Effects of RGH-237 [N-{4-[4-(3-Aminocarbonyl-phenyl)-piperazin-1-yl]-butyl}-4-bromo-benzamide], an Orally Active, Selective Dopamine D₃ Receptor Partial Agonist in Animal Models of Cocaine Abuse[^]

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ABSTRACT

Dopamine D₃ receptor partial agonism has been suggested as a potential therapeutic intervention in cocaine addiction. RGH-237 [N-{4-[4-(3-aminocarbonyl-phenyl)-piperazin-1-yl]-butyl}-4-bromo-benzamide] was identified as a novel selective dopamine D₃ receptor partial agonist and used for testing this hypothesis in animal models. The compound showed nanomolar affinity to human (Kᵢ = 6.7 nM) and rat (Kᵢ = 1.6 nM) D₃ receptors with an intrinsic activity of approximately 50%. It possessed several hundredfold selectivity over the D₂ receptor. The molecule bound with moderate (100–250 nM) affinity to 5-hydroxytryptamine 1A (5-HT₁A) and nonselectively labeled opiate receptors. RGH-237 proved to be practically inactive on more than 40 other targets, including monoaminergic, cholinergic, GABAergic, and glutamatergic receptors. In rats orally administered RGH-237 was well and rapidly absorbed yielding 41% oral bioavailability. At its pharmacologically active dose (10 mg/kg p.o.), the brain concentration of RGH-237 reached 110 ng/g. Its blood and brain levels were sustained for 3 h. RGH-237 at the oral dose of 10 mg/kg moderately but significantly inhibited the acquisition of cocaine-induced place preference, although by itself, it had no place-conditioning effect. The compound did not affect fixed ratio 1 cocaine self-administration. In a reinstatement paradigm of cocaine self-administration, the compound potently and dose-dependently blocked the cue-induced cocaine-seeking behavior of rats at 10 and 30 mg/kg oral doses. RGH-237 did not affect seeking activity for natural rewards, such as sucrose and water. It did not exert notable effect on spontaneous motor activity of rats. Our results demonstrate that selective D₃ partial agonists may be an effective therapeutic means in the treatment of cocaine abuse.

In recent years, a growing body of evidence has accumulated from animal studies demonstrating the importance of dopamine D₃ receptors in mechanisms of cocaine addiction. Nonselective D₂-like dopaminergic agonists were shown to mimic the effects of cocaine in drug discrimination (Spealman, 1996) as well as in cocaine self-administration paradigms (Caine et al., 1997, Parsons et al., 1996), and the order of potency of the compounds highly correlated with their in vitro affinity and functional activity on D₃ but not on D₂ receptors (Spealman, 1996; Caine et al., 1997). In addition, nafadotride, a modestly selective D₃ receptor antagonist (Sautel et al., 1995), decreased the reinforcing effect of cocaine in the latter method (Caine et al., 1997). Furthermore, the partial D₃ agonist compound BP-897 (Pilla et al., 1999) and the highly selective D₃ full antagonist SB-277011-A (Reavill et al., 2000) were reported to decrease cue-induced drug seeking under a second-order schedule of cocaine self-administration (Pilla et al., 1999; Di Ciano et al., 2003).

More important and clinically more relevant are the findings obtained with dopamine D₃ receptor ligands in various paradigms of reinstatement of cocaine seeking, which model

**ABBREVIATIONS:** BP-897, 1-(4-(2-naphthoylamino)butyl)-4-(2-methoxyphenyl)-1A-piperazine HCl; RGH-237, N-{4-[4-(3-aminocarbonyl-phenyl)-piperazin-1-yl]-butyl}-4-bromo-benzamide; SB-277011-A, trans-N-[4-[2-(6-cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethy]cyclohexyl]-4-quinolininecarboxamide; GTPγS, guanosine 5’-3-O-(thio)triphosphate; AUC, area under curve; ANOVA, analysis of variance; FR1, fixed ratio 1; h, human; S9, Spodoptera frugiperda (pupal ovary); PD-128907, [F(−)-trans-3,4,4a,10b-tetrahydro-4-propyl-2H,5H-furan-1[1]benzopyrano[4,3-b]-1,4-oxazin-9-ol]; S33084, (3aR,9bS)-5/4-{6-cyano-1,3a,4,9b-tetrahydro-3H-benzo[3,4-c]pyrrole-2-yl}-butyl] (4-phenyl)benzamide; spec. act., special activity; 8-OH-DPAT, 8-hydroxy-2-dipropylamino-tetralin; 7-OH-DPAT, 7-hydroxy-2-dipropylaminotetralin.
the human relapse phenomenon—the core problem of cocaine addiction (Katz and Higgins, 2003). In a discriminative cue-induced reinstatement model, nafadotride (Weiss et al., 2001) and BP-897 (Cervo et al., 2003) suppressed cocaine seeking. BP-897 was also shown to attenuate reinstatement of cocaine seeking induced by contextual and stimulus-related cues (Gál and Gyertyán, 2006). In addition, a nonselective D₃ partial agonist compound, a structural analog of BP-897, was also reported to reduce the number of active lever presses induced by reintroduction of cocaine-associated stimuli after extinction (Campiani et al., 2003). SB-277011-A was found to be effective in cocaine-triggered (Vorel et al., 2002), stress-induced (Xi et al., 2004), and cue-elicited (Gál and Gyertyán, 2006) reinstatement of cocaine-seeking paradigms.

