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RESEARCH PAPER

F15063, a potential antipsychotic with D_2/D_3 antagonist, 5-HT_{1A} agonist and D_4 partial agonist properties: (I) in vitro receptor affinity and efficacy profile

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Background and purpose: Combining 5-HT_{1A} receptor activation with dopamine D_2/D_3 receptor blockade should improve negative symptoms and cognitive deficits in schizophrenia. We describe the *in vitro* profile of F15063 (N-[(2,2-dimethyl-2,3-dihydro-benzofuran-7-yloxy)ethyl]-3-(cyclopent-1-enyl)-benzylamine).

Experimental approach: F15063 was characterised in tests of binding affinity and in cellular models of signal transduction at monoamine receptors.

Key results: Affinities (receptor and pK_i values) of F15063 were: rD_2 9.38; hD_{2L} 9.44; hD_{2S} 9.25; hD_3 8.95; hD_4 8.81; h5-HT_{1A} 8.37. F15063 had little affinity (40-fold lower than D_2) at other targets. F15063 antagonised dopamine-activated G-protein activation at hD_2, rD_2 and hD_3 receptors with potency (pK_b values 9.19, 8.29 and 8.74 in [^{35}S]GTPγS binding experiments) similar to haloperidol. F15063 did not exhibit any hD_2 receptor agonism, even in tests of ERK1/2 phosphorylation and G-protein activation in cells with high receptor expression. In contrast, like ($\pm$)8-OH-DPAT, F15063 efficaciously activated h5-HT_{1A} (E_{max} 70%, pEC_{50} 7.57) and r5-HT_{1A} receptors (52%, 7.95) in tests of [^{35}S]GTPγS binding, cAMP accumulation (90%, 7.12) and ERK1/2 phosphorylation (93%, 7.13). F15063 acted as a partial agonist for [^{35}S]GTPγS binding at hD_4 (29%, 8.15) and h5-HT_{1D} receptors (35%, 7.68). In [^{35}S]GTPγS autoradiography, F15063 activated G-proteins in hippocampus, cortex and septum (regions enriched in 5-HT_{1A} receptors), but antagonised quinelorane-induced activation of D_2/D_3 receptors in striatum.

Conclusions and implications: F15063 antagonised dopamine D_2/D_3 receptors, a property underlying its antipsychotic-like activity, whereas activation of 5-HT_{1A} and D_4 receptors mediated its actions in models of negative symptoms and cognitive deficits of schizophrenia (see companion papers).

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Keywords: antipsychotic; dopamine D_2; serotonin 5-HT_{1A}; dopamine D_4; G-protein; ERK1/2 phosphorylation; autoradiography

Abbreviations: C6, rat glioma cells; CHO, Chinese hamster ovary cells; COS7, African green monkey kidney cells; DMEM, Dulbecco's modified Eagle's medium; EPS, extrapyramidal symptoms; ERK1/2, extracellular signal-regulated kinase; F15063, *N*-[(2,2-dimethyl-2,3-dihydro-benzofuran-7-yloxy)ethyl]-3-(cyclopent-1-enyl)-benzylamine; GTPγS, guanosine 5′-*O*-(gamma-thiotriphosphate); 5-HT, 5-hydroxytryptamine, serotonin; HEK293, human embryonic kidney cells; HeLa, human carcinoma cells; NMDA, *N*-methyl-D-aspartate; Sf9, *Spodoptera frugiperda* insect cells

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Introduction

The clinical treatment of schizophrenia is based on the use of antipsychotic agents, all of which interact at dopamine D_2 receptors (Leysen, 2000). First-generation 'conventional' antipsychotics like haloperidol are effective in controlling

positive symptoms of schizophrenia, such as hallucinations, delusions and psychomotor agitation. However, they are essentially ineffective against negative symptoms, including social interaction deficits, disorganized speech and blunted affect. They also exhibit marked propensity for induction of a group of 'Parkinson-like' neuromuscular disturbances known as the extrapyramidal syndrome (EPS). Further, these drugs do not alleviate a variety of cognitive symptoms, such as working and reference memory deficits, executive function impairments and decreased vigilance (Meltzer *et al*., 1999; Silver *et al*., 2003). More recent 'atypical' antipsychotic agents, such as clozapine, risperidone, olanzapine and ziprasidone, interact at other receptors such as 5-hydroxytryptamine (5-HT) 5-$HT_{2A/2C}$ receptors, in addition to dopamine receptors. Thus, combined D_2 and 5-$HT_{2A/2C}$ antagonism is associated with lowered EPS liability and improved capacity to alleviate (but not abolish) negative and cognitive symptoms (Davis *et al*., 2003; Leucht *et al*., 2003; Meltzer *et al*., 2003).

However, many schizophrenic patients fail to respond adequately to existing medications, and exhibit continuing impairments in both social functioning and cognitive performance, as well as persistent and/or recurrent psychotic episodes. These considerations, as well as difficulties associated with side-effect management (metabolic syndrome, cardiac impact) highlight the need for antipsychotic agents that display both wider therapeutic activity and improved safety profile. One approach to respond to this need has been to develop drugs possessing partial agonist properties at D_2 receptors. The best characterized antipsychotic with this profile of activity is aripiprazole (Jordan *et al*., 2002; Shapiro *et al*., 2003), although other recent compounds, including bifeprunox and SSR181507, also display partial agonist properties (Bruins Slot *et al*., 2006; Cosi *et al*., 2006). By avoiding complete D_2 receptor blockade, such a profile should lower the incidence of EPS whilst reducing dopamine release in brain regions associated with hyperdopaminergic activity in schizophrenia, such as nucleus accumbens. Another approach is to combine 5-HT_{1A} agonist properties with D_2 antagonism (Millan, 2000; Bantick *et al*., 2001). In fact, direct or indirect 5-HT_{1A} receptor activation is implicated in the functional profiles of atypical antipsychotics, including clozapine, risperidone and aripiprazole (Cussac *et al*., 2002a; Newman-Tancredi *et al*., 2005 and see below) and a multiplicity of observations has highlighted 5-HT_{1A} receptor activation as a means to respond to unmet needs in therapy of schizophrenia. Thus, 5-HT_{1A} receptor activation reduces neuroleptic-induced catalepsy (Invernizzi *et al*., 1988; Prinssen *et al*., 2002), increases frontal cortex dopamine release (Rollema *et al*., 1997; Ichikawa and Meltzer, 2000; Assié *et al*., 2005; Diaz-Mataix *et al*., 2005), is beneficial in models of mood deficits and anxio-depressive states (Blier and Ward, 2003; Celada *et al*., 2004) and opposes dysfunctional glutamatergic transmission, consistent with activity against cognitive deficits induced by *N*-methyl-D-aspartate (NMDA) receptor hypofunction (Mauler *et al*., 2001; Czyrak *et al*., 2003; Auclair *et al*., 2006a; but see Wedzony *et al*., 2000).

It is important to note that clinical trials employing buspirone and tandospirone, drugs that act as 5-HT_{1A} receptor partial agonists, have shown an attenuation of cognitive and negative deficits observed in neuroleptic-treated schizophrenic patients, and a reduction of the incidence of EPS (Sovner and Parnell-Sovner, 1989; Goff *et al*., 1991; Sumiyoshi *et al*., 2001a, b). These observations support the notion that combining 5-HT_{1A} receptor activation with D_2 receptor blockade yields an improved 'atypical' antipsychotic profile. However, the level of 5-HT_{1A} receptor stimulation and the relative balance of 5-HT_{1A} and D_2 receptor interactions to obtain an optimal profile remain under discussion.

In view of the therapeutic potential of targeting 5-HT_{1A} receptors, several recent antipsychotic agents have been selected to include varying degrees of agonist properties at these sites. In addition to clozapine, other antipsychotics, such as ziprasidone and nemonapride, as well as aripiprazole and bifeprunox exhibit partial agonist properties at 5-HT_{1A} receptors, (Van Vliet *et al*., 2000; Cussac *et al*., 2002a; Jordan *et al*., 2002; Shapiro *et al*., 2003; Bruins Slot *et al*., 2006). Further, other drugs in various stages of development, such as SSR181507, SLV313 and the recently reported RGH-188, are specifically targeted at 5-HT_{1A} receptors, in addition to dopamine receptors (McCreary *et al*., 2002; Glennon *et al*., 2002; Claustre *et al*., 2003; Depoortère *et al*., 2003; Kiss *et al*., 2006). Nevertheless, a series of comparative studies indicates that even modest alterations in the balance of 5-HT_{1A}/D_2 receptor activity profoundly influences the profile of action in preclinical models of antipsychotic-like activity (Assié *et al*., 2005; Bruins Slot *et al*., 2005; Kleven *et al*., 2005; Newman-Tancredi *et al*., 2005; Auclair *et al*., 2006b; Bardin *et al*., 2006a). Indeed, drugs that exhibit too pronounced a preference for 5-HT_{1A} receptors, such as buspirone or the more recent anti-dyskinetic agent, sarizotan, fail to show activity in animal models of schizophrenic symptoms (Bardin *et al*., 2006a) and, correspondingly, are not clinically employed as antipsychotics. On the other hand, lower levels of 5-HT_{1A} receptor activation by, for example, nemonapride and ziprasidone, result in residual catalepsy in rodents (Kleven *et al*., 2005; Bardin *et al*., 2006a). In addition, an appropriate level of 5-HT_{1A} agonism is required for activity against PCP-induced social interaction deficit (Bruins Slot *et al*., 2005). These considerations illustrate the fundamental importance of identifying compounds that exhibit an optimal balance of D_2/5-HT_{1A} receptor properties in order to improve their therapeutic profile.

The present studies describe the *in vitro* pharmacological profile of a novel putative benzofurane antipsychotic, F15063 (*N*-[(2,2-dimethyl-2,3-dihydro-benzofuran-7-yloxy) ethyl]-3-(cyclopent-1-enyl)-benzylamine), synthesized at the Centre de Recherche Pierre Fabre (Vacher *et al*., 2002). Its activity was investigated in a series of tests of affinity and signal transduction at monoamine receptors. F15063 exhibits an innovative profile of action, with potent anti-D_2 dopaminergic and efficacious, but less potent, 5-HT_{1A} receptor agonist properties. In addition, F15063 acts as a D_4 receptor partial agonist, another property that distinguishes F15063 from established or potential new antipsychotic agents (Depoortère *et al*., 2006, 2007a, b).

