QF2004B, a potential antipsychotic butyrophenone derivative with similar pharmacological properties to clozapine

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Abstract

The aim of the present work was to characterize a lead compound displaying relevant multi-target interactions, and with an in vivo behavioral profile predictive of atypical antipsychotic activity. Synthesis, molecular modeling and in vitro and in vivo pharmacological studies were carried out for 2-[4-(6-fluorobenzisoxazol-3-yl)piperidinyl]methyl-1,2,3,4-tetrahydro-carbazol-4-one (QF2004B), a conformationally constrained butyrophenone analogue. This compound showed a multi-receptor profile with affinities similar to those of clozapine for serotonin (5-HT2A, 5-HT1A, and 5-HT2C), dopamine (D1, D2, D3, and D4), alpha-adrenergic (α1, α2), muscarinic (M1, M2) and histamine H1 receptors. In addition, QF2004B mirrored the antipsychotic activity and atypical profile of clozapine in a broad battery of in vivo tests including locomotor activity (ED50 = 1.19 mg/kg), apomorphine-induced stereotyes (ED50 = 0.75 mg/kg), catalepsy (ED50 = 2.13 mg/kg), apomorphine- and DOI (2,5-di-methoxy-4-iodoamphetamine)-induced prepulse inhibition (PPI) tests. These results point to QF2004B as a new lead compound with a relevant multi-receptor interaction profile for the discovery and development of new antipsychotics.

Keywords: Schizophrenia; Typical antipsychotics; Atypicality; Butyrophenone analogue; Prepulse inhibition test; Atypical profile

1. Introduction

Schizophrenia is a chronic and disabling psychotic disorder with an incidence between 0.17 and 0.54 per 1000 individuals per year, and the lifetime risk to develop this illness is as high as 1% in the general population (Owen et al., 2002). Conventional antipsychotics (preferential antagonists of dopamine D2 receptors) and the more recent second generation or atypical antipsychotics are being used today for the treatment of schizophrenia (Miyamoto et al., 2005). The latter compounds lack the extrapyramidal side effects (EPS) produced by conventional antipsychotics at the doses frequently used in therapy, and they present higher efficacy at improving the negative and cognitive symptoms of the illness (Leucht et al., 2003), which are primary factors to be taken into account in the choice of treatment. Clozapine, the first atypical
antipsychotic discovered nearly 50 years ago, continues to be considered as the “gold standard” among the atypical antipsychotics based on its not yet surpassed efficacy in treating schizophrenia, on its ability for reducing suicidality (Roth et al., 2004b), as well as on its relapse prevention in long term use (Geddes, 2002). In fact, clozapine remains the only licensed drug indicated for treatment-resistant schizophrenia. However, its use is restricted to these cases mainly due to the risk of agranulocytosis, its major adverse effect.

Although for a long-term blockade of dopamine D2 receptors has been considered the main mechanism responsible for the efficacy of antipsychotics, the complex pharmacological profile of clozapine challenged this assumption. In fact, clozapine exhibits activity at multiple receptors (Roth et al., 2004b). Its action mainly at 5-HT1A, 5-HT2A, and 5-HT2C receptors is thought to mediate its beneficial effects on cognition, negative symptoms, and the low incidence of EPS (Meltzer et al., 2003; Roth et al., 2004a), but it also displays affinity for dopamine receptors, related to its efficacy on positive symptoms, as well as α-adrenergic, muscarinic, and histaminergic (H1) receptors.

Unlike classical antipsychotics like haloperidol, which mainly block D2 receptors, clozapine and other atypical antipsychotics are relatively more potent at blocking 5-HT2A receptors than D2 receptors. This finding gave rise to the serotonin-dopamine hypothesis (Meltzer et al., 2003), suggesting that blockade of presynaptic 5-HT2A receptors by atypical antipsychotics is a predominant mechanism in the nigrostriatal, mesocortical, and tuberoinfundibular dopaminergic pathways where they increase dopamine release. This effect will counteract the drugs action at D2 receptors decreasing the incidence of adverse EPS, cognitive deficits, hyperprolactinemia, and/or negative symptoms. In contrast, D2 blockade would prevail over 5-HT2A antagonism in the dopaminergic mesolimbic pathway, resulting in the mitigation of the positive symptoms of psychosis. The relatively high affinity for 5-HT2A versus D2 receptors, common among atypical antipsychotics, led to the definition of the Meltzer’s index (MI) (pKᵢ[5-HT2A/pKᵢ D2 > 1.12] (Meltzer et al., 1989) as a screening criterion for candidate atypical antipsychotics (Meltzer et al., 2003).

In in vivo tests for antipsychotic activity, clozapine behaves as an atypical antipsychotic as predicted by the MI criteria. Hence, clozapine blocks the conditioned avoidance response (Barrett, 1982), and the apomorphine- and amphetamine-induced locomotor activity tests (Ellenbroek, 1993), which are considered laboratory equivalents of psychotic-like responses in humans, whereas it does not produce catalepsy, an animal screening test for EPS in humans, even at doses many fold higher than those producing an antipsychotic-like effect (Meltzer et al., 2003).

