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RESEARCH PAPER

Preclinical and clinical characterization of the selective 5-HT$_{1A}$ receptor antagonist DU-125530 for antidepressant treatment

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BACKGROUND AND PURPOSE

The antidepressant efficacy of selective 5-HT reuptake inhibitors (SSRI) and other 5-HT-enhancing drugs is compromised by a negative feedback mechanism involving 5-HT$_{1A}$ autoreceptor activation by the excess 5-HT produced by these drugs in the somatodendritic region of 5-HT neurones. 5-HT$_{1A}$ receptor antagonists augment antidepressant-like effects in rodents by preventing this negative feedback, and the mixed β-adrenoceptor/5-HT$_{1A}$ receptor antagonist pindolol improves clinical antidepressant effects by preferentially interacting with 5-HT$_{1A}$ autoreceptors. However, it is unclear whether 5-HT$_{1A}$ receptor antagonists not discriminating between pre- and post-synaptic 5-HT$_{1A}$ receptors would be clinically effective.

EXPERIMENTAL APPROACH

We characterized the pharmacological properties of the 5-HT$_{1A}$ receptor antagonist DU-125530 using receptor autoradiography, intracerebral microdialysis and electrophysiological recordings. Its capacity to accelerate/enhance the clinical effects of fluoxetine was assessed in a double-blind, randomized, 6 week placebo-controlled trial in 50 patients with major depression (clinicaltrials.gov identifier NCT01119430).

KEY RESULTS

DU-125530 showed equal (low nM) potency to displace agonist and antagonist binding to pre- and post-synaptic 5-HT$_{1A}$ receptors in rat and human brain. It antagonized suppression of 5-hydroxytryptaminergic activity evoked by 8-OH-DPAT and SSRIs *in vivo*. DU-125530 augmented SSRI-induced increases in extracellular 5-HT as effectively as in mice lacking 5-HT$_{1A}$ receptors, indicating a silent, maximal occupancy of pre-synaptic 5-HT$_{1A}$ receptors at the dose used. However, DU-125530 addition to fluoxetine did not accelerate nor augment its antidepressant effects.

CONCLUSIONS AND IMPLICATIONS

DU-125530 is an excellent pre- and post-synaptic 5-HT$_{1A}$ receptor antagonist. However, blockade of post-synaptic 5- HT$_{1A}$ receptors by DU-125530 cancels benefits obtained by enhancing pre-synaptic 5-hydroxytryptaminergic function.

Abbreviations

5-HT, serotonin; CGI, Clinical Global Impression; DR, dorsal raphe nucleus; HDRS-17, Hamilton Depression Rating Scale of 17 items; KO, knockout; LOCF, Last observation-carried- forward; MADRS, Montgomery- Asberg Depression Rating Scale; MDD, major depression diagnosis; mPFC, Medial prefrontal cortex; OC, Observed cases; SERT, 5-HT transporter; SSRI, selective 5-HT reuptake inhibitors; WT, wild type

Introduction

Major depression is a severe psychiatric syndrome with high prevalence and socioeconomic impact (Greenberg *et al.*, 2003; Andlin-Sobocki and Wittchen, 2005; Lopez *et al.*, 2006; World Health Organization, 2008). Most of the prescribed antidepressants, the selective 5-HT reuptake inhibitors (SSRI) and the dual 5-HT and noradrenaline reuptake inhibitors, block physiological reuptake mechanisms in 5-hydroxytryptaminergic axons and thereby they increase extracellular 5-HT concentration in forebrain to activate post-synaptic 5-HT receptors required for clinical effects. However, this process is severely compromised by the simultaneous activation of pre-synaptic 5-HT_{1A} receptors (receptor nomenclature follows Alexander *et al.*, 2011) located somatodendritically on 5-HT neurones (5-HT_{1A} autoreceptors) of the midbrain raphe nuclei (Pazos and Palacios, 1985; Pompeiano *et al.*, 1992). The excess 5-HT produced by reuptake inhibition in midbrain activates 5-HT_{1A} autoreceptors, thereby reducing 5-hydroxytryptaminergic neurone activity and terminal 5-HT release (Bel and Artigas, 1992; Blier and De Montigny, 1994; Romero and Artigas, 1997; Lopez *et al.*, 2006), an effect contrary to that required for therapeutic response.

The limited clinical efficacy of 5-HT-enhancing drugs and their delayed action are partly due to this negative feedback mechanism. Upon chronic treatment, 5-HT_{1A} autoreceptors desensitize, leading to the recovery of 5-hydroxytryptaminergic activity and enhanced 5-HT release (Blier and De Montigny, 1994; Artigas *et al.*, 1996). Hence, pharmacological or genetic suppression of 5-HT_{1A} autoreceptor activity enhances the neurochemical and behavioural effects of SSRI in rodents (Artigas *et al.*, 1996; Romero and Artigas, 1997; Knobelman *et al.*, 2001; Bortolozzi *et al.*, 2004; Richardson-Jones *et al.*, 2010). Moreover, patients with a gene polymorphism leading to high 5-HT_{1A} autoreceptor expression are more susceptible to depression and suicide and respond poorly to antidepressant therapy (Stockmeier *et al.*, 1998; Lemonde *et al.*, 2003; 2004; Neff *et al.*, 2009).