However, D₂-preferring antagonists like haloperidol (Gál and Gyertyán, 2006) and raclopride (Crombag et al., 2002; Cervo et al., 2003) also proved to be effective in reducing cocaine seeking in cue-induced reinstatement paradigms in rats, whereas the most D₃-selective antagonists nomadrona and eticlopride attenuated the cocaine priming-induced drug seeking in monkeys (Khroyan et al., 2000). Therefore, from an efficacy point of view, functional antagonism of both the dopamine D₂ and D₃ receptors may be a potentially usable mechanism for medications aiming to prevent relapse to cocaine seeking. However, well known central effects are coupled to D₂ receptor antagonism (e.g., anhedonia, extrapyramidal symptoms, and hyperprolactinemia), which may prove to be undesirable in the treatment of cocaine addicts. In contrast, the recently described highly selective D₃ compounds, such as SB-277011-A (Reavill et al., 2000) and S33084 (Millan et al., 2000), proved to be free from marked actions on behavior. Thus, drugs targeting the dopamine D₃ receptor may provide a more favorable clinical approach for the treatment of relapse to cocaine use.

It was also suggested that partial agonists may be even more favorable than full antagonists in preventing relapse to drug taking (Pulvirenti and Koob, 1994; Pilla et al., 1999; Childress and O’Brien, 2000). It is expected that a partial agonist compound acts with moderate agonism in states where natural transmitter tone is lacking or diminished, such as in the case of cocaine withdrawal, while it behaves as an antagonist under conditions of increased dopamine release occurring during cocaine intake or in response to cocaine-associated cues (e.g., the D₂ partial agonist terguride) (Pulvirenti et al., 1998). Thereby, a partial agonist reduces craving and/or drug seeking arising from either state. However, with regard to targeting the D₂ receptor, this concept has not yet been experimentally verified. Up to now, no superiority of BP-897 over SB-277011-A, for example, has been shown in cocaine abuse models. In addition, BP-897 has a non-negligible full D₂ antagonist character (Pilla et al., 1999; Wood et al., 2000), and its partial agonist nature on the D₃ receptor has also been questioned (Wood et al., 2000; Wicke and García-Ladona, 2001). Because a structural analog of BP-897, which had similar D₂/D₃ selectivity and proved to be a antagonist on the D₂ and partial agonist on the D₃ receptor, was also active in a cocaine reinstatement model while a much more D₂-selective partial agonist analog was not, Campiani et al. (2003) even concluded that some D₂ antagonist component might significantly contribute to the reduction of cocaine craving by D₃ partial agonism. However, brain penetrability of the inactive analog was not checked in their study. Obviously, further selective dopamine D₃ receptor partial agonist molecules can provide great help in investigating the relevance of the partial agonism hypothesis.

Here we report the pharmacological activity of RGH-237 (Fig. 1), a molecule selected from a series of compounds synthesized with the aim to obtain a potent and selective D₃ partial agonist ligand with good oral bioavailability and sufficient brain penetration.

To obtain a fair evaluation on the activity of RGH-237, we chose BP-897 and SB-277011-A as reference substances. Results obtained with the two latter compounds in the cocaine abuse models have already been published elsewhere (Gál and Gyertyán, 2003, 2006; Gyertyán and Gál, 2003); they are re-published here for the sake of comparison.

### Materials and Methods

#### Animals.

The experimental animals were rats (see strain and weight under the given method) housed in a thermostatically controlled room at 24 ± 2°C and at relative humidity of 50 ± 10% on a 12-h light/dark cycle (lights off from 6:00 PM to 6:00 AM). The animals were kept in polycarbonate cages (Lignifer Ltd., Isaszeg, Hungary) in groups of four, with the exception of those rats that underwent surgery for cocaine self-administration; they were housed individually. The rats received unlimited access to commercial pellet rat-mouse feed (sniff R/M+H; Spezialdiäten GmbH, Soest, Germany) autoclaved at 105°C and tap water throughout all experiments, except in the pretraining periods of the self-administration procedures when they were on a 23-h water-deprivation schedule.

Animal maintenance and research were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All the procedures carried out on animals had been approved by the local ethical committee and conformed to the rules and principles of the 86/609/EEC Directive.

#### Drugs.

RGH-237, SB-277011-A, and BP-897 were synthesized at Gedeon Richter Plc. (Budapest, Hungary). Cocaine was purchased from Sigma-Aldrich (St. Louis, MO). Radioligands [³H]spiperone (spec. act. 15–16 Ci/mmol), [³H]8-OH-DPAT (spec. act. 106–170 Ci/mmol), and [³H]haloxone (spec. act. 63 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences Inc. (Wellesley, MA); [7-methoxy-³H]prazosin (spec. act. 88 Ci/mmol) and [³²P]GTPγS (spec. act. 1000–1150 Ci/mmol) were purchased from Amersham Radiochemicals (Little Chalfont, Buckinghamshire, UK). In the in vivo studies, RGH-237, SB-277011-A, and BP-897 were suspended in 5% Tween 80, and cocaine was dissolved in saline.

