not attenuate scopolamine-induced deficits at any dose (1.0, 10.0 and 30.0 mg/kg), n's=20-24.

Fig. 5: Percent of bar-presses in the autoshaping task on the test day. All drugs were administered immediately after the conditioning trial, 24 hours before the test trial. [A] Treatment groups were: 0 = vehicle (n=13), or 1.0, 5.0 or 10.0 mg/kg Ro 04-6790 (n's=21-22). [B] Ro 04-6790 (5.0 mg/kg) combined with vehicle or with scopolamine (0.17 mg/kg), scopolamine alone, or vehicle alone (n's=12).

Fig. 6: Percent of bar-presses in the autoshaping task with repeated test sessions. All drugs were administered after each test session. Scopolamine (0.17 mg/kg) significantly reduced acquisition of bar-pressing, but Ro 04-6790 (5 mg/kg) only produced a non-significant trend in the direction of reduced bar-pressing (n's=12).
Fig. 7: Swim latencies during acquisition training in the Morris water maze with vehicle-treated or Ro 04-6790 (30 mg/kg), n’s=14-15. Asterisks indicate trials where Ro 04-6790-treated rats performed significantly different from vehicle-treated controls. [A] Albino Sprague-Dawley rats. [B] Hooded Long-Evans rats.

Fig. 8: Swim duration in target annulus and equivalent annulus areas in other quadrants during probe trials with vehicle or Ro 04-6790 (30 mg/kg), n’s=14-15. Probe trials were conducted 7, 10 or 14 days after the end of acquisition training. Asterisks indicate significant differences between duration in the target annulus and each of the other quadrants, as determined by planned contrasts, p’s ≤ 0.05. [A] Albino Sprague-Dawley rats. [B] Hooded Long-Evans rats.

Fig. 9: Morris water maze testing with vehicle or SB-271046 (10 mg/kg), n’s=20. [A] Swim latencies during acquisition training. [B] Percent of time spent swimming in the target quadrant during probe trials immediately after the end of acquisition training (0), or 4, 7 or 10 days after the end of acquisition training.
Table 1: Potency of compounds binding to 5-HT6 receptors (nM).

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Human 5-HT6</th>
<th>Mouse 5-HT6</th>
<th>Rat 5-HT6</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB-271046</td>
<td>0.13</td>
<td>0.81</td>
<td>0.19</td>
</tr>
<tr>
<td>Ro 04-6790</td>
<td>30.50</td>
<td>&gt;1000</td>
<td>23.07</td>
</tr>
<tr>
<td>Clozapine</td>
<td>6.37</td>
<td>3.79</td>
<td>5.96</td>
</tr>
<tr>
<td>Methiothepin</td>
<td>0.37</td>
<td>0.19</td>
<td>0.41</td>
</tr>
<tr>
<td>Serotonin</td>
<td>135.95</td>
<td>277.79</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data is expressed as $K_i$, based on an $[^3]$H-LSD concentration of 2 nM and a $K_d$ of 1.9 nM (Boess et al. 1997). ND is not determined.
FIGURE 1

SB-271046

Ro 04-6790

Clozapine

% Specific Binding

Concentration (M)

Human ▲ Mouse ■ Rat

FIGURE 1
FIGURE 2
FIGURE 3
FIGURE 4

A.

B.

% TIME FREEZING

VEHICLE
○ SCOP (1 mg/kg)
▼ Ro 04-6790 (30 mg/kg) + SCOP
▼ No-Shock Vehicle Controls

VEHICLE
○ SCOPOLAMINE (1 mg/kg)
▼ SB-271046 (1 mg/kg) + SCOP
▼ SB-271046 (10 mg/kg) + SCOP
▼ SB-271046 (30 mg/kg) + SCOP
▼ No-Shock Vehicle Controls

MINUTES
Figure 5
FIGURE 6
A. HSD-albino rats

B. L-E hooded rats

FIGURE 7
A. Albino Sprague-Dawleys

B. Hooded Long-Evans
**A. ACQUISITION**

- **DAY**
- **SWIM LATENCIES (SEC)**

**B. RETENTION**

- **DAYS AFTER ACQUISITION**
- **% Time in Target Quadrant (sec)**

**FIGURE 9**