Therefore, 5-HT_{1A} receptor antagonists might be useful to improve antidepressant therapy as they could prevent 5-HT_{1A}-autoreceptor-mediated negative feedback. Hence, the non-selective β-adrenoceptor/5-HT_{1A} receptor antagonist pindolol accelerates and, in some instances, increases the efficacy of SSRIs (Artigas *et al.*, 1994; 2001; Blier and Bergeron, 1995; Perez *et al.*, 1997; Ballesteros and Callado, 2004; Whale *et al.*, 2010). However, its complex pharmacology, including its anti-hypotensive effects, limits it clinical use. Pindolol shows a preferential affinity and occupancy of 5-HT_{1A} autoreceptors compared with post-synaptic 5-HT_{1A} receptors in rodent (Serrats *et al.*, 2004) and human (Martinez *et al.*, 2001) brains. In contrast, the prototypical 5-HT_{1A} receptor antagonist WAY-100635 (not available for human use) interacts equally with pre- and post-synaptic 5-HT_{1A} receptors (Forster *et al.*, 1995; Fletcher *et al.*, 1996). Given the requirement to activate post-synaptic 5-HT_{1A} receptors to achieve antidepressant effects in rodents (Haddjeri *et al.*, 1998; Blier and Ward, 2003), it is unclear whether selective 5-HT_{1A} receptor antagonists with equal potency at pre- and post-synaptic 5-HT_{1A} receptors would be clinically effective. Testing this working hypothesis has not been possible so far due to the lack of 5-HT_{1A} receptor antagonists available for human use.

Table 1

In vitro receptor binding profile of DU-125530 for monoaminergic receptors

Receptor	Affinity (nM)
5-HT_{1A}	0.7
5-HT_{1B}	890
5-HT_{1D}	1200
5-HT_{2A}	240
5-HT_{2C}	750
5-HT_3	1100
α_1-adrenoceptor	6.4
Dopamine D_2	5.2
Dopamine D_3	11

Data taken from Mos *et al.* (1997).

Preliminary data indicate that the 5-HT_{1A} receptor antagonist DU-125530 shows high affinity for 5-HT_{1A}- receptors and ≥10-fold selectivity versus other monoaminergic receptors (Mos *et al.*, 1997) (see also Table 1) and antagonizes behavioural effects induced by 5-HT_{1A} receptor agonists in rodents (Joordens *et al.*, 1998; Olivier *et al.*, 1998). Likewise, it occupies pre- and post-synaptic receptors in human brain, as demonstrated by PET scan studies (Rabiner *et al.*, 2002). However, a full characterization of its pharmacological properties is lacking. Therefore, we carried out a collaborative translational study in which we examined the ability of DU-125530: (i) to interact with pre- and post-synaptic 5-HT_{1A} receptors; and (ii) to accelerate or enhance the antidepressant action of fluoxetine.

Methods

Preclinical studies

Animals. All animal care and experimental procedures followed the European Union regulations (OJ of EC L358/1 18/12/1986) and were approved by the Institutional Animal Care and Use Committee. Male albino Wistar rats (230–300 g; Iffa Credo, Lyon, France; total number used: 69) and C57/Bl6J male mice (10–15 weeks old; Iffa Credo; total number used: 41) were kept in a temperature-controlled environment (12 h light–dark cycle) with food and water provided *ad libitum*. Stereotaxic coordinates (in mm) were taken from bregma and duramater according to the atlas of Paxinos and Watson (1998).

Methods. To examine the ability of DU-125530 to interact with pre- and post-synaptic 5-HT_{1A}R in rodent brain, we used receptor autoradiography, single unit extracellular recordings of 5-hydroxytryptaminergic neurones in the dorsal raphe nucleus and of pyramidal neurones in medial prefrontal cortex as well as microdialysis studies, following standard methods routinely used in our laboratory and reported elsewhere (Romero and Artigas, 1997; Amargos-Bosch *et al.*,

2004; Serrats *et al.*, 2004; Diaz-Mataix *et al.*, 2005). A detailed description can be found in the 'Supplementary Methods' section.

Data treatment. In autoradiographic studies, inhibition curves were statistically analysed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

Changes in discharge rate were quantified by averaging the values in the third minute after each drug injection. Drug effects were assessed using Student's *t*-test or one-way repeated-measures ANOVA, as appropriate. Data are expressed as the mean ± SEM. Statistical significance has been set at the 95% confidence level.

Dialysate 5-HT concentrations were measured as fmol per fraction and are expressed in the Figures as percentages of baseline (set to 100%). Statistical analysis was carried out using repeated-measures ANOVA using treatment and time as variables.

Materials. 8-OH-DPAT [8-hydroxy-2-(di-*n*-propylamino) tetralin] was from Sigma-Aldrich (St. Louis, MO). Fluoxetine [*N*-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine] was from Tocris (Bristol, UK). Paroxetine [(3*S*,4*R*)-3-[(2*H*-1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl) piperidine] was generously provided by GSK (London, UK). DU-125530 [2-[4-[4-(7-chloro-2,3-dihydro-1,4-benzodioxin-5-yl)-1-piperazinyl]butyl]-1,2-benzisothiazol-3(2*H*).-one-1, 1-dioxide] was from Solvay Pharma (Brussels, Belgium). (+/–)WAY-100635 [*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl]-*N*-(2-pyridinyl)cyclohexanecarboxamide] hydrochloride was from RBI (Natick, MA). Stock solutions were prepared, and aliquots were stored at –20°C. Working solutions were prepared daily by dilution in saline at the appropriate concentrations. Doses are expressed as weight of free bases.