#### Receptor Binding.

Assay conditions for the individual receptors are given in Table 1. Membrane preparations for the assays were made according to the references given in the table. Incubations were performed in triplicate for each concentration in the presence of [³H]RGH-237, SB-277011-A, and BP-897, which had similar D₂/D₃ selectivity and proved to be a antagonist on the D₂ and partial agonist on the D₃ receptor, was also active in a cocaine reinstatement model while a much more D₂-selective partial agonist analog was not, Campiani et al. (2003) even concluded that some D₂ antagonist component might significantly contribute to the reduction of cocaine craving by D₃ partial agonism. However, brain penetrability of the inactive analog was not checked in their study. Obviously, further selective dopamine D₃ receptor partial agonist molecules can provide great help in investigating the relevance of the partial agonism hypothesis.

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**Receptor Binding.** Assay conditions for the individual receptors are given in Table 1. Membrane preparations for the assays were made according to the references given in the table. Incubations were stopped by rapid filtration over glass fiber filters (cell harvester; Brandel, Inc., Gaithersburg, MD), filters were rapidly washed by ice-cold buffer, and radioactivity retained on the filters was determined by liquid scintillation spectrometry.

In addition to the receptor binding assays shown in Table 1, RGH-237 was tested on an additional 45 neurotransmitter receptor-
TABLE 1
Summary of binding assays conditions

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Species</th>
<th>Cell/Tissue (Amount/Assay)</th>
<th>Incubation Buffer</th>
<th>Incubation Time; Temperature; pH</th>
<th>Radioligand</th>
<th>Nonspecific Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3</td>
<td>Rat</td>
<td>SB9 (10 μg)</td>
<td>50 Tris-HCl, 5 M MgCl₂, 1 M NaCl, 0.1% ascorbic acid</td>
<td>30 min, 23°C, 7.4</td>
<td>[3H]Spiperone (0.4)</td>
<td>Haloperidol (10)</td>
</tr>
<tr>
<td>D2</td>
<td>Rat</td>
<td>CHO (10 μg)</td>
<td>50 Tris-HCl, 5 M MgCl₂, 1 M NaCl, 0.1% ascorbic acid</td>
<td>15 min, 23°C, 7.4</td>
<td>[3H]Spiperone (0.4)</td>
<td>Haloperidol (10)</td>
</tr>
<tr>
<td>D1</td>
<td>Rat</td>
<td>Striatum (1.25 mg)</td>
<td>120 mM NaCl, 5 mM Tris-HCl</td>
<td>30 min, 37°C, 7.4</td>
<td>[3H]Spiperone (0.4)</td>
<td>Haloperidol (10)</td>
</tr>
<tr>
<td>D2</td>
<td>Rat</td>
<td>Rat Brain (25 μg)</td>
<td>50 Tris-HCl, 5 M MgCl₂, 1 M NaCl, 0.1% ascorbic acid</td>
<td>60 min, 37°C, 7.4</td>
<td>[3H]Spiperone (0.4)</td>
<td>Haloperidol (10)</td>
</tr>
<tr>
<td>r5-HT1A</td>
<td>Rat</td>
<td>Hypoglossus (0.45 mg)</td>
<td>50 Tris-HCl, 5 M MgCl₂, 1 M NaCl, 0.1% ascorbic acid</td>
<td>15 min, 23°C, 7.4</td>
<td>[3H]Spiperone (0.4)</td>
<td>Haloperidol (10)</td>
</tr>
</tbody>
</table>

Incubation Buffer:
- 50 Tris-HCl, 5 M MgCl₂, 1 M NaCl, 0.1% ascorbic acid
- 50 Tris-HCl, 5 M MgCl₂, 1 M NaCl
- 50 Tris-HCl, 5 M MgCl₂
- 50 Tris-HCl
- 50 Tris-HCl

Reference:
- Haloperidol (10)
- Spiperone (0.4)
- Prazosin (0.5)
- Seeman (1983)
- Hayes et al. (1992)
- Greengrass and Bromer (1979)
- May et al. (2003)
- Prusoff (1973)

Calculation of inhibitor constants (made by Origin 6.0 (MicroCal Software Inc., Northampton, MA). For in vitro experiments in our own laboratory, nonlinear regression analysis was performed by Origin 6.0 (MicroCal Software Inc., Northampton, MA). For determination of IC₅₀ values, nonlinear least-squares regression analysis (Data Analysis Toolbox; MDL Information Systems, San Leandro, CA) was applied by MDS Pharma. For IC₅₀ determinations in binding assays and evaluation of functional activity carried out in our own laboratory, nonlinear regression analysis was made by Origin 6.0 (MicroCal Software Inc., Northampton, MA). For calculation of inhibitory constants (Ki), the equation of Cheng and Prusoff (1973) was used.

In Vivo Pharmacokinetics in Rats. Pilot pharmacokinetic investigations were performed in male Wistar rats (180–220 g) following single intravenous, oral, and subcutaneous administration of RGH-237 or reference compounds (BP-897 and SB-27011-A). The compounds were dosed in 0.4% acetic acid solution containing 5.5% glucose. The applied doses of RGH-237 and the reference compounds were 5 mg/kg for i.v. and 10 mg/kg for oral and s.c. treatment. BP-897 was administered at the dose of 10 mg/kg by all three routes of administration.

Plasma and brain concentrations of RGH-237 and the reference compounds were determined by high-performance liquid chromatography–UV method. The biological samples were extracted with Cl-butane and analyzed on Supelco Discovery C18 column (150 × 4.6 mm, 5 μ; Supelco, Bellefonte, PA) using a gradient elution. The proportion of acetone/methanol (2:1) in 0.2 M aqueous ammonium acetate increased from 40 to 70% over 15 min at a flow rate of 1 ml/min.