However, the exceptional therapeutic performance of clozapine, not completely achieved by other atypical antipsychotics that fulfill the MI criteria, is thought to arise from its interaction with a large number of molecular targets, the so called clozapine receptorome (Roth et al., 2004b). In fact, “designing non-selective drugs that interact with several molecular targets” has been suggested as a promising new paradigm in the search for better medications in schizophrenia (Roth et al., 2004b), opposite to the traditional approach aiming at drugs selective for a single molecular target. Today, while newer atypical antipsychotics like risperidone, olanzapine, quetiapine or ziprasidone have become therapeutic options in the clinic (Serretti et al., 2004), there is still a major interest in the development of new compounds with an antipsychotic profile similar to clozapine, but structurally unrelated in order to minimize its side effects, and in particular agranulocytosis.

Working with conformationally constrained butyrophenones, combining antagonism at serotonin 5-HT2A and dopamine D2 receptors in a single molecule structurally unrelated with clozapine, we have developed several compounds with a predicted atypical antipsychotic profile (MI > 1.12) (Raviña et al., 1999, 2000; Masaguer et al., 2000; Brea et al., 2002). In order to validate one of these new compounds, namely QF2004B (2-[4-(6-fluorobenzisoxazol-3-yl)piperidinyl]methyl-1,2,3,4-tetrahydrocarbazol-4-one), as a lead with potential atypical antipsychotic activity under the new perspective of non-selective drugs acting at multiple receptors, we present here an optimized synthetic procedure and a detailed study on its molecular descriptors, its in vitro multi-receptor targets and its in vivo profile.

2. Methods

2.1. QF2004B synthesis

Compound QF2004B (Fig. 1) was prepared following an alternative, shorter procedure to that previously described by us (Masaguer et al., 2000), where the key cyclocondensation step was carried out using three different
2.2. In vitro studies

2.2.1. Receptor binding assays

Human 5-HT_{2A} and 5-HT_{2C} receptor binding assays were performed as previously described (Brea et al., 2002) in membranes from CHO cells stably transfected with the receptors. Binding assays for other receptors were performed on commercially available membrane preparations expressing the human receptors (Euroscreen for 5-HT_{1A} and D_{2} receptors, Sigma for D_{3} and D_{3.4.5}, and Perkin Elmer for D_{2.3}, 5_{2A}-adrenergic, M_{1}, and M_{2} receptors) following manufacturers’ protocols. 1 nM [3H]ketanserin (5-HT_{2A}), 0.2 nM [3H]spiperone (D_{2}), 2 nM [3H]8-OH-DPAT (5-HT_{1A}), 2 nM [3H]mesulergine (M_{2}) were employed as radioligands. Apparent association constant of the antagonist-receptor complex, $K_{B}$, where $[A^{*}] / [A] = 1 = [B] / K_{B}$, where $[A^{*}] / [A]$ is the ratio of concentrations of agonist giving an equal response (50% of the maximal effect) in the presence and in the absence of a given concentration of the antagonist (B).

2.2.2. Isolated organ assays

Antagonism of norepinephrine at 5_{2A}-adrenergic receptors was assayed using denuded thoracic aorta from male Sprague–Dawley rats (250–350 g), as previously reported (Gonzalez-Gomez et al., 2003). Antagonism of histamine at H_{1} receptors was assayed using ileum from male Dunkin-Hartley guinea pigs (500–600 g), as previously reported (Elz et al., 2000). The dissociation constant of the antagonist-receptor complex, $K_{D}$ (expressed as pK_{D}), was calculated from the equation: $([A^{*}] / [A]) = 1 = [B] / K_{D}$, where $[A^{*}] / [A]$ is the ratio of concentrations of agonist giving an equal response (50% of the maximal effect) in the presence and in the absence of a given concentration of the antagonist (B).

2.2.3. IP accumulation and AA release measurements

Inhibition of basal inositol phosphate (IP) accumulation and basal arachidonic acid (AA) release by the antagonists/inverse agonists studied was measured in CHO cells stably expressing the 5-HT_{2C} receptor. Both PLC and PLA\textsubscript{2} effector pathways were simultaneously evaluated from the same experiment, following a previously described protocol (Berg et al., 1998). The potency of the compounds was measured as pIC\textsubscript{50} (-log of concentration that inhibits the 50% of the maximal stimulation) and their efficacy as the percentage of inhibition of the maximal stimulation. IC\textsubscript{50} values were obtained by fitting the data using non-linear regression (GraphPad Software).

2.2.4. Homology modeling of 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors

The numbering scheme proposed by Ballesteros and Weinstein (1994) has been used to indicate the amino acids of the two receptors modeled (5-HT\textsubscript{2A} and 5-HT\textsubscript{2C}) as well as in the description of the ligand–receptor interactions. Structural models for the most relevant serotonin receptors were built using homology modeling. The sequences of human 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors were aligned with other 5-HT receptors and the sequence of bovine rhodopsin, making sure that highly conserved residues were superimposed. The so aligned sequences of the human serotonin receptors 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} were then introduced in the program MODELER (Sali and Blundell, 1993), taking into account the published crystal structure of bovine rhodopsin 1F88 (Palczewski, 2000) and other structural information to produce candidate 3D structures. 3D structures for the loops were also generated, using appropriate structural templates searched in the ArchDB loop database (Espadaler et al., 2004). The resulting structural models were carefully validated according to geometric criteria, physicochemical consistency with agreement with mutagenesis data. Further details of the procedure used to build these structural models are available (Dezi, C., Pastor, M., Sanz, F., unpublished observations).