Methods

Competition-binding and signal transduction methods

Competition-binding and guanosine 5′-*O*-(gamma-thiotriphosphate) ([^{35}S]GTPγS)-binding experiments were carried out using the radioligands, buffer and incubation conditions outlined in Tables 1 and 2. Experiments at native rat receptors employed brains of male Sprague–Dawley rats (Ico: OFA SD (SPF Caw); Iffa Credo, France), weighing 180–200 g. Rats were killed by decapitation and brains were rapidly dissected and stored at −70°C before use in binding assays. For native 5-HT$_{2C}$ receptor-binding assays (Pazos *et al*. 1985a, b), pig cortex was obtained from the local slaughter house. All experimental procedures involving animals were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, ML, USA), and were approved by the institutional Ethical Review Committee.

Membranes from dissected brain tissues were prepared as previously described (see references in Table 1). The methodology used for rat hippocampal membranes typifies the approach: briefly, frozen brains were thawed, the hippocampi were dissected and homogenized in 20 volumes of ice-cold Tris-HCl 50 mM, pH 7.4 at 25°C. The homogenate was centrifuged at 39 000 *g* for 10 min, the pellet was resuspended in the same volume of buffer and was recentrifuged as before. Following a further resuspension, the tissue was incubated for 10 min at 37°C to favour dissociation of endogenous 5-HT and centrifuged again. The final pellet was suspended in the same buffer. The final tissue concentration was 3 mg/assay tube.

Binding experiments at recombinant human receptors were carried out using membranes from Chinese hamster ovary (CHO), human carcinoma (HeLa), C6 rat glial or human embryonic kidney (HEK293) cell lines stably expressing monoamine receptors. Alternatively, African green monkey kidney cells (COS7) or *Spodoptera frugiperda* (Sf9) insect cells transiently expressing the relevant receptors were used as described previously (see references in Tables 2 and 3). The methodology employed for HeLa-h5-HT$_{1A}$ (HA7 cells, Fargin *et al*., 1989) cells typifies the approach used for

Table 1 Summary of experimental conditions for determination of affinities at native brain monoamine binding sites *in vitro*

Binding site	*Brain tissue*	*[^{3}H]Radioligand (nM)*	*K$_d$ (nM)*	*Non-specific (μM)*	*Inc., buffer*	*Inc., time (min) & temp. (°C)*	*Literature reference*
rD$_2$	Rat striatum	Nemonapride (0.05)	0.036	(+)Butaclamol (1)	A	60, 23°	Newman-Tancredi *et al.* (2005)
rD$_1$	Rat striatum	SCH 23390 (0.3)	0.22	SKF 38393 (10)	A	30, 37°	Kleven *et al.* (1997)
r5-HT$_{1A}$	Rat cortex	8-OH-DPAT (0.2)	3.1	5-HT (10)	B	30, 23°	Newman-Tancredi *et al.* (2005)
r5-HT$_{1A}$	Rat hippocampus	8-OH-DPAT (0.2)	0.49	5-HT (10)	C	30, 23°	Kleven *et al.* (1997)
r5-HT$_{1B}$	Rat cortex	GR125,743 (0.4)	0.5	5-HT (10)	B	30, 23°	Millan *et al.* (2002b)
r5-HT$_{2A}$	Rat cortex	Ketanserin (0.2)	3.1	Methysergide (10)	B	30, 23°	Kleven *et al.* (1997)
p5-HT$_{2C}$	Pig cortex	Mesulergine (1)	4.8	Mianserin (10)	B	60, 23°	Kleven *et al.* (1997)
rα_1	Rat cortex	Prazosin (0.1)	0.063	Phentolamine (50)	C	30, 23°	Kleven *et al.* (1997)
rα_2	Rat cortex	RX 821002 (0.5)	0.50	Phentolamine (10)	C	30, 23°	Hudson *et al.* (1992)
rSERT	Rat cortex	Citalopram (1)	2.0	Paroxetine (0.5)	A	60, 23°	Assié and Koek (2000)

Abbreviations: α_1, α_2: adrenoceptors; SERT = serotonin trasporter.
Buffers A: Tris-HCl 50 mM pH 7.4, NaCl 120 mM, KCl 5 mM. Buffer B: Tris-HCl 50 mM pH 7.4, pargyline 10 μM, CaCl$_2$ 4 mM, ascorbic acid 0.1%. Buffer C: Tris-HCl 50 mM pH 7.4.

Table 2 Summary of experimental conditions for determination of affinities at recombinant human monoamine receptors *in vitro*

Receptor	*Cell line*	*[^{3}H]Radioligand (nM)*	*K$_d$ (nM)*	*Non-specific (μM)*	*Inc. buffer*	*Inc. time (min) & temp. (°C)*	*Literature reference*
hD$_{2S}$	CHO	Spiperone (0.6)	0.084	(+)butaclamol (1)	A	120, 37°	Cussac *et al.* (2000)
hD$_{2L}$	CHO	Spiperone (0.6)	0.035	(+)butaclamol (1)	A	120, 37°	Cussac *et al.* (2000)
hD$_3$	CHO	Spiperone (0.4)	0.21	Raclopride (10)	B	160, 25°	Cussac *et al.* (2000)
hD$_{4.4}$	CHO	Spiperone (0.6)	0.15	Haloperidol (1)	A	120, 37°	Newman-Tancredi *et al.* (1997)
hD$_1$	CHO	SCH23390 (0.1)	0.38	SKF38393 (10)	C	120, 25°	Pedersen *et al.* (1994)
h5-HT$_{1A}$	HeLa	8-OH-DPAT (1)	0.71	5-HT (10)	D	30, 25°	Newman-Tancredi *et al.* (2005)
h5-HT$_{1B}$	COS7	5-CT (3)	0.65	5-HT (10)	D	30, 25°	Pauwels *et al.* (1997)
h5-HT$_{1D}$	COS7	5-CT (3)	0.60	5-HT (10)	D	30, 25°	Pauwels *et al.* (1997)
h5-HT$_{2A}$	CHO	Ketanserin (0.5)	0.24	5-HT (10)	A	120, 23°	Millan *et al.* (2002a)
h5-HT$_{2B}$	CHO	Mesulergine (2)	1.68	5-HT (10)	A	120, 23°	Millan *et al.* (2002a)
h5-HT$_{2C}$	CHO	Mesulergine (2)	0.56	5-HT (10)	A	120, 23°	Millan *et al.* (2002a)
h5-HT$_{7A}$	HEK293	5-CT (1.5)	1.66	5-HT (10)	D	90, 37°	Bard *et al.* (1993)
hα_{2A}	C6 glial	RX821002 (2)	1.16	phentolamine (10)	E	60, 25°	Wurch *et al.* (1999)
hα_{2B}	C6 glial	RX821002 (10)	9.27	phentolamine (10)	E	60, 25°	Wurch *et al.* (1999)
hα_{2C}	C6 glial	RX821002 (4)	2.21	phentolamine (10)	E	60, 25°	Wurch *et al.* (1999)

Abbreviations: CHO, Chinese hamster ovary cells; HEK293, human embryonic kidney cells 293.
Buffer A: HEPES 20 mM (pH 7.4), NaCl 120 mM, KCl 5 mM, EDTA 1 mM, MgCl$_2$ 5 mM; Buffer B: Tris-HCl 50 mM (pH 7.6), NaCl 120 mM, KCl 5 mM; CaCl$_2$ 2 mM, MgCl$_2$ 5 mM, BSA 0.1%; Buffer C: HEPES 20 mM (pH 7.2); Buffer D: Tris-HCl 50 mM (pH 7.6), CaCl$_2$ 4 mM, pargyline 10 μM, ascorbic acid 0.1%; Buffer E: Tris-HCl 50 mM (pH 7.6).

Table 3 Summary of methods for determination of functional responses at native rat and recombinant human monoamine receptor *in vitro*

Receptor	*Tissue/cell line*	*Functional measure*	*Incubation conditions*	*Inc. time (min) & temp. (°C)*	*Literature reference*
rD_2	Rat striatum	[^{35}S]GTPγS binding	Buffer A	60, 37°	Newman-Tancredi *et al.* (2001)
hD_{2L}	Sf9 cells	[^{35}S]GTPγS binding	Buffer B	40, 30°	Cosi *et al.* (2006)
hD_{2S}	CHO cells	ERK1/2 phosphorylation	Ham's F12 serum-free	5, 37°	Bruins Slot *et al.* (2006)
hD_3	COS cells	Gαo [^{35}S]GTPγS binding	Buffer C	30, 30°	Pauwels *et al.* (2003)
hD_4	CHO cells	[^{35}S]GTPγS binding	Buffer D	30, 23°	Newman-Tancredi *et al.* (1997)
r5-HT_{1A}	Rat hippocampus	[^{35}S]GTPγS binding	Buffer A	60, 37°	Newman-Tancredi *et al.* (2005)
r5-HT_{1A}	Rat hippocampus	Gαo [^{35}S]GTPγS binding	Buffer E	60, 23°	Martel *et al.* (2007)
h5-HT_{1A}	HeLa cells	[^{35}S]GTPγS binding	Buffer F	60, 30°	Newman-Tancredi *et al.* (2005)
h5-HT_{1A}	HeLa cells	cAMP formation	Buffer G	10, 23°	Newman-Tancredi *et al.* (2005)
h5-HT_{1A}	CHO cells	ERK1/2 phosphorylation	RPMI serum-free	5, 37°	Bruins Slot *et al.* (2006)
h5-HT_{1D}	C6 glial cells	[^{35}S]GTPγS binding	Buffer C	30, 23°	Pauwels *et al.* (1997)
h5-HT_{2A}	CHO cells	Gαq [^{35}S]GTPγS binding	Buffer H	60, 23°	Cussac *et al.* (2002b)
h5-HT_{2B}	CHO cells	Gαq [^{35}S]GTPγS binding	Buffer H	60, 23°	Cussac *et al.* (2002b)
h5-HT_{2C}	CHO cells	Gαq [^{35}S]GTPγS binding	Buffer H	60, 23°	Cussac *et al.* (2002b)
h5-HT_{7A}	HEK293 cells	cAMP formation	Buffer I	5, 37°	Rauly-Lestienne *et al.* (2004)
$h\alpha_{2A}$	C6 glial cells	[^{35}S]GTPγS binding	Buffer C	30, 23°	Pauwels *et al.* (2003)
$h\alpha_{2C}$	C6 glial cells	[^{35}S]GTPγS binding	Buffer C	30, 23°	Pauwels *et al.* (2003)

Abbreviations: CHO, Chinese hamster ovary cells; HeLa, human carcinoma cells; DTT, dithiothreitol.
Buffer A: 50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 100 μM GDP, 0.2 mM EDTA, 0.2 mM DTT; Buffer B: 20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 μM GDP, 0.1 mM DTT; Buffer C: 20mM HEPES, pH 7.4, 100 mM NaCl, 3 mM $MgCl_2$, 30 μM GDP; Buffer D: 20 mM HEPES, pH 7.4, 30 mM NaCl, 3 mM $MgCl_2$, 3 μM GDP; Buffer E: 20 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 50 μM GDP, 0.2 mM EDTA, 0.2 mM DTT; Buffer F: 20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 30 μM GDP, 10 μM pargyline; Buffer G: DMEM + 10 mM HEPES, pH 7.4, 100 μM forskolin, 100 μM isobutylmethylxanthine; Buffer H: 20 mM HEPES, pH 7.4, 150 mM NaCl, 50 mM $MgCl_2$, 0.1 μM GDP; Buffer I: 25 mM Tris-hCl, pH 7.4, 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 5 mM glucose, 1 mM isobutylmethylxanthine.
When drugs were tested for antagonist properties, an additional 30 min pre-incubation was performed before addition of the agonist, except for ERK1/2 phosphorylation experiments (15 min pre-incubation).
G-protein activation experiments were carried out by [^{35}S]GTPγS binding to cell membrane preparations. Other measures (cAMP formation and ERK1/2 phosphorylation) were carried out on whole cells.

recombinant cell lines: briefly, HeLa-h5-HT_{1A} cells were grown in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal calf serum, gentamicin (100 μg/ml), and geneticin (G418) (400 μg/ml), in 5% CO2 at 37°C in a water-saturated atmosphere. The cells were plated in 150 cm^2 Petri dishes until they reached a 90–100% confluence, after which they were washed with phosphate-buffered saline and stored at −80°C until used for [^{35}S]GTPγS binding.