Clinical trial

Patients. Consecutive eligible patients aged 18 to 70, referred by general practitioners of primary care centers or from psychiatric emergency services (Catalonian Public Health Service), were recruited. Inclusion criteria were as follows: diagnosis of unipolar major depression using DSM-IV criteria with moderate to severe symptoms (≥18 on the Hamilton Depression Rating Scale of 17 items, HDRS-17). There was a wash-out of 1 week of any antidepressant drug (except for fluoxetine, 28 days) before entering the study. Written informed consent was obtained from all participants. Exclusion criteria were as follows: concurrent psychiatric disorders (DSM IV axis I, II cluster A or B); failure to respond to drug treatment in current depressive episode; previous resistance to antidepressant drugs, including SSRI; suicide risk score ≥3 on the HDRS; participation in other drug trials within the previous month; presence of delusions or hallucinations; history of substance abuse (including alcohol) in the past three months; pregnancy or lactation; serious organic illnesses in the past 6 months; frequent or severe allergic reactions; concomitant use of other psychotropic drugs (benzodiazepines were allowed) and blockers or catecholamine-depleting agents; current structured psychotherapy.

Study variables. Demographic and clinical data were collected. Likewise, any other relevant clinical information to the study was recorded: number of previous episodes, age at first depressive episode, melancholic features and medical history.

The primary variable of the clinical trial was the HDRS score. Sustained response was defined as a 50% or greater decrease in the admission HDRS score maintained until day 42, allowing a 5% variation during intermediate visits. Sustained remission was defined as an HDRS score of 8 or less maintained until endpoint. Secondary variables were the Montgomery-Asberg Depression Rating Scale (MADRS) and the Clinical Global Impression (CGI). Safety was assessed by means of biochemical variables and vital signs. ECGs were performed at admission, 2 weeks after beginning active treatment and at the end of the study. Plasma concentration of fluoxetine was obtained at 3 weeks of treatment and at the end of the trial.

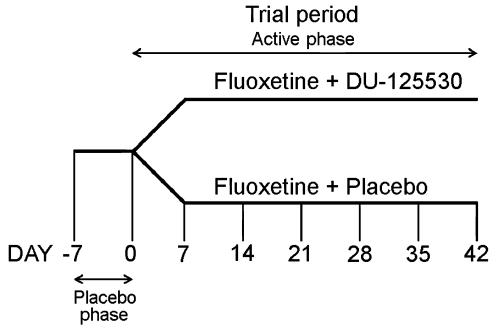

Figure 1
Clinical study design. Patients entered in a single-blind placebo phase of 3–7 days. Patients showing a reduction of 25% or greater of their HDRS score or with a decrease to below 18 at day 0 were excluded. Patients entering the study were randomized on day 0 to one of two treatment arms: fluoxetine + placebo or fluoxetine + DU-125530.

Study design. The design of the study (Figure 1) was the same as that of a previous study assessing the effect of pindolol addition to fluoxetine treatment (Perez *et al.*, 1997) and had two active treatment arms: fluoxetine + placebo and fluoxetine + DU-125530 after a placebo run-in phase.

Placebo phase. After obtaining informed consent, patients entered a single-blind placebo run-in period of 3–7 days. Patients showing a ≥25% reduction of their admission HDRS score or an HDRS score lower than 18 during this period were excluded.

Active phase. Patients entering the study were randomized and assigned (day 1) to one of two treatment arms: fluoxetine 20 mg·day^{-1} plus placebo or fluoxetine 20 mg·day^{-1} plus DU-125530 (20 mg·day^{-1}). Patients, investigators and all personal participating in the study were unaware of the treatments (double-blind). The active phase lasted for 6 weeks. Clinical assessments were carried out on day 1 and every 7 days (±3 days) until day 42. Compliance was assessed by direct questioning patients and by counting returned pills and capsules at follow-up visits. Side effects were requested at each visit.

The study was approved by the Ethics Committee of the Hospital de Sant Pau and was registered with the US National Institutes of Health Protocol Registration System (NCT01119430). An independent researcher (Ignasi Gich, MD, Department of Clinical Pharmacology, Hospital de Sant Pau), not involved in the clinical trial, carried out the randomization by means of computer-generated random numbers.

Data treatment and statistical analyses.

The planned sample size for this study was 100 randomly assigned patients (50 in each treatment group), chosen to provide approximately 75% power to detect a difference in the percentage of responders at endpoint of 60% for fluoxetine plus placebo an 80% for fluoxetine plus DU-125530 using a one-sided 0.05 level test. Given the absence of previous trials using DU-125530, the use of one-sided test was considered to be more appropriate than increasing the sample size. Thus, one-sided *P*-values were used in safety and efficacy analyses. Data are given as means (SD). All scores were computed using a last observation-carried-forward (LOCF) approach. All analyses were done by intention to treat. Additional analysis of the observed cases (OC) was carried out. An interim analysis was performed at $n = 50$ (half of the planned sample), which met the criteria to stop the trial.