2.2.5. Docking of QF2004B

The binding site of the serotonin receptors was prepared using a method involving several successive steps, in which ligands of growing size were docked within the receptor, preserving the well-known interaction between the ligand charged nitrogen and the Asp3.32. The program GOLD (Verdonk et al., 2003) was used to dock the ligands with the program GOLD (Verdonk et al., 2003), forcing the interaction involving several successive steps, in which ligands of growing size were docked within the receptor, preserving the well-known interaction between the ligand charged nitrogen and the Asp3.32. The program GOLD (Verdonk et al., 2003) was used to dock the ligands with the program GOLD (Verdonk et al., 2003), forcing the interaction between the above mentioned atoms, and then the structure of the complex was relaxed during a 1ns molecular dynamic simulation, using the program AMBER 6.0 (Case et al., 1999). The docking procedure docked first the structure of the natural agonist (serotonin) and then the structure of ketanserin, an inverse agonist structurally related to QF2004B. A detailed description of the docking procedure is available (Dezi, C., Pastor, M., Sanz, F., unpublished observations). Once the structure of the binding site was ready, the structure of QF2004B was docked within the binding site using software GOLD, taking into account the published crystal structure of bovine rhodopsin 1F88 (Palczewski, 2000) and other structural information to produce candidate 3D structures. 3D structures for the loops were also generated, using appropriate structural templates searched in the ArchDB loop database (Espadaler et al., 2004). The resulting structural models were carefully validated according to geometric criteria, physicochemical consistency with agreement with mutagenesis data. Further details of the procedure used to build these structural models are available (Dezi, C., Pastor, M., Sanz, F., unpublished observations).

2.3. In vivo studies

2.3.1. Animals

Male CD1 mice (Charles River, France), and male Swiss albino mice (University of Sevilla, Spain), weighing 22 ± 2 g were housed in groups of...
five animals per cage in standard laboratory conditions (12 h light/dark cycle, 21 ± 1 °C room temperature, 55 ± 10% humidity). Food and water were available ad libitum. Behavioral tests and animal care were conducted in accordance with the standard ethical guidelines (National Institutes of Health, 1995; Council of Europe, 1996) and approved by the local ethical committees.

2.3.2. Drugs
Apomorphine sulfate, DOI, clozapine, and haloperidol were purchased from Sigma. Apomorphine was dissolved in double distilled water containing 0.1% sodium bisulfate, and injected subcutaneously (s.c.). QF2004B, clozapine, and haloperidol were dissolved in a double-distilled water solution containing 1% lactic acid (vehicle), and injected intraperitoneally (i.p.). DOI was dissolved in 0.9% saline solution. All drugs were administered in a volume of 0.1 ml per 10 g of body weight.

2.3.3. Locomotor activity test
Locomotor activity was measured by placing the animals in individual actimeters (9 × 20 × 11 cm) (Imetronic, Bordeaux, France) equipped with two lines of six infrared beams under a dim light (15 lux). Mice were treated with vehicle (double-distilled water containing 1% lactic acid), QF2004B, clozapine, or haloperidol (0.33, 1.0 and 3.3 mg/kg, i.p.), and 30 min later, ambulatory activity was recorded every 10 min during a 30-min period.

2.3.4. Catalepsy
The ring catalepsy test was used (Valverde et al., 2000). This test consisted of a vertical transparent open cylinder (9 cm diameter × 16 cm height) fitted on the upper side with a wire ring (5.5 cm diameter). Mice were placed with all four paws on the ring and habituated for 5 min to this test before the drug administration. Vehicle, QF2004B, clozapine, or haloperidol (0.33, 1.0, 3.3 and 10.0 mg/kg, i.p.) were administered, and 30 min later the animals were again placed on the ring. An immobility index was calculated as the percentage of time that the animal spends motionless divided by the duration of the test (300 s). If the animal fell down or jumped off the ring, it was immediately placed on the ring again. After a maximum of five such escapes, the test was terminated.

2.3.5. Apomorphine-induced stereotypies test
Mice were randomly injected with vehicle, QF2004B, clozapine, or haloperidol (0.1, 0.33 and 1.0 mg/kg i.p.), and the emergence of stereotypes or other behavioral responses was evaluated for 30 min under blind conditions. Subsequently, apomorphine (2 mg/kg s.c.) was injected and animal behavior was evaluated again for 30 min under blind conditions. A time sampling model was used to score behavior such that the presence or absence of a pattern was scored every 5 s, and the total frequency of each pattern was later quantified. The behavioral repertoire included stereotyped and non-stereotyped movements. Stereotypical movements were: head-bobbing (repetitive upswings and downswings movements of the head) and oral movements (repetitive mastication, chewing or gnawing). Non-stereotypical movements were: exploring the cage (the mouse moves around the cage, including rearing posture), self-grooming (the mouse grooms its hands or hind legs), and quiet (lying on the floor).