Experiments were carried out in duplicate or triplicate and repeated at least three times. All binding experiments terminated by rapid filtration, through Whatman GF-B fibre filters. Radioactivity retained on the filters was measured by liquid scintillation spectroscopy. Data from all experiments were analysed using non-linear curve fitting programs. Data from native tissue receptors were analysed using KELL RADLIG version 6 (Biosoft, Cambridge, UK) and pK_i values are given as mean $\pm$ s.e.m. of at least three experiments, each comprising six to seven concentrations differing by one log unit interval. The K_d values of the different ligands are reported in Table 2. Data from human cloned receptor-binding experiments were analysed using GraphPad Prism, version 4 (GraphPad Software Inc., San Diego, CA, USA), and pK_i values are expressed as mean $\pm$ s.e.m. of at least three experiments each comprising seven to 10 concentrations differing by 0.5 or 1 log unit interval. All data were analysed using a 4-parameter logistic equation:

$$Y = \text{minimum} + (\text{maximum} - \text{minimum})/ (1 + 10((\text{LogIC}_{50} - X) * \text{HillSlope}))$$

where the maximum is defined by the values observed in the absence of competitor (100% value) and the minimum is defined in the presence of an excess of competing ligand (non-specific binding) respectively.

Functional responses at native rat and recombinant human receptors

The agonist/antagonist properties of F15063 at a range of rat and human receptors were determined *in vitro* for several measures of signal transduction representing different levels of intracellular responses: activation of G-proteins, inhibition of cyclic adenylyl cyclase accumulation and phosphorylation of extra-cellular signal regulated kinase (ERK1/2). An outline of methodologies, together with relevant literature references are shown in Table 3. In the case of recombinant human receptors, G-protein activation was monitored by [^{35}S]GTPγS binding to membranes from CHO, HeLa, C6 glial and Sf9 insect cells.

Cyclic AMP accumulation was determined in HeLa-h5-HT_{1A} cells as previously described (Newman-Tancredi *et al.*, 2005). Briefly, cells were incubated (10 min, room temperature) with compounds in DMEM, 10 mM HEPES, 100 μM forskolin, and 100 μM 3-isobutyl-1-methylxanthine (IBMX). The reaction was stopped by aspiration of the medium and addition of 0.1 N HCl. cAMP content was measured using a radioimmunoassay kit (Dupont NEN: NEK-033). Basal cAMP levels were 10 ± 0.9 pmol/well ($n = 8$). E_{max} values are expressed as % of the response obtained with 5-HT 10^{-5} M.

Extracellular signal regulated kinase (ERK)1/2 phosphorylation was examined using whole CHO-h5-HT_{1A} or

CHO-hD_{25} cells. Briefly, cells were grown until 90% confluent, washed once and starved overnight in serum-free medium. Cells were stimulated for 5 min with compounds diluted in serum-free medium. In antagonist studies, cells were incubated for 15 min with the relevant compound and then stimulated for 5 min with agonist. Reaction was stopped by lysis (15 min, room temperature) with RIPA buffer supplemented with protease and phosphatase inhibitors. Cell lysates were assayed for phospho-ERK (pERK) 1/2 content using an immunometric kit (Biosource, catalogue no. KHO0091, Camarillo, CA, USA), as described previously (Bruins Slot *et al*., 2006).

Isotherms were analysed by non-linear regression, using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) and a four-parameter logistic equation (see above). The value of the minimum and maximum asymptotes was not fixed. The latter (E_{max}) is expressed as a percentage of the effect observed with a reference agonist, as indicated in Table 6. K_B values of antagonists for inhibition of agonist action were calculated according to Lazareno and Birdsall (1993): $K_B = IC_{50}$: $[1 + (\text{Agonist}/EC_{50})]$, where IC_{50} = inhibitory concentration$_{50}$ of antagonist, agonist = concentration of agonist in the test and EC_{50} = effective concentration$_{50}$ of agonist.

Functional autoradiography
[^{35}S]Guanosine 5′-*O*-(gamma-thiotriphosphate ([^{35}S]GTPγS) autoradiography was carried out essentially as described by Newman-Tancredi *et al*. (2003). Frozen rat brains were cut horizontally in 20 μm thick serial sections using a cryostat at −20°C, and fixed on microscope slides. Sections were kept frozen at −20°C until assayed. The assay was performed by pre-incubating the slides at room temperature for 15 min in buffer A (50 mM HEPES buffer containing 150 mM NaCl, 0.2 mM EGTA and 0.2 mM dithiothreitol) plus 2.5 mM GTP, 15 min in buffer A plus 2.5 mM GDP, and 15 min in buffer A with 2.5 mM GDP, 10 mM $MgCl_2$, and 100 mU/ml adenosine deaminase (= buffer B) plus antagonist. Sections were then incubated for 60 min at 37°C in buffer B containing 0.05 nM [^{35}S]-GTPγS and drugs. Basal [^{35}S]GTPγS binding was defined as that observed in the absence of drugs, and non-specific binding was defined as the binding in the presence of 100 μM unlabelled GTPγS. At the end of the incubation period, sections were rapidly washed twice for 2 min in cold buffer B (4°C), and rapidly dried under a flow of cold air. Dried sections, together with [^{14}C]radioactivity standards were placed in X-ray cassettes, apposed to Biomax films and exposed for $4\frac{1}{2}$ days. Films were developed and radioactivity on sections was quantified using an image analysis system (AIS system, InterFocus Ltd, Linton, UK). Grey levels were converted to nC_i/g equivalents using [^{14}C]radioactivity standards, and radioactivity was measured on each structures/sections. Sections were analysed in series of six adjacent sections, each having received a different treatments as described in Figure 7. Non-specific labelling for each structure was subtracted from the corresponding values determined under basal and ligand-treated conditions. The effect of ligand treatments on changes in radioactivity were expressed as percent change from specific basal values. Thus, 100% represents a doubling of labelling (Figure 8).

Drugs
F15063 (*N*-[(2,2-dimethyl-2,3-dihydro-benzofuran-7-yloxy) ethyl]-3-(cyclopent-1-enyl)-benzylamine mono-tartrate; Vacher *et al*., 2002; Cuisiat *et al*., in press) was synthesized by Medicinal Chemistry Division 1, Centre de Recherche Pierre Fabre (Castres, France). The following radioligands were purchased from Amersham Bioscience (manufacturer reference in brackets with specific activity): [^{3}H]8-OH-DPAT (TRK.850: 5.92–8.88 TBq mmol^{-1}), [^{3}H]GR125,743 (TRK. 1046: 1.85–3.18 TBq mmol^{-1}), [^{3}H]mesulergine (TRK.845: 2.59–3.15 TBq mmol^{-1}), [^{3}H]SCH 23390 (TRK.876: 2.22–3.33 T Bq mmol^{-1}), [^{3}H]RX 821002 (TRK.914: 1.48–2.59 TBq mmol^{-1}), [^{3}H]citalopram (TRK.1068: 2.22–3.18 TBq mmol^{-1}), [^{3}H]prazosin (TRK.843: 2.41–3.15 TBq mmol^{-1}), [^{3}H]spiperone (TRK818: 2.89–3.44 TBq mmol^{-1}), [^{35}S]GTPγS (37–44 TBq mmol^{-1}). The following radioligands were purchased from Perkin-Elmer Life Sciences (Courtaboeuf, France): [^{3}H]ketanserin (NET-791: 2.22–3.33 TBq mmol^{-1}), [^{3}H]5-CT (NET-1071: 0.74–2.22 TBq mmol^{-1}), and [^{3}H]YM-09151–2 (i.e. [^{3}H]nemonapride; NET-1004: 2.59–3.22 TBq mmol^{-1}). Apomorphine HBr, dihydroergotamine mesylate, dopamine HCl, haloperidol, 5-HT creatinine sulphate, (±) 8-hydroxy-dipropylaminotryptamine ((±)8-OH-DPAT) bromohydrate, phentolamine mesylate, raclopride tartrate, methysergide maleate, mianserin HCl, SB269970 HCl, SKF38393 HCl and (+)butaclamol HCl were purchased from Sigma RBI (St Quentin Fallavier, France). GR127935 HCl, paroxetine, MDL100907, RS127445 HCl, RX821002 HCl, SB242084 HCl and sumatriptan HCl were synthesized by Jean-Louis Maurel, the Chemistry Dept., Centre de Recherche Pierre Fabre. Drugs were dissolved in distilled water or 10% DMSO at 10^{-3} M, and subsequent dilutions were prepared in the appropriate assay buffer (Figure 1).

Results

F15063 interacts at native brain and recombinant human D_2 and 5-HT_{1A} receptors
F15063 possessed high affinity at rat (r) brain D_2 receptors and 14-fold lower affinity at r5-HT_{1A} receptors (Table 4. Figure 2b and d). Correspondingly, F15063 exhibited high affinity at recombinant human (*h*) D_2 and D_3 receptors (pK_i values >9) and about 10-fold lower affinity at hD_4 and h5-HT_{1A} receptors (Figure 2, Table 5). In comparison (data from Newman-Tancredi *et al*., 2005), haloperidol exhibited high affinity at rat striatal D_2 receptors (pK_i rD_2 = 9.01;

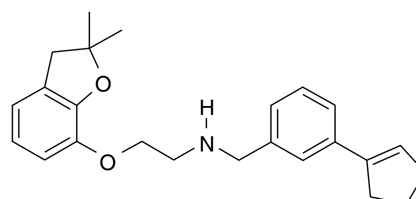

Figure 1 Chemical structure of F15063.