Main analysis was performed using repeated-measures ANOVA, with time (eight time points) as the within-subjects factor and group (fluoxetine + placebo vs. fluoxetine + DU-125530) as the between-subjects factor. A Huynh–Feldt correction was used where the assumption of sphericity was violated (uncorrected d.f. reported). Further differences were assessed by means of *post hoc* analyses. All randomized patients who had a baseline and at least one post-baseline score were included in the analyses. One-way ANOVA (treatment group as the between-subjects factor) was used to examine group differences with other clinical variables. Additionally, a survival analysis was done to establish the velocity of each treatment arm. All statistics were performed by means of statistical package for Windows SPSS 17.0.

Results

Characterization of DU-125530 as a 5-HT$_{1A}$ receptor antagonist: preclinical studies

Quantitative receptor autoradiography. The binding of the 5-HT$_{1A}$ receptor agonist [^{3}H]8-OH-DPAT and the corresponding antagonist [^{3}H]WAY-100635 to rat brain structures was inhibited by DU-125530 at low nanomolar concentrations, as illustrated in Figure 2A. Displacement curves of DU-125530 against the two radioligands, as generated from microdensitometric data, fitted to the one site binding competition model (Figure 3). The calculated pIC$_{50}$ values of DU-125530 for both ligands did not differ among the regions examined (Table 2).

In the human hippocampus (Figure 2B), DU-125530 also displaced [^{3}H]WAY-100635 binding with high affinity and produced monophasic displacement curves (not shown). The pIC$_{50}$ values calculated for the CA1 hippocampal field and the perirhinal cortex are reported in Table 3.

Electrophysiological studies. We examined the ability of DU-125530 to reverse the inhibition of the discharge rate of DR 5-hydroxytryptaminergic neurones induced by the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT and the SSRI fluoxetine. The administration of DU-125530 (67–134 μg·kg^{-1} i.v.) did not alter the firing rate of 5-hydroxytryptaminergic neurones by itself (1.9 ± 0.3 spikes per second vs. 1.7 ± 0.3 spikes per second in baseline conditions; n.s.; $n = 7$). However, DU-125530 fully reversed the decrease in firing rate induced by 8-OH-DPAT (1.2–4.8 μg·kg^{-1} i.v.) in all neurones examined ($F_{3,12} = 3.9$; $P < 0.04$; $n = 5$; Figure 4A,C). The subsequent administration of increasing doses of WAY-100635 (5–60 μg·kg^{-1} i.v.) did not increase the 5-hydroxytryptaminergic firing rate further, indicating full antagonism by DU-125530.

Similarly, DU-125530 (67–134 μg·kg^{-1} i.v.) reversed the reduction in DR 5-hydroxytryptaminergic firing rate produced by fluoxetine (0.8–4 mg·kg^{-1} i.v.) ($F_{3,15} = 4.0$; $P < 0.03$; $n = 6$; Figure 4B,D). Likewise, the subsequent administration of WAY-100635 (5–20 μg·kg^{-1} i.v.) did not augment the reversal elicited by DU-122530.

DU-12530 also reversed the effect produced by 8-OH-DPAT on medial prefrontal cortex pyramidal neurones in the few cases examined (see two examples in Figure 4E,F).

Microdialysis studies. We assessed the putative antagonist properties of DU-125530 at pre- and post-synaptic 5-HT$_{1A}$ receptors controlling 5-HT release *in vivo* in rats and mice (WT and 5-HT$_{1A}$ receptor *knockout*-KO) using four different experimental models: (i) antagonism of systemic 8-OH-DPAT-induced reduction of 5-HT release; (ii) reversal of paroxetine-induced reduction of 5-HT release (with local 5-HT reuptake inhibition in medial prefrontal cortex; mPFC); (iii) reversal of local 8-OH-DPAT application in mPFC; and (iv) augmentation of SSRI effect on extracellular 5-HT in mPFC.

Rat experiments. The systemic administration of DU-125530 (3 mg·kg^{-1} s.c.) did not significantly modify 5-HT release in mPFC (vehicle + vehicle, $n = 6$; vehicle + DU-125530, $n = 7$). However, its administration (3 mg·kg^{-1} s.c.) prevented the reduction of 5-HT release evoked by 50 μg·kg^{-1} s.c. 8-OH-DPAT (treatment $F_{1,7} = 7.8$, $P < 0.03$; time $F_{15,105} = 2.4$, $P < 0.01$ and treatment × time interaction $F_{15,105} = 5.3$; $P < 0.0001$; Figure 5A). To test the capacity of DU-125530 to antagonize the actions of 5-HT at somatodendritic 5-HT$_{1A}$ autoreceptors, we used an experimental paradigm in which an SSRI (e.g. paroxetine) is administered systemically while locally blocking the 5-HT transporter (SERT) with citalopram in the sampling forebrain area. In these experimental conditions, the systemically administered SSRI cannot further block SERT in the sampling area (e.g. mPFC), but it does in midbrain, where the increase in extracellular 5-HT activates 5-HT$_{1A}$ autoreceptors, thus reducing terminal 5-HT release (Romero and Artigas, 1997). In these conditions, paroxetine (3 mg·kg^{-1} s.c.) significantly reduced 5-HT release in mPFC, an effect significantly antagonized by DU-125530 administration (3 mg·kg^{-1} s.c.) (time $F_{15,120} = 38.7$; $P < 0.0001$ and treatment × time interaction $F_{15,120} = 3.5$; $P < 0.0001$; Figure 5B).