Place Conditioning and Effect on the Acquisition of Cocaine-Induced Conditioned Place Preference. The method is described in detail in Gyertyán and Gal (2003). Male Sprague-Dawley rats obtained from LATI (Gödöllő, Hungary) weighing 200 to 220 g were used in the experiments. The place-conditioning box consisted of two end compartments of different colors and floor textures (for the sake of simplicity, named hereon as black compartment and white compartment) and a narrow middle transition zone separated by removable partitions. During the preconditioning phase, baseline place preference of the rats was determined in three daily sessions when animals were allowed to move freely in all of the three compartments of the place-conditioning box for 15 min. Time
spent by the rat in each compartment of the place-conditioning box was measured. Data from the three preconditioning sessions were averaged for each animal and used as a measure of its baseline place preference. During the next 4 days (conditioning phase), alternate sessions of saline and drug pairing took place in the morning and in the afternoon; animals received saline or drug injections and, 30 min later, were confined in the black (saline treatment) or white compartment (drug treatment) for 30 min. On the last day (test session), untreated rats were allowed free access to both sides of the box for 15 min. Time spent by the rat in each compartment was measured again.

Individual differences between test session and baseline values were calculated for the drug-paired side with positive or negative differences reflecting place preference or place aversion, respectively. Mean ± S.E.M. of individual differences was calculated, and Student’s t test for dependent samples was performed for determining statistical significance.

Cocaine-induced place preference was carried out as described above with modification in which, in the conditioning phase, animals were confined to the appropriate compartment immediately after cocaine (10 mg/kg i.p.) or saline administration. When effects of compounds on acquisition of cocaine-induced place preference were studied following the preconditioning phase on the basis of baseline data, animals were divided in a balanced way into two groups. Both groups underwent place conditioning with cocaine; one of them received drug treatment before each cocaine injection (DRUG+ group), whereas the other was given saline (COG group). Both groups were treated with saline before saline pairings. The pretreatment times of the compounds were the same as in the place-conditioning experiments. On the last day (test phase), each rat was placed uninjected into the box and allowed free access to both sides of the box for 15 min.

Data from the COG group were evaluated as described above to verify the existence of cocaine-induced place preference. Only in the case of a statistically significant cocaine effect was further analysis carried out. Times spent on the cocaine-paired side in the postconditioning test by the COG group and the COG+DRUG group were then compared. Group means were calculated for both groups, and Student’s t test for independent samples was used for statistical evaluation.

Cocaine Self-Administration Model. The experiments were performed as described in Gál and Gyertyán (2003). In brief, male Long-Evans rats (Toxicoop Ltd., Budapest, Hungary) weighing between 220 and 250 g upon arrival were used. Training and testing took place in computer-controlled operant chambers (Coulbourn Instruments, Allentown, PA) equipped with two levers and a house light. First, animals were trained to lever press on the “active” lever for water drops in the operant chamber. Responding on the other, “inactive lever” was registered but was without consequences. Training consisted of a daily 30-min magazine training session on FR1 schedule until all of the animals had learned the task.

After acquiring the operant behavior, all animals were implanted with a catheter into the right jugular vein under chloral hydrate (400 mg/kg) anesthesia. Self-administration sessions began on the 5th to 7th day after the surgery. Acquisition of cocaine self-administration (0.25 mg of cocaine/infusion) was established on a FR1 schedule of reinforcement in daily sessions lasting 2 h. When the self-administration behavior became stable (defined as no more than 15% variation in the number of self-infusions during 3 consecutive days), rats were challenged with the dopamine D3 receptor ligands. Animals received the drugs 30 min before the self-administration session by the same route as in the place-conditioning studies. Animals could be reused for drug testing when their performance again met the stability criterion.

The individual drug infusions on the 3 consecutive pretest days were averaged and taken as pretest baseline performance, and drug effect (number of drug infusions on the test day) was expressed as percentage changes compared with the baseline. Group means were then calculated from these individual percentage data. For statistical evaluation, paired t test was used. Group size was five to seven.

Cue-Induced Reinstatement of Cocaine Seeking. The relapse model was carried out as described in Gál and Gyertyán (2006). In brief, the method required five phases. The first three phases [1, learning the operant task (FR1); 2, surgery and recovery; 3, establishing stable cocaine self-administration] were the same as in the cocaine-self administration model. Phase 4 was the period of abstinence. After a minimum of 10 days of cocaine self-administration, the sessions were suspended for 3 weeks. Animals went through the abstinence period in another room, which was free from cocaine and any environmental or technical cues that could be associated with drug intake. Phase 5 was considered reinstatement. When the abstinence period expired, animals were reintroduced to a 30-min long extinction session where all the environmental and reinforcer-contingent cues were the same as in the acquisition phase, with the exception that the lever presses were not reinforced with cocaine infusions. Animals were assigned to separate treatment groups and, 30 min before the reinstatement session, received saline p.o. or various doses of the tested D3 ligands.

All drug-treated groups consisted of eight rats (saline-treated group, n = 14) matched for their stable cocaine intake during training. Mean ± S.E.M. of number oflever pressings on the reinforced lever during the test session was calculated in each treatment group. Data were analyzed by one-way ANOVA with treatment groups as the between group factor. Whenever a significant effect was found, post hoc comparison was done using Duncan’s multiple range test.