Newman-Tancredi *et al*., 2005). Using the protocol described in Table 2, haloperidol also exhibited high affinity at cloned human D_2 receptors: pK_i $hD_{2L}=8.96\pm0.03$; pK_i $hD_{2S}=8.56\pm0.02$; but not at rat cortex or cloned human 5-HT_{1A} receptors (pK_i <6). In contrast, (±)8-OH-DPAT exhibited high affinity at rat cortex and cloned human 5-HT_{1A} receptors (pK_i=8.85 and 8.92; Newman-Tancredi *et al*., 2005). Using the protocols described in Table 1, (±)8-OH-DPAT also had high affinity at rat hippocampal 5-HT_{1A} receptors (p$K_i=9.00\pm0.03$), but not at rat striatal D_2 receptors (p$K_i=6.26\pm0.03$).

Table 4 Affinities of F15063 at native brain monoamine sites

Receptor	*pK_i±s.e.m.*	*K_i (95% CI)*	*K_i ratio vs rD_2*
rD_2	9.38±0.05	0.42 (0.26–0.66)	1
rD_1	5.89±0.03	1284 (950–1738)	3057
r5-HT_{1A} (cortex)	8.24±0.07	5.9 (3.0–11.2)	14
r5-HT_{1A} (hippocampus)	8.65±0.09	2.3 (1.0–5.3)	5
r5-HT_{1B}	6.31±0.10	487 (180–1315)	1159
r5-HT_{2A}	6.57±0.01	270 (237–306)	643
p5-HT_{2C}	6.50±0.17	318 (57–1781)	757
rα_1	7.29±0.01	51 (46–56)	121
rα_2	6.52±0.02	303 (255–360)	721
rSERT	5.98±0.03	1045 (776–1406)	2488

Abbreviation: CI, confidence interval.
Affinity values were calculated from competition binding experiments and derived pK_i values are shown±s.e.m. The corresponding geometric mean of K_i values (in nanomolar) are shown with their 95% confidence intervals.

F15063 interacted much more weakly (>30-fold less affinity than for D_2 receptors; see Tables 4 and 5) with a range of other targets. Thus, F15063 exhibited low or negligible affinity for D_1 receptors, as well as only modest affinity at α_1 and α_2 adrenoceptors both in rat tissue and in cloned systems (Tables 4 and 5). The affinity of F15063 for native rat 5-$HT_{2A/2C}$ receptors was low, relative to its affinity at D_2 receptors. F15063 also had only modest affinity at h5-$HT_{2A/2B/2C}$ receptors (at least 47-fold lower than that at hD_{2L} receptors). F15063 exhibited modest affinity at h5-HT_{1D} receptors (36-fold less than at hD_{2L} receptors, Table 5) but very little at human or rat 5-HT_{1B}. F15063 interacted weakly with serotonin transporters in rat cortex.

In a receptor screen carried out on F15063 by Cerep (Courtaboeuf, France; data on file), weak interactions were detected with sigma sites (pK_i=7.0), rat cerebral cortex verapamil site Ca^{2+} (6.68), site-2 Na^+ channels (6.54), histamine H2 (6.49) and dopamine hD_5 receptors (6.16). In addition, F15063 did not interact (less than 50% inhibition of radioligand binding at 1 μM) with a series of other sites, including 5-HT_3, 5-HT_4, 5-HT_6, muscarinic (M1, M2, M3, M4 and M5), histaminergic (H1 and H3), adenosine (A1, A2A), β_1 adrenoceptors, opiate, benzodiazepine, GABA-A, GABA-B, AMPA, kainate, PCP and NMDA receptors, dopamine or noradrenaline transporters, ATP-sensitive, voltage-sensitive or Ca^{2+}-dependent K^+ channels, diltiazem site or DHP site Ca^{2+} channels or site-1 Na^+ channels. F15063 did not inhibit acetylcholinesterase, MAO-A or MAO-B enzyme activities.

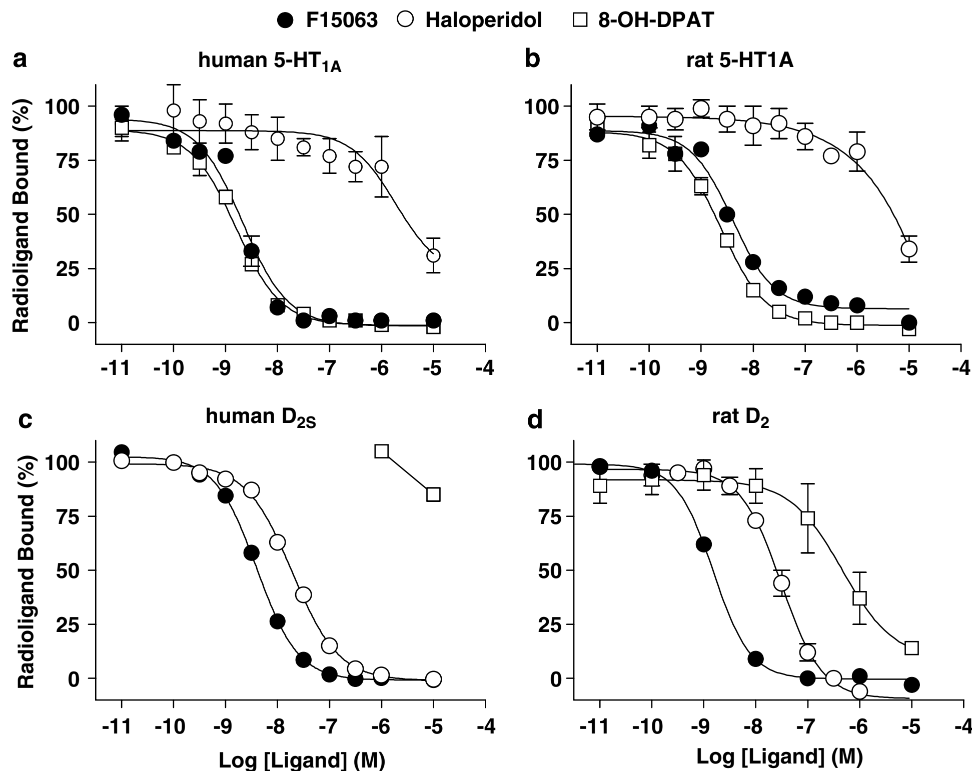

Figure 2 F15063 inhibits radioligand binding at 5-HT_{1A} and D_2 receptors. Competition-binding curves for F15063 in comparison with haloperidol and (±)8-OH-DPAT at: (**a**) human 5-HT_{1A} receptors expressed in HeLa cells; (**b**) rat hippocampal 5-HT_{1A} receptors; (**c**) human D_{2S} receptors expressed in CHO cells; (**d**) rat striatal D_2 receptors. Binding conditions are described in Tables 1 and 2 and values are mean±s.e.m. from three experiments performed in triplicate or in duplicate. Data from these experiments are shown in Tables 4 and 5.

F15063 activates native rat and recombinant human 5-HT$_{1A}$ receptors

In G-protein activation measures, determined by binding of [^{35}S]GTPγS to HeLa cell membranes expressing recombinant h5-HT$_{1A}$ receptors, F15063 markedly increased G-protein activation with a maximal response of 70% relative to that induced by 5-HT and of a similar magnitude to that exhibited by ($\pm$)8-OH-DPAT (82%, Table 6; Figure 3a).

In the same cell line, F15063 also inhibited cyclic AMP formation, demonstrating an influence on second messenger signal transduction in living cells. The maximal response to F15063 was 90% of that observed with 5-HT, similar to that observed with ($\pm$)8-OH-DPAT (Figure 3).

In CHO cells stably expressing h5-HT$_{1A}$ receptors, F15063 concentration-dependently stimulated phosphorylation of ERK1/2, a down-stream response to 5-HT$_{1A}$ receptor activation. The maximal efficacy was 93% relative to that of 5-HT and was slightly greater than that observed with ($\pm$)8-OH-DPAT in this system (Table 6).

F15063 also stimulated total G-protein activation in rat hippocampal membranes, indicating that it activates native 5-HT$_{1A}$ receptors in a brain region relevant to potential therapeutic properties (Table 6, Figure 3). The maximal stimulation in this system was 36% relative to that of 5-HT. The influence of F15063 in hippocampal membranes was entirely mediated by 5-HT$_{1A}$ receptors, as demonstrated by its complete blockade with the selective 5-HT$_{1A}$ receptor antagonist, WAY100 635. The latter abolished F15063 (1 μM)-stimulated [^{35}S]GTPγS binding with a pIC$_{50}$ of 7.23 ± 0.08 and a pK_b of 8.71 ± 0.08 ($n = 3$).

In a G-protein subtype targeting procedure ([^{35}S]GTPγS binding coupled to antibody-capture and SPA detection), F15063 stimulated Gαo activation in rat hippocampal membranes by 52% relative to 5-HT, similar to ($\pm$)8-OH-DPAT (Table 6).

Table 5 Affinities of F15063 at recombinant human monoamine receptors

Receptor	p$K_i \pm$ *s.e.m.*	K_i *(95% CI)*	K_i *ratio vs rD$_2$*
hD$_{2L}$	9.44 ± 0.01	0.36 (0.32–0.42)	1
hD$_{2S}$	9.25 ± 0.01	0.56 (0.55–0.58)	1.6
hD$_3$	8.95 ± 0.05	1.12 (0.68–1.58)	3.1
hD$_{4.4}$	8.81 ± 0.08	1.53 (0.68–3.48)	4.3
hD$_1$	6.51 ± 0.16	312 (67–1454)	867
h5-HT$_{1A}$	8.37 ± 0.02	4.23 (3.36–5.34)	12
h5-HT$_{1B}$	7.04 ± 0.05	91 (56–145)	253
h5-HT$_{1D}$	7.89 ± 0.05	13 (7.5–21)	36
h5-HT$_{2A}$	7.77 ± 0.08	17 (7–38)	47
h5-HT$_{2B}$	7.71 ± 0.05	19 (12–32)	53
h5-HT$_{2C}$	7.27 ± 0.08	53 (24–118)	147
h5-HT$_{7A}$	6.60 ± 0.08	237 (140–400)	658
hα_{2A}	6.81 ± 0.02	156 (136–179)	433
hα_{2B}	7.02 ± 0.04	96 (65–141)	266
hα_{2C}	7.20 ± 0.06	60 (33–108)	166

Abbreviations: CI, confidence interval.
Affinity values were calculated from competition binding experiments and derived pK_i values are shown ± s.e.m. The corresponding geometric mean of K_i values (in nanomolar) are shown with their 95% confidence intervals.