To examine the ability of DU-125530 to block post-synaptic 5-HT$_{1A}$ receptors, we locally applied 8-OH-DPAT in

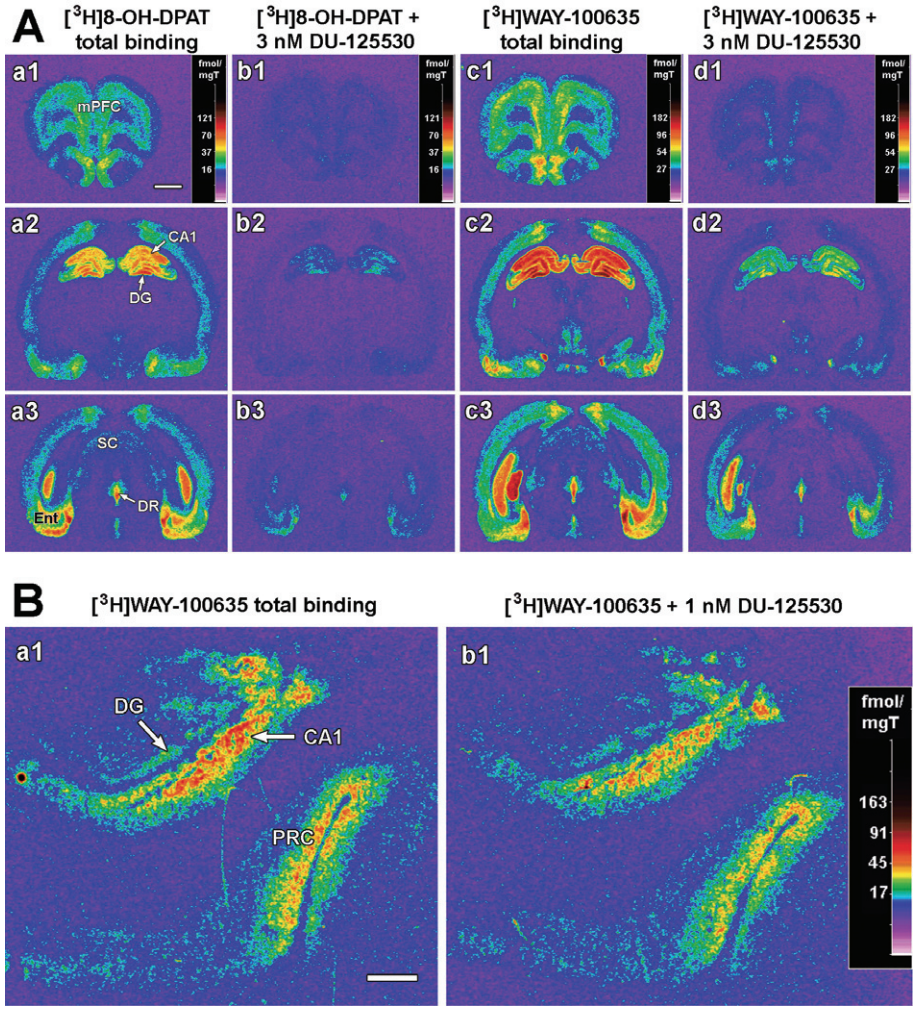

Figure 2

(A) Pseudocolour images from autoradiograms obtained from rat brain sections at different brain levels (prefrontal cortex, hippocampus and midbrain-upper pons) incubated with 0.5 nM [^{3}H]8-OH-DPAT alone (a1–a3) and in the presence of 3×10^{-9} M DU1255530 (b1–b3), or incubated with 0.5 nM [^{3}H]WAY-100635 alone (c1–c3) and in the presence of 3×10^{-9} M DU1255530 (d1–d3). Note that DU-1255530 inhibits [^{3}H]8-OH-DPAT and [^{3}H]WAY-100635 binding in all structures, including CA1, DG (dentate gyrus), DR (dorsal raphe), Ent (entorhinal cortex), mPFC (medial prefrontal cortex) and SC (superior colliculus). Bar = 2 mm. (B) Pseudocolour images from autoradiograms obtained from human hippocampal sections incubated with 0.5 nM [^{3}H]WAY-100635 alone (a1) or in the presence of 10^{-9} M DU-1255530 (b1). CA1, CA1 hippocampal field; DG, dentate gyrus; PRC, perirhinal cortex. Bar = 2 mm.

the mPFC by inverse microdialysis. The extensive occupancy of 5-HT$_{1A}$ receptors in mPFC by local 8-OH-DPAT inhibits excitatory inputs to the dorsal raphe (DR), thereby reducing 5-HT neuronal activity and terminal 5-HT release (Celada *et al.*, 2001). The local application of 100 μM 8-OH-DPAT in mPFC markedly reduced local extracellular 5-HT concentration. Subsequent systemic administration of DU-125530 (3 mg·kg^{-1} s.c.) significantly attenuated this reduction (time $F_{15,135} = 14.5$; $P < 0.001$ and treatment × time interaction $F_{15,135} = 2.7$; $P < 0.002$; Figure 5C) [note that saline rapidly increased extracellular 5-HT due to the injection stress (Adell *et al.*, 1997), yet the effect disappeared rapidly].