2.3.6. PPI studies
This test consists in a reduction of the startle reflex when a weak prepulse stimulus immediately precedes the pulse that triggers the startle response. PPI was measured as previously described (Fernandez-Espejo and Galan-Rodriguez, 2004), in startle chambers (Cybertec Co., Madrid, Spain) consisting of a Plexiglas non-restrictive enclosure (28.5 × 15 × 3.5 cm) positioned in a ventilated and well-lit chamber. In this enclosure, the mice could move horizontally, but vertical movements were fully restricted by a ceiling. High frequency speakers, mounted 15 cm above the enclosure, produced all acoustic stimuli. The floor platform had a piezoelectric accelerometer mounted under it that transduced animal movements. Sound levels for different stimuli and background noise were calibrated with a digital sound level meter. Startle response amplitude was measured in mNw, and a dynamic calibration system was used to ensure comparable sensitivities. After a period of 5-min habituation in the enclosure (only with 65 dB background noise), mice were exposed to 67 trials randomly presented within a 20-min session. Trials were: 18 PULSE alone trials in which 30-ms broadband 120 dB stimuli were presented; 8 × 3 PREPULSE + PULSE trials in which 20-ms long 75 or 85 dB stimuli (10 and 20 dB above background noise) preceded the 120-dB pulse by 100 ms (onset to onset); 5 × 3 PREPULSE alone trials in which 20-ms long 10 and 20 dB stimuli (above background noise) were presented, and 7 NO STIMULUS trial, in which only the background noise (65 dB) was presented. The test session began with three trials of 120-dB pulses 100 ms apart each, for quantifying basal startle response. Trials were presented in pseudo-random order. Percent PPI was calculated as a percentage score: 

\[
\%PPI = \frac{[100 – \text{startle response to PREPULSE + PULSE trial}]}{\text{[startle response to PULSE ALONE trial]}} \times 100
\]

2.3.7. Statistical analysis
The locomotor activity data were analyzed using a two-way ANOVA with repeated measures (time as within subjects and dose as between subjects factors). When significant interactions were observed, a one-way ANOVA was used to analyze the effect of individual factors followed by a Dunnett’s post hoc test for individual comparisons. The catalepsy data were analyzed using one-way ANOVA for the effect of dose administered. The apomorphine-induced stereotypes data were analyzed using the non-parametric Kruskal-Wallis test followed by Mann–Whitney test for comparison between two groups since the data did not follow a normal distribution. The data for the startle response and the PPI were analyzed with one-way ANOVA (treatment as between subjects factor) followed by the Newman–Keuls post hoc test.

3. Results
3.1. Molecular modeling
The structure of QF2004B has been modeled and docked inside homology models obtained for two of the most relevant receptors for the antipsychotic effects; the 5-HT2A and 5-HT2C receptors. The structures of the complexes and the most relevant interactions are shown in Fig. 2. The ligand occupies the binding site in an orientation lining the helix 3 of the receptors, as previously found for ketanserin and other ligands sharing a common scaffold with the QF2004B molecule (conformationally constrained butyrophenones moieties linked to a substituted piperidine ring) (Ravina et al., 2000). Among all the interactions observed in both receptors, the most relevant reproduces the key interactions published for similar compounds (Roth et al., 1998), which are in agreement with mutagenesis data for such ligands (i.e. ketanserin).

In the structure of QF2004B we can distinguish three characteristic moieties: a butyrophenone moiety, a piperidine ring, and a 6-fluorobenzisoxazol substituent. Regarding its docking into the serotonin 5-HT2A receptor, the nitrogen of the piperidine ring (positively charged at physiological pH) interacts with the negatively charged Asp3.32 (2.90 Å distance between the ligand nitrogen and the protein oxygen), and the side chain of Phe6.51 adopts a conformation nearly parallel to the piperidine ring. The nitrogen and the oxygen of the fluorobenzisoxazol
ring form hydrogen bonds with the nitrogen of Trp6.48 (3.5 Å) and the side chain of Ser3.34 (2.7 Å). Additionally, the tetrahydrocarbazol ring interacts with the side chain of Trp3.28 while its carbonyl forms a hydrogen bond with the side chain of Tyr7.43 (3.2 Å).

Regarding the 5-HT$_{2C}$ receptor, the best docking solutions found reproduce nearly the same interaction pattern, showing a few differences with respect to the solutions found for 5-HT$_{2A}$. In its interaction with Tyr7.43, the ligand tetrahydrocarbazol ring is placed more in parallel with respect to the residue side chain, probably establishing a π-stacking interaction, instead of forming a hydrogen bond and the Ser2.71 forms a hydrogen bond with the carbonyl of the butyrophenone moiety (3.8 Å), which was not observed in 5-HT$_{2A}$. However, the most remarkable difference found was the absence of the hydrogen bond between the ligand and the Trp6.48, located at the bottom of the binding pocket.

3.2. In vitro receptor binding profile of QF2004B

On the evaluation of the serotonin/dopamine antagonist activity, QF2004B showed a pK$_i$ value at 5-HT$_{2A}$ receptors of 8.93 and a pK$_i$ value at D$_2$ receptors of 6.85 (Fig. 3; Table 1). In these experiments, clozapine showed pK$_i$ values of 8.12 and 6.58 at 5-HT$_{2A}$ and D$_2$ receptors, respectively, whereas haloperidol showed pK$_i$ values of 7.28 and 8.48 at 5-HT$_{2A}$ and D$_2$ receptors, respectively (Table 1). Thus, following the classical prediction of in vivo activity based on the MI (see Introduction), QF2004B possesses a MI predictive of atypical antipsychotic effect slightly higher than clozapine (1.28 for

![Fig. 2. The image on the right hand side represents a general view of the binding sites found for the QF2004B compound on the models of 5-HT$_{2A}$ (in green) and of 5-HT$_{2C}$ (in orange). On the left hand side, the same sites are depicted in more detail, showing the residues exhibiting the more relevant interactions.](image1)