F15063 antagonizes native rat and recombinant human D$_2$ receptors

In Sf9 cells expressing recombinant human D$_{2L}$ (long isoform) receptors, F15063 did not induce any increase in [^{35}S]GTPγS labelling to endogenous G-proteins, consistent with absence of agonist properties for G-protein activation at this site (Table 6, Figure 4). In contrast, the reference agonist, apomorphine, induced a potent stimulation of G-protein

Table 6 Agonist and antagonist properties of F15063 at monoamine receptors determined by transduction assays *in vitro*

		F15063			*Reference ligand*			
Receptor	*Efficacy model*	E_{max}	*pEC$_{50}$*	pK_b	*Drug*	E_{max}	*pEC$_{50}$*	pK_b
rD$_2$	G-protein activation	0		8.29 ± 0.11	Haloperidol	0		8.45 ± 0.07
hD$_{2L}$	G-protein activation	0		9.19 (pA$_2$)	Haloperidol[a]	0		9.12 ± 0.10
hD$_{2S}$	ERK1/2 phosphorylation	0		8.18 ± 0.16	Haloperidol[b]	0		8.13 ± 0.04
hD$_3$	Gαo-protein activation	0		8.74 ± 0.04	Haloperidol	0		7.67 ± 0.01
hD$_{4.4}$	G-protein activation	29 ± 4	8.15 ± 0.13	9.89 ± 0.17	Apomorphine	42 ± 4	7.99 ± 0.06	
r5-HT$_{1A}$	G-protein activation	36 ± 4	7.47 ± 0.11		(±)8-OH-DPAT[c]	50 ± 4	7.00 ± 0.09	
r5-HT$_{1A}$	Gαo-protein activation	52 ± 6	7.95 ± 0.10		(±)8-OH-DPAT	63 ± 6	7.27 ± 0.12	
h5-HT$_{1A}$	G-protein activation	70 ± 2	7.57 ± 0.10		(±)8-OH-DPAT[c]	82 ± 4	7.59 ± 0.04	
h5-HT$_{1A}$	cAMP formation	90 ± 4	7.12 ± 0.27		(±)8-OH-DPAT[c]	82 ± 5	7.65 ± 0.25	
h5-HT$_{1A}$	ERK1/2 phosphorylation	93 ± 8	7.13 ± 0.14		(±)8-OH-DPAT	82 ± 5	7.81 ± 0.19	
h5-HT$_{1D}$	G-protein activation	35 ± 2	7.68 ± 0.02	8.02 ± 0.12	5-HT	99 ± 2	8.36 ± 0.06	
h5-HT$_{2A}$	Gαq-protein activation	12 ± 1	6.79 ± 0.12	6.78 ± 0.05	MDL100907	0		9.36 ± 0.12
h5-HT$_{2B}$	Gαq-protein activation	0		6.78 ± 0.11	RS127445	0		8.36 ± 0.17
h5-HT$_{2C}$	Gαq-protein activation	23 ± 3[d]		7.17 ± 0.05	SB242084	0		9.06 ± 0.06
h5-HT$_{7A}$	cAMP formation	0		6.22 ± 0.05	SB269970	0		7.79 ± 0.17
hα_{2A}	G-protein activation	0		6.90 ± 0.06	(±)RX821002	0		9.51 ± 0.12
hα_{2C}	G-protein activation	0		7.16 ± 0.10	(±)RX821002	0		8.89 ± 0.01

Efficacy (E_{max}) values are expressed as % of the stimulation induced by saturating concentrations (10 μM) of 5-HT (for 5-HT receptors), dopamine (for dopamine receptors) or noradrenaline (for hα_{2A} and hα_{2C} receptors). Comparative data are shown for reference ligands.
[a]Cosi *et al*. (2006).
[b]Bruins Slot *et al*. (2006).
[c]Newman-Tancredi *et al*. (2005).
[d]Activation observed at 10 μM.

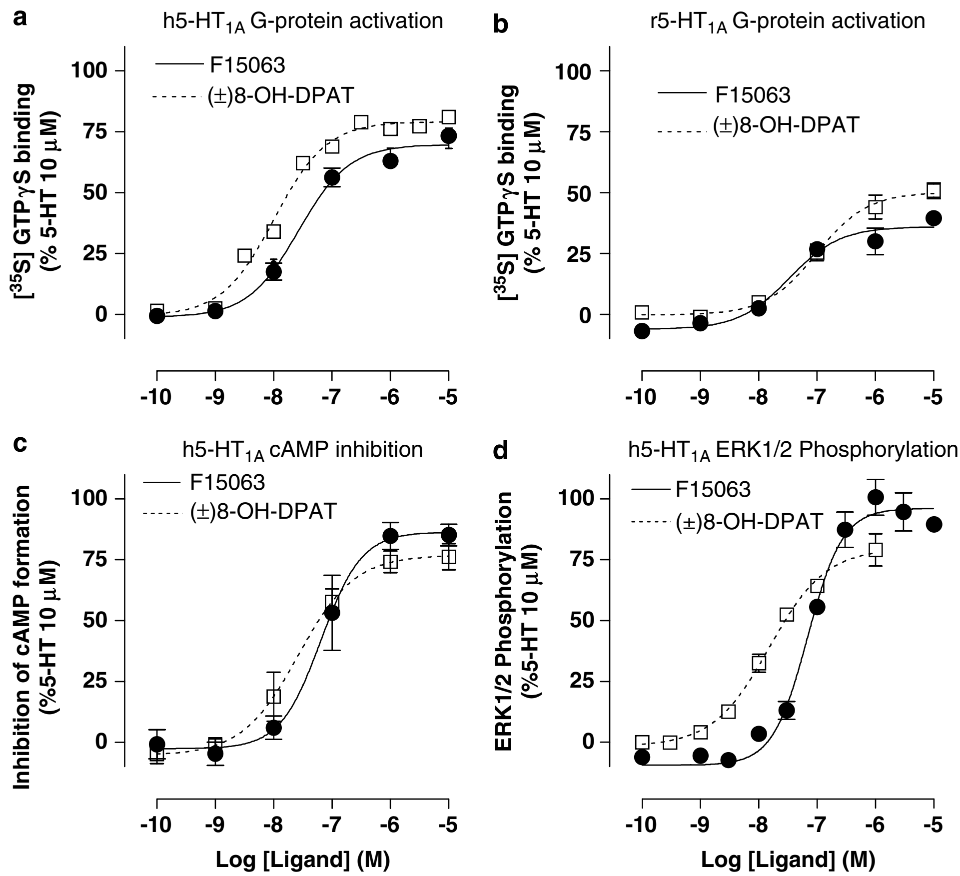

Figure 3 F15063 efficaciously activates cloned human and native rat 5-HT_{1A} receptors. (**a**) Stimulation by F15063 of G-protein activation determined using [^{35}S]GTPγS binding at recombinant human (HeLa-h5-HT_{1A}) and (**b**) native rat hippocampal 5-HT_{1A} receptors. (**c**) Inhibition of cAMP accumulation in HeLa-h5-HT_{1A} cells. (**d**) stimulation of ERK1/2 phosphorylation at h5-HT_{1A} receptors expressed in CHO cells. Data are expressed as percentage of the effect induced by a saturating concentration of 5-HT (10 μM). Values are mean $\pm$ s.e.m. from three experiments performed in triplicate or in duplicate. For comparison, the dotted line represents results obtained under the same conditions for ($\pm$)8-OH-DPAT (Newman-Tancredi *et al.*, 2005; Bruins Slot *et al.*, 2006). Data from these experiments are shown in Table 6.

activation. The apomorphine stimulation curve was progressively shifted to the right by the addition of increasing concentrations of F15063, consistent with competitive antagonist actions at hD_2 receptors (Figure 4a).

The EC_{50} value of apomorphine alone was 8.7 ± 1.1 nM; EC_{50} value in the presence of 3.16 nM F15063: 116 ± 26 nM; with 10 nM F15063: 1500 ± 55 nM; with 31.6 nM F15063: 5516 ± 995 nM. The pA_2 value derived from the Schild plot of these data (Figure 4b) was $pA_2 = 9.19$ with a slope of 1.71. The antagonist potency of haloperidol was similar (Table 6).

In CHO cells stably expressing hD_{2S} (short isoform) receptors, F15063 did not induce any ERK1/2 phosphorylation when tested alone, but potently antagonized ERK1/2 phosphorylation induced by dopamine, indicating that F15063 behaves as a potent antagonist at dopamine D_2 receptors (Figure 4c; Table 6). Haloperidol likewise blocked ERK1/2 phosphorylation without inducing any by itself.

In rat striatal membranes F15063 did not stimulate [^{35}S]GTPγS binding, but abolished the stimulation induced by the dopaminergic agonist, quinelorane, demonstrating antagonist properties at native rat D_2 receptors (Figure 4d). The antagonist potency of F15063 at rD_2 dopamine receptors was similar to that of haloperidol (Table 6).

F15063 antagonizes hD_3, but has partial agonist properties at hD_4 receptors

When tested alone, F15063 did not modify activation of Gαo G-proteins transiently co-expressed with hD_3 receptors in Cos7 cells, consistent with an absence of agonist properties (Figure 5a). In contrast, F15063 antagonized dopamine-induced [^{35}S]GTPγS binding to Gαo proteins (Table 6), consistent with potent antagonist properties at D_3 receptors.

In membranes of CHO cells expressing hD_4 (4-repeat isoform) receptors, F15063 moderately increased G-protein activation ($\sim$30% relative to that of the full agonist, dopamine), as measured in [^{35}S]GTPγS-binding experiments. In the presence of dopamine, F15063 potently reduced dopamine-induced [^{35}S]GTPγS binding to the same level as that seen with F15063 alone (Figure 5b). These data demonstrate partial agonist properties of F15063 at dopamine D_4 receptors (Table 6). Under the same conditions, apomorphine yielded an E_{max} value of 42%.