Finally, DU-125530 augmented the increase of extracellular 5-HT in mPFC evoked by (i) 3 mg·kg^{-1} paroxetine (time $F_{15,270} = 6.7$; $P < 0.0001$; treatment × time interaction $F_{15,270} = 2.3$; $P < 0.005$; Figure 5D); and (ii) 10 mg·kg^{-1} s.c. fluoxetine (time $F_{15,300} = 41.9$; $P < 0.0001$; treatment × time interaction $F_{15,300} = 1.8$; $P < 0.04$; fluoxetine + vehicle, $n = 9$; fluoxetine + DU-125530, $n = 13$; data not shown).

Mouse experiments. The systemic administration of DU-125530 (3 mg·kg^{-1} s.c.) alone had no effect on the extracellular 5-HT concentration in mPFC of wild-type mice (WT) (vehicle + vehicle, $n = 5$; vehicle + DU-125530, $n = 5$) nor in 5-HT$_{1A}$ receptor knock-out mice (KO) (vehicle + vehicle, $n = 5$; vehicle + DU-125530, $n = 4$). However, DU-125530 prevented the reduction of 5-HT release induced by 0.5 mg·kg^{-1} s.c. 8-OH-DPAT in WT mice (treatment $F_{1,10} = 16.4$; $P < 0.005$; time $F_{15,150} = 4.1$; $P < 0.0001$; treatment × time interaction $F_{15,150} = 2.7$; $P < 0.005$; Figure 6A).

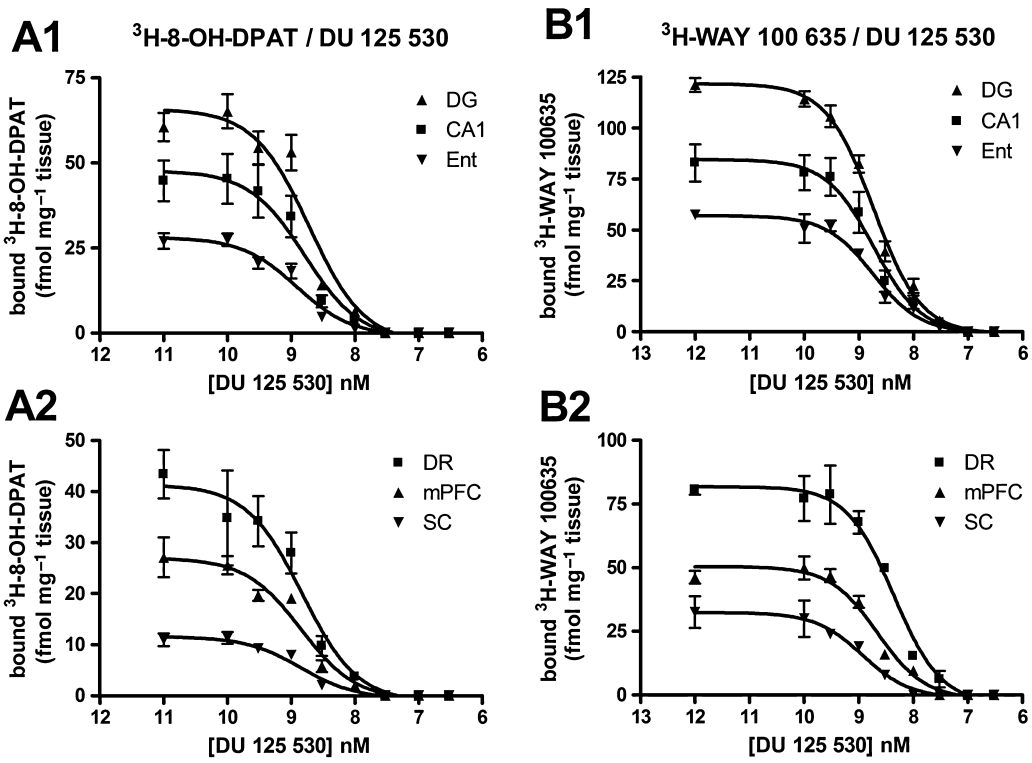

Figure 3

Displacement of [^{3}H]8-OH-DPAT (A1,A2) and [^{3}H]WAY-100635 (B1,B2) binding by DU1255530 in the hippocampus (CA1), DG, DR (Ent), mFPC and SC of the rat. Data points are means ± SEM of three animals and were obtained by microdensitometric analysis of autoradiograms.

Table 2

Relative binding affinities (pIC_{50}) of DU-125530 for [^{3}H]8-OH-DPAT and [^{3}H]WAY-100635 binding sites in various regions of the rat brain

Area	[^{3}H]8-OH-DPAT pIC_{50} ± SD	[^{3}H]WAY-100635 pIC_{50} ± SD
CA1	8.8 ± 0.1	8.7 ± 0.1
DG	8.7 ± 0.1	8.7 ± 0.1
Ent	8.9 ± 0.1	8.7 ± 0.1
DR	8.8 ± 0.2	8.4 ± 0.1
mPFC	8.9 ± 0.1	8.7 ± 0.1
SC	8.9 ± 0.1	8.9 ± 0.2

CA1, Ammon's horn area 1 of hippocampus; DG, dentate gyrus; DR, dorsal raphe nucleus; Ent, entorhinal cortex; mPFC, medial prefrontal cortex; SC, superior colliculus.