![Fig. 3. Competition of QF2004B for the binding of specific radioligands at the 5-HT$_{2A}$ and D$_2$ receptors. Non-specific binding was subtracted from total binding and normalized to the maximal binding in absence of QF2004B. Data are the mean ± S.E.M. of three independent experiments performed in triplicate.](image2)
operidol at several CNS-located receptors in in vitro and ex vivo assays. This was particularly evident at the D2 and 5-HT2C receptors. Interestingly, compound QF2004B, similarly to clozapine, behaved as an inverse agonist at 5-HT2C receptors, both on the PLC (pIC\textsubscript{50} = 6.40 ± 0.12 and 7.28 ± 0.16 for QF2004B and clozapine, respectively), and PLA\textsubscript{2} (pIC\textsubscript{50} = 6.50 ± 0.21 and 7.51 ± 0.13 for QF2004B and clozapine, respectively) signaling pathways (Table 1), whereas haloperidol was a neutral antagonist at these receptors.

QF2004B displayed an affinity similar to that reported for clozapine and haloperidol for D1 receptors (Table 1). The same was true for α1- and α2-adrenergic receptors, as well as for muscarinic M2 receptors. Regarding other receptors of the clozapine receptorome, QF2004B displayed minimal affinity for muscarinic M1 receptors (pKi < 5), whereas in the case of H1 receptors, this compound showed an affinity intermediate between that of clozapine and haloperidol (Table 1).

Considering the similar profile observed for QF2004B and clozapine in vitro assays, we evaluated the potential behavior of this compound as an atypical antipsychotic in different in vivo tests.

### 3.3. In vivo studies

#### 3.3.1. Locomotor activity

Spontaneous locomotor activity was evaluated in order to investigate the possible induction of motor side effects by QF2004B. The reference drugs clozapine and haloperidol, as well as compound QF2004B dose-dependently reduced locomotor activity as compared to vehicle (Fig. 4). In the case of haloperidol treatment, two-way ANOVA revealed a significant effect of time of measurement [F(2,56) = 17.44, P < 0.0001], a significant effect of dose [F(3,28) = 53.92, P < 0.0001], and a significant interaction between these two factors [F(6,56) = 4.93, P < 0.0001]. Subsequent one-way ANOVA showed a significant effect of haloperidol dose at each of the three time points measured (10, 20 and 30 min): [F(3,31) = 28.78, P < 0.0001], [F(3,31) = 31.4, P < 0.0001], [F(3,31) = 30.78, P < 0.0001], respectively. The Dunnett’s post hoc test showed that haloperidol significantly reduced horizontal locomotor activity at all time points and doses tested (0.33, 1.0 and 3.3 mg/kg) (P < 0.0001) with respect to vehicle administration (Fig. 4). Regarding clozapine, two-way ANOVA revealed a significant effect of time of measurement [F(2,56) = 25.97, P < 0.0001], a significant effect of dose [F(3,28) = 17.44, P < 0.0001], and a significant interaction between these two factors [F(6,56) = 2.45, P < 0.05].

![Fig. 4. Horizontal activity measured during 30 min at 10-min intervals in animals treated with vehicle, QF2004B, clozapine or haloperidol at different doses (0.33, 0.1 and 3.3 mg/kg). ***P < 0.0001 compared to vehicle administration (Dunnett’s post hoc test).](image-url)
Subsequent one-way ANOVA showed a significant effect of clozapine dose at each of the three time points measured (10, 20 and 30 min): \( F(3,31) = 10.26, P < 0.0001 \), \( F(3,31) = 8.62, P < 0.0001 \), \( F(3,31) = 8.39, P < 0.0001 \), respectively. The Dunnett’s post hoc test showed that clozapine significantly reduced horizontal locomotor activity at all time points as compared to vehicle administration, but only at the dose of 3.3 mg/kg (Fig. 4). For QF2004B, the two-way ANOVA revealed a significant effect of time of measurement \( F(2,74) = 30.24, P < 0.0001 \), a significant effect of dose \( F(3,37) = 27.49, P < 0.0001 \), and a significant interaction between these two factors \( F(6,74) = 5.05, P < 0.0001 \). Subsequent one-way ANOVA showed a significant effect of QF2004B dose at each of the three time points measured (10, 20 and 30 min): \( F(3,40) = 14.95, P < 0.0001 \), \( F(3,40) = 21.50, P < 0.0001 \), \( F(3,40) = 23.10, P < 0.0001 \), respectively. The Dunnett’s post hoc test showed that QF2004B at the doses of 1.0 and 3.0 mg/kg significantly reduced horizontal locomotor activity during the first 10 min of measurement. At 20 and 30 min, all doses of QF2004B significantly reduced locomotor activity with respect to vehicle administration (Fig. 4). The ED50 values for clozapine and haloperidol were 1.85 and 0.31 mg/kg, respectively (Table 2). Compound QF2004B showed an intermediate ED50 value of 1.19 mg/kg (Table 2).