F15063 has modest or weak actions at other receptor subtypes

In contrast to its potent actions at D2-like and 5-HT_{1A} receptors (responses in the nanomolar range), F15063

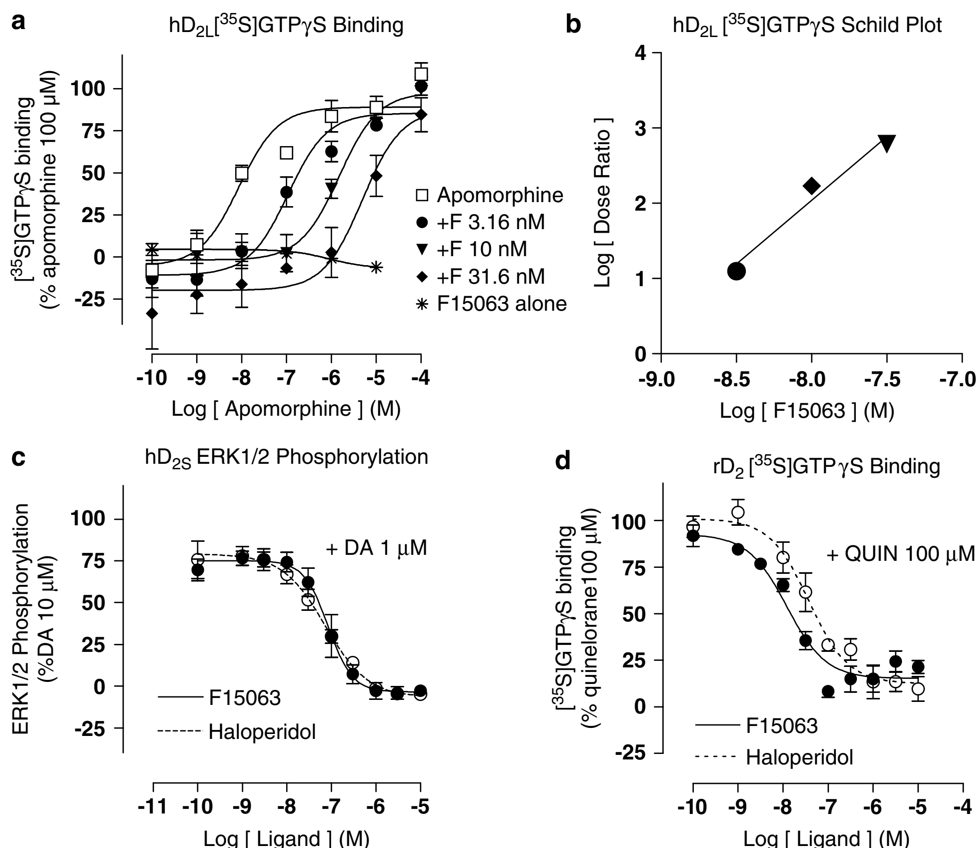

Figure 4 F15063 antagonizes cloned human and native rat D_2 receptors. (**a**) F15063 induces a rightward shift of a concentration-effect curve of apomorphine-induced stimulation of [^{35}S]GTPγS binding to membranes of Sf9 cells expressing recombinant hD_{2L} receptors. Points are mean$\pm$s.e.m. of values from three experiments performed in triplicate. (**b**) Schild plot of the data from (**a**). (**c**) F15063 reverses the stimulation of ERK1/2 phosphorylation induced by dopamine in CHO cells expressing recombinant hD_{2S} receptors. (**d**) F15063 antagonizes quinelorane-induced [^{35}S]GTPγS binding to rat striatal membranes. Points are mean$\pm$s.e.m. of values from three experiments performed in triplicate. For comparative purposes, the dotted line represents the results obtained for haloperidol under the same conditions. Data from these experiments are shown in Table 6.

elicited modest or weak actions at other receptors. At h5-HT_{1D} receptors expressed in C6 glioma cells, F15063 induced a modest stimulation of [^{35}S]GTPγS binding, but reduced the stimulation induced by 5-HT (Table 6, Figure 6). These data demonstrate partial agonism of F15063 at h5-HT_{1D} receptors. For comparative purposes, three additional compounds were tested in the same system: the anti-migraine agents, dihydroergotamine (E_{max} 75$\pm$2%, pEC_{50} 9.13$\pm$0.07, $n=3$) and sumatriptan (87$\pm$3%, 7.75$\pm$0.09, $n=8$), and the weak partial agonist, GR127935 (50$\pm$4%, 8.07$\pm$0.07, $n=3$).

F15063 acted as a low-potency antagonist (p$K_b$$\sim$7) at h5-$HT_{2A/2B/2C}$ receptors in a model of Gαq protein activation (Table 6), although a very slight increase in Gαq activation was detected at high concentrations at h5-HT_{2A} and h5-HT_{2C} receptors (Table 6). At micromolar concentrations, F15063 also blocked noradrenaline-induced G-protein activation of hα_{2A} and hα_{2C} receptors (Table 6). At h5-HT_7 receptors, F15063 had no influence on cyclic AMP formation when tested alone, but reversed the stimulation of cAMP formation induced by 5-HT, indicating low-potency neutral antagonist properties at h5-HT_7 receptors (Table 6).

F15063 exhibits dual 5-HT_{1A}/D_2 properties in functional autoradiography

Incubation of rat brain horizontal sections with a 100 μM of F15063 induced an increase in [^{35}S]GTPγS binding in brain areas rich in 5-HT_{1A} receptors, including hippocampus, lateral septum and limbic cortex (Figure 7). This effect of F15063 was abolished by co-incubation of F15063 with WAY100 635 (10 μM), confirming that the stimulation is specifically mediated by 5-HT_{1A} receptor activation. F15063 did not stimulate labelling of brain regions such as striatum, that are rich in D_2 receptors, indicating absence of agonist properties at native D_2 receptors under these conditions (Figure 7). F15063 even tended to reduce labelling of striatum below basal values, suggestive of potential inverse agonism at high concentrations (see quantification of autoradiograms by densitometry, Figure 8). In comparison, [^{35}S]GTPγS labelling of striatum was strongly increased by incubation of sections with the dopaminergic agonist, quinelorane (100 μM). F15063 abolished quinelorane-induced binding in striatum, indicating antagonist actions (Figure 8). When sections are incubated with both quinelorane and F15063, the increase in [^{35}S]GTPγS binding in brain structures responding to 5-HT_{1A} activation by F15063 can be observed (Figure 7).

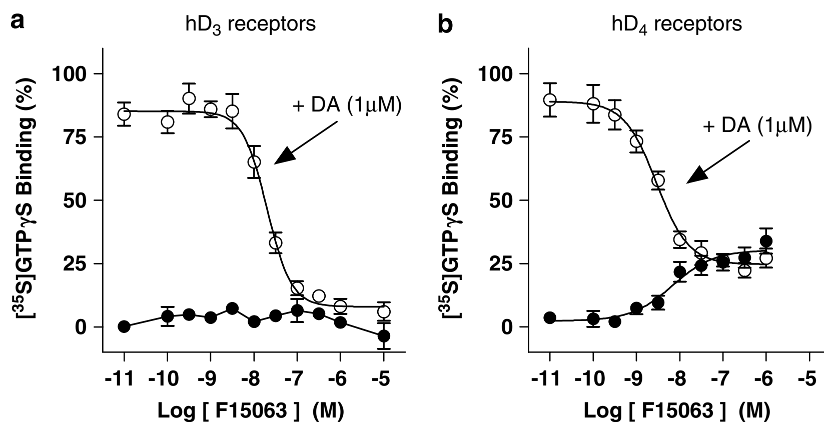

Figure 5 F15063 antagonizes hD_3 but shows partial agonism at hD_4 receptors. (**a**) Blockade by F15063 of dopamine-stimulated hD_3 receptor-mediated Gαo protein activation. Cos7 cells were co-transfected with hD_3 receptors and Gαo (C351I) subunits. Gαo-protein activation was determined by [^{35}S]GTPγS binding. (**b**) Stimulation by F15063 of hD_4 receptor-mediated G-protein activation in stably transfected CHO cells. Values are mean ± s.e.m. from at least three experiments performed in triplicate or in duplicate of F15063 tested alone (filled circles) and in the presence of 1 μM dopamine (empty circles). Data from these experiments are shown in Table 6.

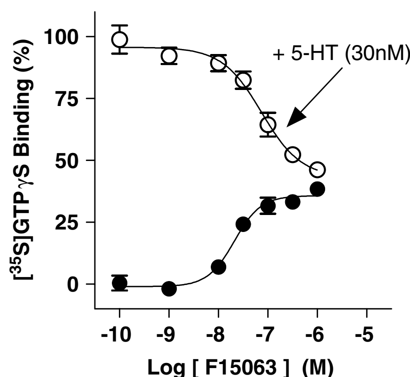

Figure 6 F15063 shows partial agonism at h5-HT_{1D} receptors. Stimulation by F15063 of h5-HT_{1D} receptor-mediated G-protein activation in stably transfected C6-glial cells. Values are mean ± s.e.m. from at least three experiments performed in triplicate or in duplicate of F15063 tested alone (filled circles) and in the presence of 30 nM 5-HT (empty circles). Data from these experiments are shown in Table 6.

Discussion

Balance of affinity of F15063 at dopamine D_2-like and 5-HT_{1A} receptors in vitro

F15063 is a member of a new generation of antipsychotic agents that combine dopamine D_2 receptor blockade and activation of 5-HT_{1A} receptors. Ample evidence indicates that such a profile should result in a favourable 'atypical' antipsychotic profile, but the balance of 5-HT_{1A} and D_2 interactions can profoundly influence the *in vivo* actions of such drugs (see Introduction and Assié *et al.*, 2005; Bruins Slot *et al.* 2005; Kleven *et al.* 2005; Newman-Tancredi *et al.*, 2005; Auclair *et al.*, 2006b; Bardin *et al.*, 2006a). Thus, antipsychotics that lack 5-HT_{1A} receptor activation, as in the case of haloperidol, are associated with EPS induction and lack of beneficial influence against negative symptoms. On the other hand, excessive 5-HT_{1A} receptor activation negates the dopamine D_2 antagonism necessary for antipsychotic action and disrupts pre-pulse inhibition, a model of sensorymotor gating (Auclair *et al.*, 2006a; Bardin *et al.*, 2006a). In the case of F15063, the balance of dopamine D_2 receptor blockade and 5-HT_{1A} receptor agonism results in a favourable profile of pharmacological activities with potent actions *in vivo* in models of positive symptoms, negative symptoms and cognitive deficits (Depoortère *et al.*, 2007a, b). F15063 possesses high affinity at both native rat and cloned human D_2 receptors, (pK_i value>9) and 10–20-fold lower affinity at rat/human 5-HT_{1A} receptors (Tables 4 and 5). The affinity of F15063 at D_2 receptors is comparable to that of other potent antipsychotics at this receptor, such as haloperidol or risperidone (Newman-Tancredi *et al.*, 2005). In comparison, the affinity of F15063 at 5-HT_{1A} receptors is similar to that of the prototypic 5-HT_{1A} receptor agonist, (±)8-OH-DPAT. The ratio of affinity at these receptors is an important consideration in the profile of activity of the compound. Indeed, other 'new generation' drugs, such as SSR181507 and SLV313 (currently undergoing clinical evaluation), have higher affinity at 5-HT_{1A} relative to D_2 receptors whereas bifeprunox has a reversed balance of affinity (Newman-Tancredi *et al.*, 2005). Although clinical data relating to 5-HT_{1A}/D_2 properties are as yet unavailable for these drugs, it may be speculated that these compounds do not fall within an 'optimal activity window' of 5-HT_{1A}/D_2 balance, because they are not as active in a variety of *in vivo* behavioural models of social withdrawal (Bruins Slot *et al.*, 2005), dopamine release in frontal cortex (Assié *et al.*, 2005) or cognitive deficits (Auclair *et al.*, 2006a; Bardin *et al.*, 2006b).