Table 3

Relative binding affinities (pIC_{50}) of DU-125530 for [^{3}H]WAY-100635 binding sites the CA1 hippocampal field and the perirhinal cortex of two human control cases

Area	[^{3}H]WAY-100635 pIC_{50} ± SD
CA1 case A	8.6 ± 0.1
CA1 case B	8.7 ± 0.2
PRC case A	8.8 ± 0.1
PRC case B	8.4 ± 0.1

CA1, Ammon's horn area 1; PRC, perirhinal cortex.

As expected, 0.5 mg·kg^{-1} s.c. 8-OH-DPAT did not reduce 5-HT release in the mPFC of 5-HT_{1A} receptor KO mice, and the change in 5-HT concentration produced by vehicle + 8-OH-DPAT was identical to that produced by DU-125530 + 8-OH-DPAT (Figure 6B) [a moderate, fast increase in extracellular 5-HT was produced, as a result of handling and injection stress (Adell *et al.*, 1997)].

The systemic administration of fluoxetine (20 mg·kg^{-1} s.c.) increased extracellular mPFC 5-HT concentration significantly more in KO mice than in WT mice (genotype effect $F_{1,8} = 6.0$; $P < 0.05$; time effect $F_{15,120} = 14.7$; $P < 0.0001$; time × genotype interaction $F_{15,120} = 2.5$; $P < 0.005$; Figure 6C). The subsequent administration of DU-125530 (3 mg·kg^{-1} s.c.) significantly enhanced extracellular 5-HT concentration in WT mice, up to the level seen in 5-HT_{1A} receptor KO mice after fluoxetine administration (time $F_{15,135} = 20.3$; $P < 0.0001$; genotype × time interaction $F_{15,135} = 2.6$; $P < 0.002$; Figure 6D).