3.3.2. Apomorphine-induced stereotypies

Apomorphine (2 mg/kg) induced head-bobbing, oral stereotypies, and self-grooming, while haloperidol, clozapine nor QF2004B induce stereotypical movements per se (data not shown). Apomorphine-induced head bobbing was significantly reduced by clozapine (0.3 mg/kg, \( P < 0.05 \); 1 mg/kg, \( P < 0.05 \)) and haloperidol (1 mg/kg, \( P < 0.01 \)), with ED50 values of 0.78 and 0.48 mg/kg, respectively (Table 2). Compound QF2004B significantly reduced apomorphine-induced head-bobbing at the dose of 1 mg/kg (\( P < 0.01 \)), with an ED50 value of 0.75 mg/kg (Table 2). None of the three compounds showed significant effects on apomorphine-induced oral stereotypies or self-grooming (data not shown). Additionally, haloperidol elicited a strong sedative effect as revealed by a significant reduction of non-stereotypical movements and a significant enhancement of quiet responses (Table 2). QF2004B and clozapine, however, were devoid of sedative effects.

Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Locomotor activity</th>
<th>APO-induced stereotypies test</th>
<th>Quiet response</th>
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<td></td>
<td>Head-bobbing</td>
<td>Non stereotypies</td>
<td>Quiet</td>
</tr>
<tr>
<td>QF2004B</td>
<td>1.19</td>
<td>0.75</td>
<td>n.s.</td>
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<tr>
<td>Clozapine</td>
<td>1.85</td>
<td>0.78</td>
<td>n.s.</td>
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<tr>
<td>Haloperidol</td>
<td>0.31</td>
<td>0.48</td>
<td>0.51</td>
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n.s., no significant difference with respect to mice pretreated with apomorphine (APO) plus vehicle.

3.3.3. Catalepsy test

The catalepsy test was carried out in order to evaluate the possible extra pyramidal side effects (EPS) profile of compound QF2004B compared to the reference compounds haloperidol and clozapine. Statistical analysis showed a significant main effect of haloperidol \( F(4,53) = 103.65, P < 0.0001 \), clozapine \( F(4,55) = 51.29, P < 0.0001 \), and QF2004B \( F(4,54) = 75.19, P < 0.0001 \) (data not shown). The potency of QF2004B to induce catalepsy in mice was similar to that of clozapine (ED50 values of 2.13 and 1.53 mg/kg, respectively), and lower to that of haloperidol (ED50 value of 0.38 mg/kg) (Table 2).

3.3.4. Effects of QF2004B, haloperidol and clozapine alone in the PPI test

Before testing the compounds in apomorphine- and DOI-treated mice, the effects of QF2004B, haloperidol and clozapine alone on startle and PPI were studied, in comparison to vehicle-treated mice. QF2004B, clozapine and haloperidol alone significantly reduced startle amplitude only after the highest dose tested (1 mg/kg, \( P < 0.01 \) vs. vehicle; haloperidol, \( P < 0.05 \)), as shown in Table 3. Normal PPI was not affected by any dose tested of QF2004B, haloperidol or clozapine, as shown in Fig. 5.

3.3.5. Apomorphine-induced disruptions in PPI

Apomorphine did not reduce the startle response as compared to vehicle (Table 3). QF2004B and clozapine significantly reduced the startle response in apomorphine-treated mice after all the doses tested (treatment effect, \( F(5,74) = 4.20, P < 0.01 \)) for QF2004B, \( F(5,72) = 3.4, P < 0.05 \) for clozapine), indicating a strong hyporeactive effect of these compounds in mice treated with apomorphine (Table 3). Haloperidol injection however, induced a significant reduction of the startle response in apomorphine-treated mice only at the highest dose tested (1 mg/kg, \( P < 0.01 \)) (Table 3). Since there was a dramatic reduction in the startle response after QF2004B and clozapine (see Table 3), subgroups equalized for startle amplitude were selected for the PPI study. By using these equalized subgroups, apomorphine disrupted PPI at 10 dB \( P < 0.05 \) and 20 dB prepulses \( P < 0.01 \), and this disruption of the PPI response was not significantly affected by QF2004B or clozapine (Fig. 6A). However, haloperidol counteracted the apomorphine-induced disruption of PPI at the three doses tested with 20 dB prepulse intensity \( F(5,72) = 4.40, P < 0.01 \) (Fig. 6A). Therefore, only haloperidol was able to reverse the apomorphine-induced disruption of PPI at doses not inducing hyporeactive effects on the basal startle response of mice, whereas both QF2004B and clozapine did not affect PPI disruption by apomorphine.

3.3.6. DOI-induced disruptions in PPI

DOI did not significantly reduce the startle response as compared to vehicle (Table 3). Neither haloperidol nor clozapine injection induced significant changes in the startle response at any of the doses tested in DOI-treated mice (Table 3). QF2004B significantly reduced the startle response...
only at the dose of 1 mg/kg (F(5,74) = 3.3, P < 0.01); P < 0.05 versus vehicle plus DOI; P < 0.01 versus vehicle plus vehicle), but not at the dose of 0.1 or 0.33 mg/kg (Table 3). DOI disrupted PPI at 10 (P < 0.01) and 20 dB prepulses (P < 0.01). QF2004B pre-treatment counteracted the DOI-induced disruption of PPI at the dose of 0.1 mg/kg at both prepulse intensities [F(5,74) = 3.58, P < 0.01; 20 and 30 dB, P < 0.05], and at the dose of 0.33 mg/kg at 10 dB prepulse (P < 0.05) (Fig. 6B). Clozapine counteracted DOI-induced disruption of PPI at the dose of 0.33 and 1 mg/kg at 10 dB prepulse [F(5,74) = 3.2, P < 0.05], and at all three doses tested at 20 dB prepulse [F(5,74) = 3.9, P < 0.05]. The DOI-induced disruption of PPI was counteracted by all doses of haloperidol tested at 10 dB prepulse [F(5,74) = 4.7, P < 0.05], and by 0.1 mg/kg at 20 dB prepulse [F(5,74) = 3.3, P < 0.05] (Fig. 6B). Thus, QF2004B, clozapine, and haloperidol were able to counteract DOI-induced PPI disruption at doses that did not produce hyporeactive effects on the basal startle response in mice.