Cue-Induced Reinstatement of Sucrose Seeking. The experimental procedure mirrored the previous one in every respect with the exception of the surgery. Animals “self-administered” 10% sucrose drops orally under FR1 schedule in the operant boxes. All the environmental cues were the same as in the cocaine self-administration model, with the exception that the infusion harness and pump were absent and the rewards were given through a liquid delivery system. After the stable sucrose administration behavior had been established, rats went through a 3-week long abstinence period from sucrose. In the reinstatement phase when drug effects were tested, all the environmental cues were the same, except that lever pressing was not reinforced with sucrose. Animals were assigned to separate treatment groups and, 30 min before the reinstatement session, received saline p.o. or various doses of the tested D3 ligands.

All drug-treated groups consisted of eight rats (in the pooled saline-treated group, n = 35) matched for their stable cocaine intake during training. Mean ± S.E.M. of number oflever pressings on the reinforced lever during the test session was calculated in each treatment group. Data were analyzed by one-way ANOVA with treatment groups as the between group factor. Whenever a significant effect was found, post hoc comparison was done using Duncan’s multiple range test.

Cue-Induced Reinstatement of Water Seeking. In the experiment with water reward, water-deprived animals learned to self-administer water drops under FR1 schedule during the 30-min session as it was detailed in description of reinstatement phase above. After stable responding had been established, animals had unlimited access to water for 24 h in their home cages. They then were reintroduced the operant chambers (test session) and allowed to press the previously active lever; however, responding was not reinforced, but the empty liquid dipper was lifted up.

Thirty minutes before the test session, animals were assigned to separate treatment groups and received saline p.o. or various doses of the test compounds. Group size was seven to eight in the drug-treated groups and 35 in the saline-treated (pooled) control group. Mean ± S.E.M. of number oflever pressings on the reinforced lever during the test session was calculated in each treatment group. Data were analyzed by one-way ANOVA with treatment groups as the between group factor. Whenever a significant effect was found, post hoc comparison was done using Duncan’s multiple range test.
Effect on Spontaneous Motor Activity. Male Wistar rats weighing 160 to 180 g were used in the experiments. Spontaneous locomotor activity was measured in an activity monitor working with infrared photobeams, which detected both horizontal and vertical (rearing) activity. Thirty minutes after the oral administration of RGH-237, SB-277011-A, or vehicle, animals were individually placed in the experimental cages, and horizontal and vertical movements were recorded for 30 min.

Means ± S.E.M. of horizontal and vertical activity counts were calculated in each treatment group. Statistical significance between group means was determined by ANOVA followed by post hoc Duncan test. The percentage inhibition of horizontal or vertical activity was also calculated for each dose.

Results

Receptor Binding Profile of RGH-237. The compound RGH-237 showed nanomolar affinity to rat dopamine D₃ receptors with more than 1000-fold selectivity over the D₂ receptor (Table 2). It showed negligible activity on α₁-adrenergic receptors. SB-277011-A exhibited similar profile, whereas BP-897—although it had stronger binding to rat D₃ receptor than the other two molecules—showed weaker selectivity over the dopamine D₂ and especially over the α₁ receptor. RGH-237 bound with moderate affinity to nonselectively labeled populations of rat opiate receptors and weakly to rat 5-HT₁A receptors (Table 2).

Among human targets RGH-237 showed nanomolar affinity to cloned human D₃ dopamine receptors (Ki = 6.7 nM), whereas it was found to be inactive on human D₂ receptors (IC₅₀ >1000 nM). Besides its D₃ receptor activity, the compound possessed weak to moderate affinity to human 5-HT₁A receptors (Ki = 136 nM).

In addition, RGH-237 was examined on additional 45 neurotransmitter receptor-, ion channel-, neurotransmitter transporter-, and enzyme-binding sites (MDS Pharma Service; study no. PT 1023692; data on file at Gedeon Richter Plc.), and at a concentration of 1 μM, it produced less than 60% displacement at guinea pig H₁ receptors (58%), human serotonin transporter (57%), human α₁ receptors (50%), and human serotonin 5-HT₂ and 5-HT₂B (45–45%) receptors and less than 30% displacement at the remaining binding sites. The complete list of binding sites can be found in Supplemental Table 1.

Functional Activity of RGH-237 on Dopamine D₂ and D₃ Receptors. The results of a typical [³⁵S]GTPγS binding experiment with RGH-237 and the natural agonist dopamine are demonstrated in Fig. 2. In Table 3, data obtained with BP-897 and SB-277011-A are summarized. The data indicate that RGH-237, in comparison with dopamine, behaved as a partial agonist at both the D₂ and the D₃ receptors. Its intrinsic activity (Eₘₐₓ) was found to be half of that of dopamine (0.54 and 0.52 for D₂ and D₃ receptors, respectively). RGH-237 showed 40-fold selectivity toward D₃ receptors.

As a partial agonist, RGH-237 was able to inhibit the dopamine-induced [³⁵S]GTPγS binding but only up to the level of its own intrinsic activity (Fig. 3). Its IC₅₀ value was found to be 28 nM (Table 3).

In our test system, BP-897 proved to be pure antagonist on both the D₂ and the D₃ receptor with 10-fold selectivity in favor of the latter (Table 3). The compound SB-277011-A was found to be a highly selective (~120-fold) full D₃ receptor antagonist.