F15063 activates 5-HT_{1A} receptors and antagonizes D_2 receptors

Relative affinities at D_2 and 5-HT_{1A} receptors must be interpreted in the context of the agonist/antagonist properties at these sites. In the case of F15063, potent antagonism at D_2 receptors is demonstrated *in vitro* in rat striatal membranes, where it reversed quinelorane-induced G-protein activation. F15063 also behaved as a neutral antagonist

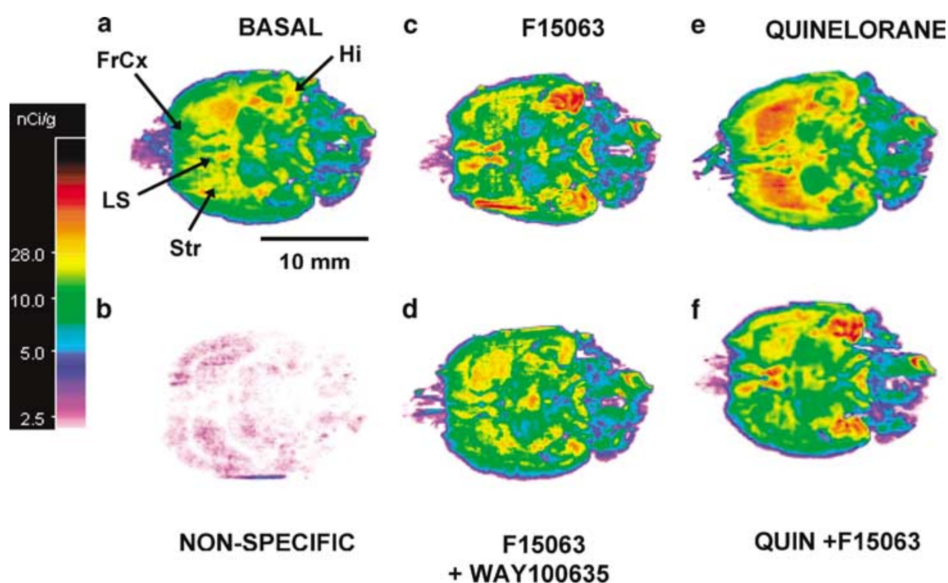

Figure 7 F15063 exhibits dual 5-HT_{1A}/D_2 properties in functional autoradiography. Influence on G-protein activation in various regions of horizontal rat brain sections as determined by [^{35}S]GTPγS autoradiography. (**a**) Basal conditions, that is no drugs; (**b**) non-specific binding was determined in the presence of 10 μM unlabelled GTPγS; (**c**) F15063 (100 μM); (**d**) co-incubation of F15063 (100 μM) with the selective 5-HT_{1A} receptor antagonist, WAY100 635 (10 μM). (**e**) Quinelorane (100 μM); (**f**) co-incubation of quinelorane (100 μM) with F15063 (100 μM). The lower end of spectrum (blue/pink) represents the lowest G-protein activation while the upper end of spectrum (red) represents the maximal response. Abbreviations: FrCx: frontal cortex; Hi: hippocampus; LS: lateral septum; Str: striatum.

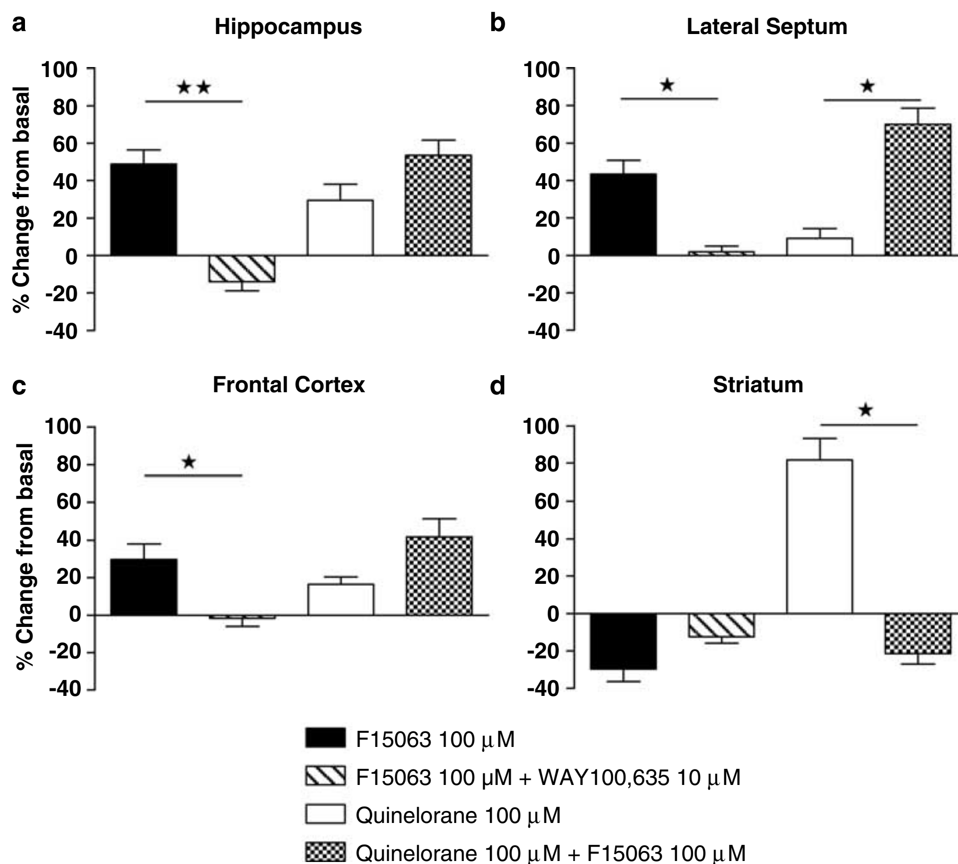

Figure 8 F15063 increases 5-HT_{1A}-mediated but blocks D_2 receptor-mediated G-protein activation. Quantification of G-protein activation by F15063, as determined by [^{35}S]GTPγS autoradiography, in (**a**) hippocampus, (**b**) lateral septum, (**c**) frontal cortex, (**d**) striatum. Bars represent the mean$\pm$s.e.m. values determined from quantification of autoradiograms of six animals. G-protein activation is expressed as percent changes from basal labelling. Statistical analyses were carried out by a Kruskall–Wallis test followed by Dunn's *post hoc* test. *$P<0.05$; **$P<0.01$.

in downstream transduction systems that are sensitive to weak partial agonist properties, such as activation of G-proteins at hD_2 receptors in a high-expressing Sf9 cell system (Cosi *et al*., 2006). In contrast, SSR181507, aripiprazole and bifeprunox behaved as D_2 partial agonists in this system. F15063 also behaved as a 'silent' antagonist for stimulation of ERK1/2 phosphorylation in CHO cells whereas aripiprazole, bifeprunox and SSR181507 acted as partial agonists (Bruins Slot *et al*., 2006). The latter observation is particularly interesting in view of the involvement of this pathway in the antipsychotic-like actions of clozapine *in vivo* (Browning *et al*., 2005). The potent D_2 receptor antagonist properties of F15063 are of the same order of magnitude as those of haloperidol (Figure 4) and manifest themselves *in vivo* in models of hyperdopaminergic activity induced by methylphenidate or amphetamine in rats, or by apomorphine, in mice (Depoortère *et al*., 2007b). Thus, the activity of F15063 contrasts with that of other antipsychotics targeting D_2 and 5-HT_{1A} receptors, such as bifeprunox, SSR181507 and aripiprazole, (although the latter has multiple additional interactions). These three drugs all act as partial agonists at D_2 receptors, a property that is associated with reduced liability to induce EPS and hyperprolactinaemia (Cosi *et al*., 2006). However, D_2 partial agonists, such as aripiprazole and bifeprunox, are also somewhat less potent in models of positive symptoms of schizophrenia and have less influence on frontal cortex dopamine release (Li *et al*., 2004; Assié *et al*., 2005; Bardin *et al*., 2006a) and recent data show that aripiprazole fails to reverse PCP-induced deficits in reversal learning, whereas F15063 does (Auclair *et al*., 2006a; Depoortère *et al*., 2006, 2007b). Further, D_2 receptor partial agonist activity, in combination with 5-HT_{1A} receptor activation, disrupts basal pre-pulse inhibition in rats, suggesting that such drugs may interfere with sensorimotor gating response (Auclair *et al*., 2006b). Hence, the relative benefits of partial agonist properties at D_2 receptors in antipsychotic therapy remain under discussion and F15063 provides, with SLV313 (also a pure D_2 receptor antagonist; Bruins Slot *et al*., 2006; Cosi *et al*., 2006), a different balance of efficacy at D_2 versus 5-HT_{1A} receptors compared with that of other new generation antipsychotics. An earlier antipsychotic, ziprasidone, also displays 5-HT_{1A} receptor partial agonism but is also potently active at other sites, including 5-HT_{2A} receptors (Leysen, 2000). Its balance of receptor activity appears less favourable than that of F15063, as indicated by the propensity of ziprasidone to dose-dependently induce catalepsy in rats and mice (Kleven *et al*., 2005; Bardin *et al*., 2006a) and its failure to reverse PCP-induced social interaction deficits in rats (Bruins Slot *et al*., 2005).