4. Discussion

In this work we present the in vitro and in vivo pharmacological characterization of a new antipsychotic lead compound, QF2004B for which we also describe a more efficient synthetic route. This compound originated from a line of investigation aimed at generating leads for new antipsychotics with pharmacological activity at multiple receptors.

The results obtained in the in vitro studies demonstrate that QF2004B is a serotonin/dopamine antagonist with a MI similar to clozapine (MI = 1.28 for QF2004B and 1.23 for clozapine). This is one of the first criteria used for selecting antipsychotic candidates with atypical profile in drug discovery. However, this index results insufficient for prediction of an atypical antipsychotic profile similar to clozapine, since it does not take into account the affinity displayed by clozapine at other receptors apart from 5-HT2A/D2 receptors (Meltzer et al., 2003). Therefore, we evaluated the pharmacological profile of compound QF2004B, from a comprehensive perspective, on a battery of G-protein coupled receptors on which clozapine is active (5-HT2C, and 5-HT1A serotonergic, D1, D3, and D2 dopaminergic, α1- and α2-adrenergic M1, and M2 muscarinic, and H1 histaminergic receptors). The results revealed a profile for QF2004B similar to clozapine at most of these receptors.

Although some studies have implicated 5-HT2C receptors in the therapeutic efficacy of atypical antipsychotics, recently it has been proposed that a combination of 5-HT2A and 5-HT2C

![Fig. 5. Effects of QF2004B, clozapine, and haloperidol (0, 0.1, 0.33 and 1.0 mg/kg) per se on the PPI test. Data are mean ± S.E.M percent PPI (%) at prepulse intensities of 10 and 20 dB.](image-url)
blockade is a more efficient mechanism for increasing antipsychotic effects than blockade of either receptor alone (Meltzer et al., 2003). Hence, 5-HT2C antagonism can prevent the EPS induced by haloperidol, suggesting that 5-HT2C receptors contribute to the observation of relatively mild EPS by atypical antipsychotics. In addition, by enhancing dopamine release in the cortex, 5-HT2C antagonists would efficiently counteract the hypofrontality that contributes to the negative symptoms of schizophrenia (Knable and Weinberger, 1997). Therefore, the affinity of QF2004B at 5-HT2C receptors could confer a beneficial component to its antipsychotic profile. In particular, the observed profile of QF2004B as an inverse agonist at constitutive 5-HT2C receptors could be relevant for its possible therapeutic effects on the negative symptoms of schizophrenia, and for preventing EPS (Herrick-Davis et al., 2000).

Due to the involvement of 5-HT2C receptors in schizophrenia, and taking into account their pharmacological similarity with 5-HT2A receptors, we performed docking studies of QF2004B inside the homology models of both 5-HT2A and 5-HT2C receptors. The most important finding in these studies is that QF2004B interacts differently with residues suspected important for the activation mechanism of G protein-coupled receptors, namely Trp6.48 at 5-HT2A and 5-HT2C receptors, and which could have implications in the different pharmacological behavior of compounds with a similar scaffold at 5-HT2A/5-HT2C receptors.

Similar to clozapine, QF2004B showed high affinity for 5-HT1A receptors by QF2004B, coupled with a relatively weak D2 receptor antagonism may enhance the atypical antipsychotic profile of this compound.

QF2004B showed affinity for dopamine D1, D3 and D4 receptors. D1 receptors are predominant in the prefrontal cortex, an area involved in cognitive functions and related to the negative symptoms in schizophrenia (Lynch, 1992), two aspects for which atypical antipsychotic therapy can be advantageous in comparison with conventional antipsychotics. However, D1 affinity does not seem to be a discriminative factor between typical and atypical antipsychotics, as drugs from both groups possess similar affinities for this receptor. D3 and D4 receptors are highly expressed in limbic and cortical areas, and the fact that clozapine has preferential affinity for the D4 receptors supports an involvement of these receptors in schizophrenia. Compound QF2004B showed a similar profile to that of clozapine at blocking D3 and D4 receptors as well.

Based on the observed minimal affinity of QF2004B for muscarinic M1 receptors (pKᵢ < 5), neither cardiac nor gastrointestinal side effects associated with anticholinergic activity (Owens, 1996), nor cognitive impairments are expected for this compound. In the case of H1 receptors, the affinity of QF2004B for this receptor, lower than that of clozapine, could be promising considering that antipsychotics with low affinity for H1 receptors are less likely to induce weight gain, somnolence, or metabolic side effects (Roth et al., 2004b).