In Vivo Pharmacokinetics of RGH-237 in Rats. Following i.v. administration of RGH-237 to rats, the compound exhibited moderate plasma clearance of approximately 7.0 ml/min/kg. The volume of distribution of 0.9 l/kg did not indicate extensive tissue binding of the compound. In accordance with these parameters, the plasma concentrations were very high, even at the last investigated time point of 5 h. Elimination of RGH-237 was characterized with apparent
Pharmacokinetic parameters of RGH-237, BP-897, and SB-277011-A in the in vitro [35S]GTPγS binding assay

| Compound   | Dose (mg/kg) | t1/2, plasma (h) | C_max, plasma (ng/ml) | C_max, brain (ng/g) | T_max, plasma (h) | Bioavailability (%) | Brain/Plasma Ratio
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<tbody>
<tr>
<td>RGH-237</td>
<td>10 (p.o.)</td>
<td>2.0 ± 0.7</td>
<td>2876 ± 371</td>
<td>110</td>
<td>0.8 ± 0.4</td>
<td>41</td>
<td>0.047</td>
</tr>
<tr>
<td>RGH-237</td>
<td>10 (s.c.)</td>
<td>1.8 ± 0.5</td>
<td>4397 ± 2041</td>
<td>n.d.</td>
<td>1.0 ± 0</td>
<td>51</td>
<td>n.d.</td>
</tr>
<tr>
<td>BP-897</td>
<td>10 (p.o.)</td>
<td>n.c.</td>
<td>29.4 ± 10.6</td>
<td>n.d.</td>
<td>0.55 ± 0.41</td>
<td>n.c.</td>
<td>n.c.</td>
</tr>
<tr>
<td>BP-897</td>
<td>10 (s.c.)</td>
<td>1.3</td>
<td>344 ± 39.5</td>
<td>0.25 ± 0</td>
<td>36</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>SB-277011-A</td>
<td>10 (p.o.)</td>
<td>1.9 ± 0.4</td>
<td>2166 ± 197</td>
<td>n.d.</td>
<td>1.0 ± 0</td>
<td>63</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.c., not calculated; n.d., not determined.

a Area under the curve in brain/area under the curve in plasma.

Pharmacokinetic parameters of RGH-237, BP-897, and SB-277011-A

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose</th>
<th>t1/2, plasma (h)</th>
<th>C_max, plasma (ng/ml)</th>
<th>C_max, brain (ng/g)</th>
<th>T_max, plasma (h)</th>
<th>Bioavailability (%)</th>
<th>Brain/Plasma Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGH-237</td>
<td>10</td>
<td>2.0 ± 0.7</td>
<td>2876 ± 371</td>
<td>110</td>
<td>0.8 ± 0.4</td>
<td>41</td>
<td>0.047</td>
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a Area under the curve in brain/area under the curve in plasma.
tation of the 10 mg/kg and the 30-mg/kg doses of RGH-237 significantly and dose-dependently inhibited the secondary cues-induced cocaine seeking compared with the control group. BP-897 at the dose of 1 mg/kg also significantly attenuated the number of lever presses, although there was no significant effect of the lower dose of this compound. Both doses of SB-277011-A produced significant inhibition in the drug-seeking behavior but without dose dependence (Fig. 7).

Cue-Induced Reinstatement of Sucrose Seeking. After 3 weeks of abstinence, secondary cues paired with previous sucrose-taking behavior were also able to elicit reward seeking in the reinstatement session but to a lesser extent than cocaine (44.9 ± 4.1 responses/session, Fig. 8). However, in contrast to their effects in the cocaine reinstatement model, neither RG-237 nor BP-897 and SB-277011-A significantly influenced this behavior [F(5,68) = 1.10, p = 0.422] (Fig. 8).

Cue-Induced Reinstatement of Water Seeking. In spite of the fact that the rats were not water-deprived during the test session, animals were actively engaged in pressing the previously reinforced lever (47.4 ± 5.0 presses/session) (Fig. 9).

Similar to their effects on sucrose seeking behavior, none of the tested compounds could significantly influence the lever presses induced by secondary cues associated with previous water self-administration [F(5,68) = 0.840, p = 0.526] (Fig. 9). Only the 1-mg/kg dose of BP-897 caused a moderate (44%) but nonsignificant inhibition.

Effect on Spontaneous Motor Activity. RGH-237 did not affect the locomotor activity of rats up to the dose of 30 mg/kg (Table 5, F(3,56) = 0.817). At 10 mg/kg p.o., it produced a moderate, nonsignificant reduction in vertical movements [F(3,56) = 0.158]. SB-277011-A did not affect any component of motor activity of rats in the dose range of 13.5 to 30 mg/kg p.o. [F(3,36) = 0.955 for horizontal and F(3,36) = 0.375 for vertical activity].
Discussion

The results demonstrate that RGH-237 is an orally active, selective dopamine D₃ receptor partial agonist that can effectively inhibit the cue-induced reinstatement of cocaine seeking in a relapse model. The action of the compound is cocaine-specific.

According to the receptor binding data, RGH-237 has high affinity and selectivity for either human or rat D₃ receptors. In rat, the compound showed excellent (>1000-fold) selectivity over the D₂ and adrenergic α₁ receptors and still considerable selectivity over opiate (80-fold) and 5-HT₁A serotonergic (150-fold) receptors. The human receptor profile of RGH-237 was similar to that in the rat but with proportionally less selectivity ratios (D₃/D₂ > 150, D₃/5-HT₁A = 20). No appreciable affinity (IC₅₀ > 1000 nM) was noted at 45 other receptors, ion channels, and enzymes.