As concerns 5-HT_{1A} receptors, F15063 consistently exhibited marked efficacy at 5-HT_{1A} receptors, similar to that of the prototypical agonist, ($\pm$)8-OH-DPAT, and greater than that of other antipsychotics, including clozapine, ziprasidone, aripiprazole as well as SLV313 (Newman-Tancredi *et al*., 2005; Bruins Slot *et al*., 2006; Table 6). Thus, F15063 efficaciously stimulated 5-HT_{1A} receptor signalling at different levels of signal transduction: G-protein activation, cyclic AMP accumulation and ERK1/2 phosphorylation. It is notable that, in the latter two systems, downstream of G-protein activation, the efficacy of F15063 relative to that of 5-HT was amplified, consistent with substantial agonist properties (Figure 3). In comparison, F15063 also exhibited agonist activity at rat brain 5-HT_{1A} receptors in tests of total and Gαo-specific G-protein activation in rat hippocampal membranes. In these experiments the E_{max} values of F15063 were lower than those observed in cell lines, an observation attributable to two factors: first, receptor expression levels are lower in hippocampus than in recombinant cell lines, and, second, hippocampal membranes express a multiplicity of serotonin receptors: about 85% of the 5-HT-mediated effect is due to 5-HT_{1A} receptors, the rest is due to activation of other sites (Newman-Tancredi *et al*., 2005). In contrast, the totality of the influence of F15063 in hippocampal membranes is mediated by 5-HT_{1A} receptors, as was demonstrated by its complete blockade with the selective 5-HT_{1A} receptor antagonist, WAY100 635.

The dual D_2 antagonist and 5-HT_{1A} agonist properties of F15063 result in a complementary pattern of receptor activation in brain regions expressing these sites. Thus, in functional autoradiography experiments, and consistent with other studies using this methodology (Newman-Tancredi *et al*., 2001, 2003), F15063 stimulated 5-HT_{1A} receptor-coupled G-protein activation in the hippocampus, septum and frontal cortex, structures with substantial expression levels of 5-HT_{1A} receptors. In contrast, F15063 did not stimulate G-protein activation in striatum but blocked quinelorane-induced activation in this brain region that expresses high levels of D_2 receptors. Although these data demonstrate the dual D_2/5-HT_{1A} properties of F15063, further studies need to address the issue of concentration–response relationships for these effects. Indeed, it is likely that the concentrations of F15063 producing 5-HT_{1A}/D_2 responses in various brain regions will differ from those of other recent antipsychotics, such as ziprasidone and aripiprazole, that have weaker partial agonist actions at 5-HT_{1A} receptors (Newman-Tancredi *et al*., 2005; Bruins Slot *et al*., 2006). In contrast, some new drugs in development, such as SSR181507 and SLV313, have higher potency and/or efficacy at 5-HT_{1A} receptors but display marked diversity in their behavioural and neurochemical profiles (see Introduction), illustrating the profound influence that modifications in D_2/5-HT_{1A} receptor interactions can have on *in vivo* responses.

Taken together, these data provide a comprehensive *in vitro* picture of the agonist/antagonist activities of F15063 at 5-HT_{1A} and D_2 receptors, respectively, allowing interpretation of the influence of these pharmacological properties. Indeed, the combined D_2/5-HT_{1A} properties of F15063 account for many of the actions of F15063 in neurochemical and behavioural models in rodents. Thus, F15063 potently reversed apomorphine-induced climbing behaviour in mice and methylphenidate-induced behaviours in rats, two measures reflecting blockade of central dopamine D_2 receptors (Depoortère *et al*., 2007a, b). Conversely, the 5-HT_{1A} agonist properties of F15063 *in vivo* are demonstrated by unmasking of catalepsy when 5-HT_{1A} receptors are occluded by the selective antagonist, WAY100 635. Further, the 5-HT_{1A} agonist properties of F15063 are responsible for the increase in extracellular levels of dopamine in the frontal

cortex and attenuation of PCP-induced social interaction deficits by this compound (Newman-Tancredi *et al*., 2006; Depoortère *et al*., 2006, 2007a, b).

F15063 blocks D_3 receptors but activates D_4 receptors in vitro

Whilst D_2 antagonism and 5-HT_{1A} agonism are key components of the profile of action of F15063, other activities make important contributions to its actions. Thus, F15063 possesses marked D_3 receptor affinity (Table 5), at which it behaves as a potent antagonist ($pK_b = 8.74$, Figure 5). Blockade of D_3 receptors is a common effect of antipsychotics, whether conventional or atypical (Joyce and Millan, 2005; Sokoloff *et al*., 2006) and considerable effort has been invested in examining the role of D_3 antagonism in the actions of antipsychotics in pre-clinical models. While selective D_3 antagonists do not show activity in rodent models of dopaminergic hyperstimulation (Millan *et al*., 2000; Reavill *et al*., 2000), the influence of BP897, a D_3 partial agonist, in models of NMDA blockade in mice has raised the possibility that D_3 interactions may contribute to antipsychotic efficacy (Leriche *et al*., 2003; Sokoloff *et al*., 2006). Recent reports have shown that selective D_3 antagonists, S33084 and SB277011, increase acetylcholine release in rats and are active in models of scopolamine-induced cognitive deficits (Dekeyne *et al*., 2004; Laszy *et al*., 2005). Thus, the potent D_3 blocking properties of F15063 may represent a favourable element in its cognitive profile.

A feature of F15063 that differentiates it from current antipsychotic agents is the combination of D_2/D_3 antagonist properties with partial agonism at the other member of the D2-like receptor family, the D_4 subtype. Using an approach similar to that employed previously (Newman-Tancredi *et al*., 1997), F15063 exhibited modest stimulation of G-protein activation in CHO cell membranes expressing the 4-repeat isoform of the D_4 receptor, while reducing dopamine-induced [^{35}S]GTPγS binding, demonstrating partial agonist properties of F15063 at D_4 receptors. In comparison, apomorphine, a drug that possesses substantial efficacy at D_4 receptors (Newman-Tancredi *et al*., 1997) exhibited an E_{max} value no more than 1.5-fold greater than that of F15063. It is interesting that D_4 receptor activation is implicated in pro-cognitive actions in rodents (Bernaerts and Tirelli, 2003; Browman *et al*., 2005). In contrast, some reports indicate that D_4 receptor blockade is necessary for activity in other models of cognitive deficits (Jentsch *et al*., 1999). It may be that an intermediate (i.e. partial agonist) influence is necessary to provide the optimal profile of action, as suggested by some authors (Zhang *et al*., 2004). In the case of F15063, its reversal of scopolamine-induced memory deficit in a social recognition paradigm was completely abolished by pre-treatment with a D_4 receptor antagonist, L745870 (Bardin *et al*., 2006b; Depoortère *et al*., 2006, 2007b). These results indicate that the D_4 partial agonist properties of F15063 detected *in vitro* are reflected in an *in vivo* behavioural model of cholinergic deficits and suggest that incorporating D_4 partial agonist properties in novel antipsychotic candidates could lead to improved influence on mnesic functions.

F15063 has minimal interaction at other monoamine receptors

A distinguishing feature of F15063 is its minimal interactions with a range of other receptors. In particular, F15063 has only modest affinity for h5-$HT_{2A/2B/2C}$ receptors (47- to 147-fold lower than at hD_2 receptors) whereas atypical antipsychotics such as risperidone, olanzapine and ziprasidone have markedly higher affinity at these sites than at D_2 receptors (Leysen, 2000). While blockade of 5-HT_{2A} and/or 5-HT_{2C} receptors is associated with anti-cataleptic properties and facilitation of cortical dopamine release (see Introduction), these desirable responses are also robustly induced by activation of 5-HT_{1A} receptors, suggesting that a D_2/5-HT_{1A} profile may be sufficient to produce an 'atypical' antipsychotic profile (Millan, 2000; Bantick *et al*., 2001; Meltzer *et al*., 2003). Interestingly, F15063 exhibited partial agonism for stimulation of [^{35}S]GTPγS binding at h5-HT_{1D} receptors (Figure 6) with a potency ($pEC_{50} = 7.68$) similar to that for stimulation of [^{35}S]GTPγS binding at 5-HT_{1A} receptors ($pEC_{50} = 7.57$; Table 6). In contrast, other antipsychotics, including clozapine, olanzapine, risperidone and haloperidol, display inverse agonism at 5-HT_{1D} receptors (Audinot *et al*., 2001). Thus, activity at 5-HT_{1D} receptors may play a role in the distinctive pharmacological profile of F15063, although the therapeutic relevance of this observation is unclear. Indeed, the *in vivo* responses to F15063 (Depoortère *et al*., 2007a, b) can be satisfactorily attributed to actions at D_2-like and 5-HT_{1A} receptors. Further, the efficacy of F15063 at 5-HT_{1D} receptors (E_{max} 35%, Table 6) is low relative to that of the antimigraine agents sumatriptan (87%) and dihydroergotamine (75%). The E_{max} of F15063 is also inferior to that of GR127935 (50%), a partial agonist that has little, if any, agonist activity *in vivo* (Skingle *et al*., 1996; De Vries *et al*., 1997). Taken together, these data suggest that the predominant action of F15063 at 5-HT_{1D} receptors will be as an antagonist. While this is unlikely to constitute a prominent property of F15063, it underlines the latter's novel profile at monoamine receptors.

F15063 does not interact with a variety of sites associated with undesirable effects associated with current antipsychotics such as olanzapine and clozapine. These include antagonism of α_1 adrenoceptors, muscarinic M_1 receptors and histamine H_1 receptors, sites associated with autonomic side effects, sedation, weight gain and potential metabolic disturbance. Thus a 'selectively non-selective' profile, as displayed by F15063, may avoid a number of potential side effects, whilst retaining the desired pharmacological properties underlying efficacious antipsychotic actions (Leysen, 2000; Shapiro *et al*., 2003; Roth *et al*., 2004).

Conclusions

Abundant *in vitro* and *in vivo* results, together with clinical evidence from add-on studies with 5-HT_{1A} receptor partial agonists, indicate that appropriate targeting of D_2 and 5-HT_{1A} receptors should produce a promising 'atypical' antipsychotic with improved potential for the management of negative and cognitive symptoms of schizophrenia. However, a fundamental issue in the characterization of the new generation of antipsychotics is the definition of an

'optimal balance' of activity at D_2 and 5-HT_{1A} receptors (see Assié *et al*., 2005; Bruins Slot *et al*., 2005; Newman-Tancredi *et al*., 2005; Bardin *et al*., 2006a, b; Auclair *et al*., 2006a, b). Thus, while potent $D_{2/3}$ receptor antagonism is desirable for robust antipsychotic actions, the presence of sufficient 5-HT_{1A} receptor activation should alleviate negative symptoms, favour cognitive function and diminish EPS liability. The potent $D_{2/3}$ antagonist properties of F15063, combined with its less potent, but high efficacy, 5-HT_{1A} receptor agonism and D_4 receptor partial agonism confer on F15063 a novel profile. The absence of marked interactions of F15063 with targets associated with potential side effects, including histaminergic and muscarinic receptors suggests improved safety profile. In view of the favourable activity of F15063 in a series of *in vivo* models of positive, negative and cognitive symptoms of schizophrenia (Depoortère *et al*., 2006, 2007a, b), it appears that its balance of receptor activities is promising for improved treatment of this pathology.

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Conflict of interest

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