First-line atypical antipsychotics chemically unrelated to clozapine, such as ziprasidone or risperidone display particular multi-receptor profiles, that in some cases could explain their differential clinical behavior. Hence, patients benefit from the
lack of weight gain side effects of ziprasidone in comparison with other atypical antipsychotics due to its low affinity for H1 and α1-adrenergic receptors (Roth et al., 2004b). The lower affinity of risperidone for muscarinic receptors as compared to clozapine could account for its lack of anticholinergic side effects, especially advantageous for the treatment of psychosis associated with dementia in the elderly. Thus, the fact that QF2004B is a butyrophenone derivative, chemically unrelated to clozapine underscores the potential interest of this compound as a lead for a new line of antipsychotics lacking the more severe side effect of clozapine, namely agranulocytosis, which would be elicited through an unknown mechanism apparently not related with any of the receptor targets assessed in this study.

In behavioral studies, compound QF2004B showed an intermediate ED50 value (1.19 mg/kg) for reducing locomotor activity as compared to clozapine (ED50 value 1.85 mg/kg) and haloperidol (ED50 value 0.31 mg/kg). Effects of antipsychotic drugs on motor activity are generally attributed to blockade of D2 receptors, which are involved in the initiation of movement (Hauber, 1996). In this regard, a good association between D2 blockade and reduction in motor activity was found for the three compounds tested (haloperidol > QF2004B > clozapine).

Apomorphine-induced stereotypies are considered as a “psychotic-like” sign in rodents, (Giuliani and Ferrari, 1997), and stereotypical movements are observed in psychotic patients, which can be exacerbated by dopaminergic agonists and attenuated by antipsychotics. Compound QF2004B significantly reduced apomorphine-induced head-bobbing at the dose of 1 mg/kg, with an ED50 value of 0.75 mg/kg. In addition, similar to clozapine it was devoid of sedative effects, which could be at least partially explained by the low affinity for H1 receptors observed for both compounds.

In the catalepsy test, a helpful tool to predict the EPS produced by antipsychotics, QF2004B and clozapine showed lower potencies than that of haloperidol, in agreement with an atypical profile of both compounds. However, under the experimental conditions of our study, we found that clozapine produced catalepsy at lower doses that those described in the literature (Morimoto et al., 2002). This discrepancy may be due to differences in the methodology used. For example, we have used the “ring test” to measure catalepsy, which may be a more sensitive test than the more common “horizontal bar-test catalepsy” (Meschler et al., 2000).

To characterize the antipsychotic activity of QF2004B, we carried out experiments using the prepulse inhibition (PPI) test. While this test has been extensively validated in rats as a behavioral model for predicting antipsychotic effects (see review by Geyer et al., 2001), studies in mice using this model are more recent (Dulawa and Geyer, 1996; McCaughran et al., 1997; Curzon and Decker, 1998). In addition, species differences regarding the pharmacology of PPI have been suggested. Thus, D1 receptors appear to be primarily responsible for the PPI-disruptive effects of direct DA agonists in mice, while D2 receptors appear to be more critically involved in rats (Ralph-Williams et al., 2002, 2003).

Our findings indicated that neither haloperidol, clozapine nor QF2004B administered alone altered PPI. In contrast to the present result, other studies have shown that PPI is enhanced after haloperidol and clozapine in different inbred mouse strains (McCaughran et al., 1997; Olivier et al., 2001; Ouagazzal et al., 2001). However, our experiments were carried out in Swiss albino mice, an outbred strain, which may account for this apparent discrepancy. Indeed, in agreement with our results, no significant intrinsic effects of haloperidol and clozapine were found in the PPI test when using CD1 mice, another outbred strain (Curzon and Decker, 1998).

It is worth noting that in the startle experiments, clozapine and QF2004B administered by themselves induced hyperreactivity only after the highest dose, but when these compounds were administered with apomorphine, all three doses of clozapine and QF2004B reduced startle. This unexpected result contrasts with the finding that haloperidol reduced startle only at the highest dose when injected with either vehicle or apomorphine.

In accordance with several other studies in mice (Curzon and Decker, 1998; Geyer et al., 2001, 2002; Malone et al., 2004; Russig et al., 2004), we show that haloperidol significantly counteracted the apomorphine-induced disruption of PPI, and in addition we show for the first time that haloperidol significantly modified the DOI-induced disruption of PPI. On the other hand, under our experimental conditions, clozapine and QF2004B were able to counteract DOI-induced PPI disruption only. Despite the fact that there are no data in mice looking at the effects of antipsychotics in DOI-induced deficits in PPI, studies in rats suggest that this model can provide evidence for the involvement of the serotonergic system in the action of new antipsychotics (Sipes and Geyer, 1995; Geyer et al., 2001). Based on these data, together with our other in vivo findings, we can predict an atypical antipsychotic activity for QF2004B with low propensity to induce EPS at therapeutic doses.

In conclusion, we describe an efficient synthesis pathway for QF2004B, a compound that mirrored the characteristics of clozapine in terms of its receptor binding profile and inverse agonist activity at 5-HT2C receptors. This compound showed a profile very similar to that of clozapine in a broad battery of in vivo tests predictive of antipsychotic activity and atypical profile. Considering that QF2004B is a butyrophenone analogue with a chemical structure unrelated to clozapine, we do not expect for this compound severe adverse effects specifically associated to the clozapine molecule, like agranulocytosis. Therefore, we propose QF2004B as a new lead compound in the discovery and development of new antipsychotics with a profile similar to clozapine.

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