With respect to selectivity over dopaminergic and adren-
ing acidification rate as endpoint. However, BP-897, described as a partial agonist at D₃ receptors with 53% intrinsic activity (Pilla et al., 1999), behaved as a high-affinity antagonist in our experiments.

A possible explanation for the discrepancy may be the use of a different endpoint (thymidine incorporation versus GTP·S binding in our laboratory). However, three other studies also showed the compound to be a full D₃ receptor antagonist measured either by extracellular acidification rate (Wood et al., 2000) or by GTP·S binding (Cussac et al., 2000; Wicke and Garcia-Ladona, 2001). The latter authors even demonstrated the antagonist character of BP-897 in vivo, namely on quipirilo-induced inhibition of firing rate of dopaminergic neurons in the substantia nigra (Wicke and Garcia-Ladona, 2001). On the other hand, BP-897 was unequivocally shown to be a full antagonist on the D₃ receptor (Pilla et al., 1999; Cussac et al., 2000; Wood et al., 2000) similarly to our own results.

The diverse findings with BP-897 may reflect the phenomenon of “functional selectivity” (Urban et al., 2007); as it has similarly to our own results.

Taking all the above data into account, we consider RGH-237 as a selective D₃ partial agonist but SB-277011-A as a selective D₃ full antagonist compound. BP-897 probably behaves as a D₃-preferring D₃/D₂ dual antagonist in native conditions, although its partial agonist-like action cannot be excluded on certain cell types and/or in certain physiological states.

In rats, RGH-237 was absorbed well and rapidly, not only after parenteral but also after oral administration. Its blood level was sustained up to 3 h. In contrast, BP-897 practically proved to be an orally nonabsorbable compound. However, following subcutaneous injection, the two compounds yielded similar bioavailability values. SB-277011-A showed excellent oral absorption in accordance with the results of Reavill et al. (2000).

In contrast to its good intestinal penetration, cerebral penetration of RGH-237 was rather poor. The resulting low brain concentration explains the relatively low effective doses of RGH-237 in the cocaine models despite its low Kᵢ value on the D₃ receptor.

BP-897 showed much better brain penetration than RGH-237, yielding a brain/plasma concentration ratio of five. The high brain level not only justifies the relatively low effective dose of BP-897 in the cocaine models but also raises the possibility that the observed effects of the compound may have involved the dopamine D₂ receptor as well. From the concentration measured at a 10-mg/kg s.c. dose, a brain concentration of 510 nM can roughly be estimated at the 1-mg/kg s.c. dose of the compound. This is more than 10-fold higher than its Kᵢ value (33 nM) on the D₂ receptor. Even if we take into consideration the practical and theoretical limits of such calculations, it seems likely that not only D₃ but also D₂ receptor-mediated actions may underlie the observed in vivo effects of BP-897.

In contrast, in the case of RGH-237, the pharmacokinetic measurements suggest that the compound penetrates into the brain at concentrations providing sufficient occupancy of the D₃ receptors while maintaining selectivity over D₂ receptors. Therefore, RGH-237 is especially suitable for testing the effect of D₃ partial agonism in various models of cocaine use.

RGH-237, like SB-277011-A, did not produce significant place-conditioning effects, whereas BP-897 at its higher dose induced moderate but significant place aversion. Because the receptor profile of BP-897 is not very clean, it is difficult to tell what mechanism(s) may lie behind its effect. Because D₃ agonist compounds, such as PD-128907 and 7-OH-DPAT, were shown to cause significant place aversion (Gyertyán and Gál, 2003), a possibility may be that, in this peculiar paradigm, BP-897 behaves as a D₃ partial agonist. However, RGH-237, which is undoubtedly a partial agonist, showed only a slight tendency to induce place aversion. Another receptor candidate is the adrenergic α₁ receptor to which BP-897 showed affinity comparable with that of D₃ with pure antagonist functional activity (Cussac et al., 2000). However, the selective α₁ antagonist prazosin was shown to be inactive in the place-conditioning paradigm (Tzschenke, 1998; Sahraei et al., 2004). BP-897 has still considerable affinity to 5-HT₁A and α₂ receptors, being partial agonist on the former and antagonist on the latter receptor (Cussac et al., 2000). However, 5-HT₁A agonists and partial agonists caused place preference (Tzschenke, 1998), whereas there are conflicting data with α₂ agonists; idazoxan induced place preference, whereas yohimbine was shown to produce place aversion or no effect (Tzschenke, 1998; Morales et al., 2001; Sahraei et al., 2004). Finally, even the D₃ antagonist character of BP-897 does not give a clue because the highly potent D₃ antagonist haloperidol reproducibly showed inefficacy in place conditioning (Tzschenke, 1998).

In the cocaine-induced place-preference model where the compounds were administered in the acquisition phase of conditioning, both doses of RGH-237 produced small inhibition, which proved to be statistically significant at the lower dose. SB-277011-A and BP-897 did not induce significant changes in cocaine-induced place preference, although the lower dose of BP-897 nearly significantly increased the action of cocaine. However, its effect size, just as that of RGH-237, was quite small. Because of the low magnitude and the lack of dose dependence, we do not assign biological significance to either of these effects. According both to the literature (for review see Tzschenke, 1998) and our other results (Gyertyán and Gál, 2003), neither the various D₃ antagonists nor D₃ agonists could notably influence the cocaine-induced place preference. It seems that RGH-237 does not differ from them.

RGH-237, like SB-277011-A, did not influence the FR1 cocaine self-administration behavior. In contrast, the 1-mg/kg dose of BP-897 slightly but significantly increased the number of infusions, which means that it inhibited the rewarding effect of cocaine. Although for SB-277011-A, our results are in accordance with findings published in the literature (Di Ciano et al., 2003), it is not the case with BP-897. Pilla et al. (1999) reported BP-897 to be ineffective on cocaine self-administration under continuous reinforcement at the dose of 1 mg/kg i.p. and below. Because the effect of BP-897 was not very robust in our study, the discrepancy between the results obtained in the two laboratories may well be due to some methodological differences. Given that both the full an-
tagonist SB-277011-A and the partial agonist RGH-237 were ineffective in the same paradigm, it seems unlikely that the effect of BP-897 on cocaine self-administration was mediated via the D3 receptor. On the other hand, because haloperidol and other D2 antagonists do increase cocaine self-administration under fixed ratio schedule (Corigall and Coen, 1991; Caine et al., 2002; Gál and Gyertyán, 2003), an action of this nature may provide a more plausible explanation for the observed effect of BP-897. However, because this compound was shown to have similar or higher affinity to α1, α2, and 5-HT1A receptors than to the D2 receptor, these actions also cannot be excluded. Findings in the cocaine place preference and cocaine self-administration models suggest that RGH-237, like SB-277011-A, does not exert a direct blocking action on the interoceptive cue/rewarding property of cocaine.

RGH-237 produced dose-dependent and robust inhibition in the secondary cue-induced reinstatement of cocaine self-administration. The compound (30 mg/kg) decreased the drug seeking to 17% of the control. BP-897 also dose-dependently reduced cocaine seeking but with weaker efficacy. SB-277011-A proved to be active as well, exerting 70% inhibition at its lower dose.

Our results with SB-277011-A add up to the growing list of findings with this molecule demonstrating the efficacy of selective D3 antagonism in various forms of cocaine-seeking reinstatement models: cocaine-triggered (Vorel et al., 2002), cue-induced (Cervo et al., 2006), and stress-induced (Xi et al., 2004) reinstatement. But what about the idea of D3 partial agonism? Although BP-897 was also shown to prevent cue-induced reinstatement of cocaine seeking (Cervo et al., 2003, Gál and Gyertyán, 2006), results obtained with this compound do not provide convincing evidence for the use of D3 partial agonism, because (as we pointed out above) both its partial agonist nature and its in vivo D3 selectivity are questionable. The D3 antagonist as well as the α1 antagonist character of BP-897 may also have played role in its reinstatement inhibitory action, because both types of antagonists were shown to decrease reinstatement of cocaine seeking (Zhang and Kosten, 2005; Gál and Gyertyán, 2006). Moreover, because a much more D3-selective structural analog of BP-897, which proved to act as a partial agonist (measured by GTP\(\gamma\)S binding), was not able to inhibit cue-induced reinstatement of cocaine seeking (however, brain levels of the compound were not checked), it has been questioned whether D3 partial agonism alone could be an effective mechanism for preventing reinstatement of cocaine seeking (Campiani et al., 2003).

Within this context, it is of great importance that RGH-237, a selective D3 partial agonist, was shown to be highly effective in this model, thereby providing supporting evidence for the applicability of partial D3 agonism in preventing reinstatement of cocaine seeking.

To check the cocaine specificity of the reinstatement inhibitory actions of RGH-237, we studied the effect of the compound on secondary cue-induced natural reward seeking. For reinforcement, we used sucrose as natural reward with hedonic property and water as natural reward with homeostatic property. RGH-237, similarly to SB-277011-A and BP-897, did not influence either type of reward-seeking behavior. These results suggest that, in the reward-seeking models, the effect of RGH-237, similar to those of the other two D3 compounds, is cocaine-specific.

RGH-237, like SB-277011-A, did not exert notable effect on spontaneous motor activity in rats (BP-897 was not tested in this method). Selective D3 antagonists including SB-277011-A were shown to have negligible effect on motor activity in rats (Millan et al., 2000; Reavill et al., 2000; Gyertyán and Sághy, 2004). According to our results with RGH-237, this observation can be extended to D3 partial agonists as well. Furthermore, these results further confirm that the decrease in response rate caused by the compounds in the cocaine reinstatement paradigm did not result from a nonspecific sedative action.

Our results demonstrate that not only dopamine D3 full agonists but selective D3 partial agonists could be effective in preventing the cue-induced relapse in the clinic and may have therapeutic potential for the treatment of cocaine abuse. However, the assumed advantage of partial agonists would manifest itself in the stage of cocaine withdrawal when the dopaminergic tone is low. Further comparative experiments are needed to show this benefit.

In conclusion, RGH-237 is a real partial agonist on dopamine D3 receptors with an intrinsic activity of approximately 50% and with greater than 1000-fold selectivity over the D2 receptor. Orally administered the compound penetrates the brain at sufficient concentration. Accordingly, it proved to be highly effective in a cue-induced reinstatement of cocaine-seeking model. RGH-237 may be considered as a useful pharmacological tool for studying the consequences of partially stimulating the dopamine D3 receptor.

References
Cheng YC and Prusoff WH (1973) Relationship between the inhibition constant (K\textsubscript{i}) and the concentration of inhibitor which causes 50 percent inhibition (IC\textsubscript{50}) of an enzymatic reaction. Biochem Pharmacol 20:2999–3108.