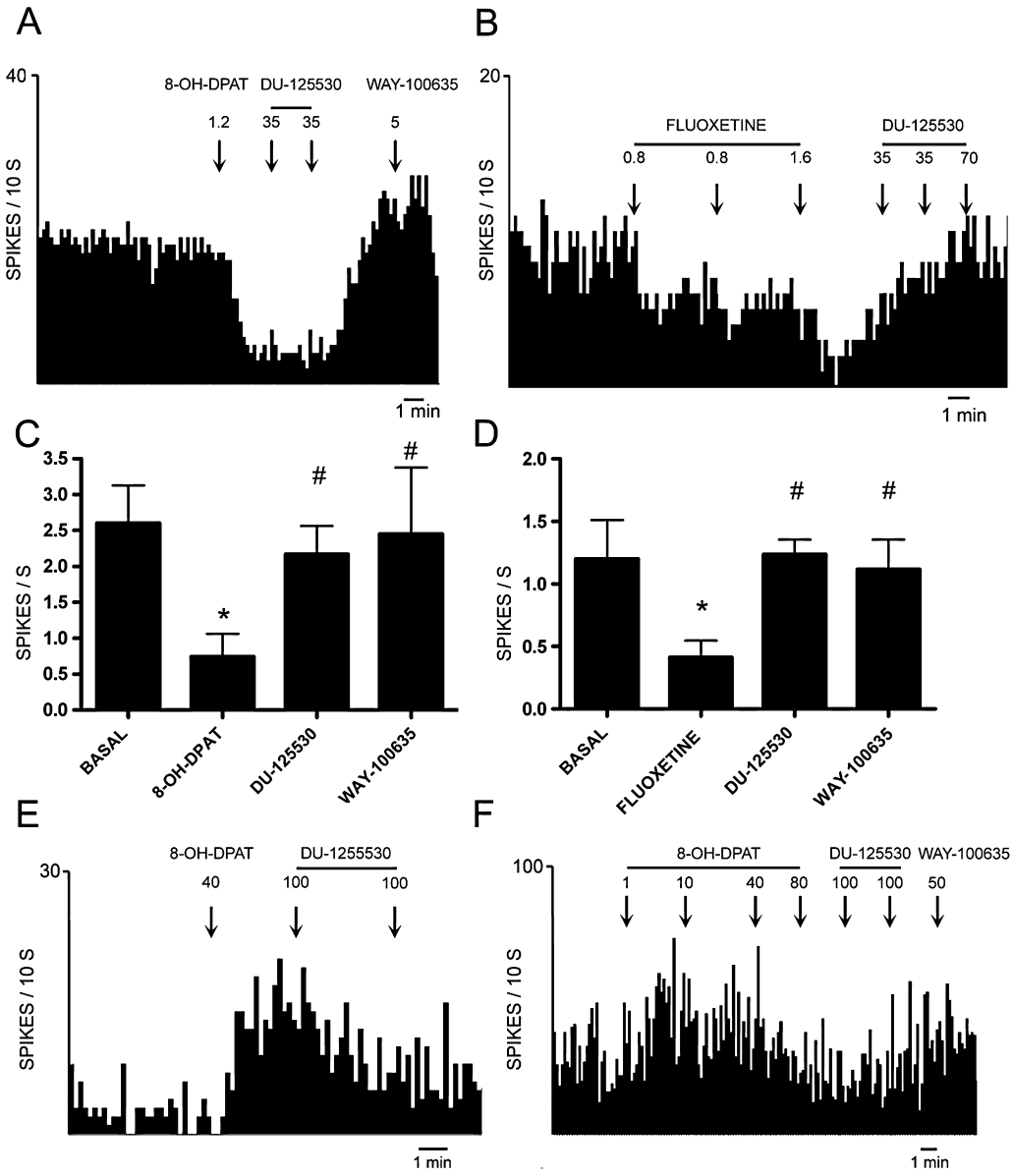

Figure 4

(A,B) Representative integrated firing rate histograms of two 5-hydroxytryptaminergic neurones showing the inhibition of discharge rate induced by the i.v. administration of 8-OH-DPAT (A) and fluoxetine (B) as well as the reversal of the effect by the subsequent administration of DU-125530 in both cases. Note that the administration of the prototypical 5-HT$_{1A}$ receptor antagonist WAY-100635 after DU-125530 did not evoke any further effect on firing rate indicating a complete reversal of the action of 8-OH-DPAT by DU-125530. (C,D) Bar graphs showing the inhibitory effect on 5-HT cell firing produced by 8-OH-DPAT (C) or fluoxetine (D) and the reversal of these effects by DU-125530. (E,F) Integrated firing rate histograms of two pyramidal cells in mPFC, which were identified by antidromic stimulation from the DR. The administration of the 5-HT$_{1A}$ agonist 8-OH-DPAT evokes excitations (E) or excitations at low doses followed by inhibitions at higher doses (F). Both effects are reversed by the subsequent administration of DU-125530, showing its antagonist properties at post-synaptic 5-HT$_{1A}$ heteroreceptors. Arrows mark the time of drug administration.

Clinical characterization of DU-125530 in accelerating/enhancing fluoxetine antidepressant response

Fifty-seven patients were screened and entered the study between May 2004 and November 2007. Seven patients were excluded before randomization due to placebo response. Therefore, 50 patients with major depression diagnosis (MDD) finally entered the active phase (Figure 7). Twenty-five were randomly assigned to fluoxetine plus DU-125530 arm and 25 to fluoxetine plus placebo arm (Figure 1). No differences were found in demographic or clinical variables between the two groups (Table 4).

Neither the percentage of patients with first depressive episode (51% receiving DU-125530, 48% receiving placebo) nor the percentage of melancholic features (23% and 14%.

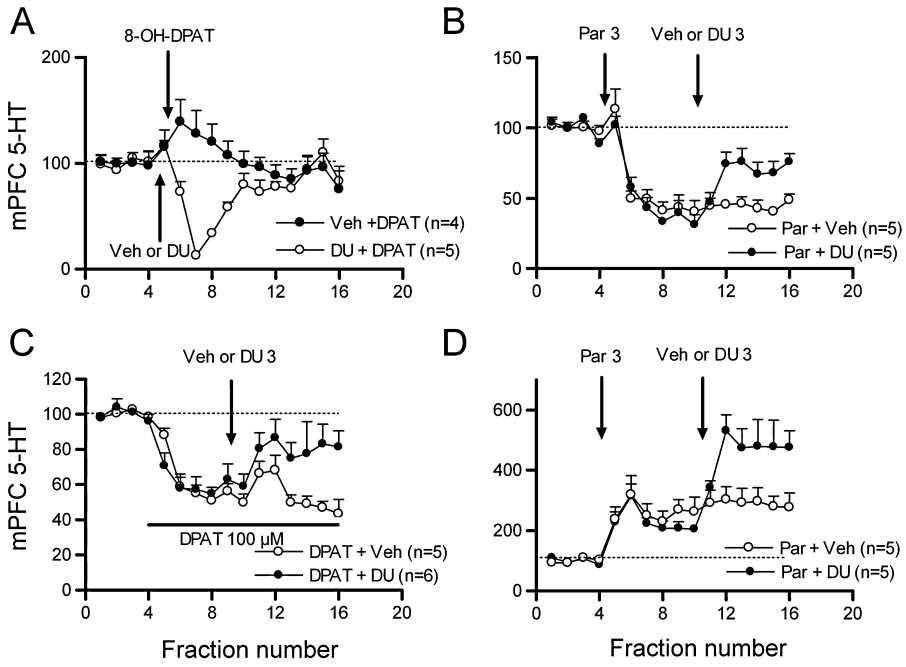

Figure 5

In vivo microdialysis experiments showing the antagonism/reversal exerted by DU-125530 in the different experimental models used in rats. The extracellular 5-HT concentration (shown as percentages of baseline; set to 100, dotted line) in mPFC was used in all instances. (A) Prevention by DU-125530 of the 8-OH-DPAT-induced reduction of 5-HT release. (B) Reversal by systemic administration of DU-125530 of the paroxetine (Par)-induced decrease in 5-HT release in mPFC during the local perfusion of citalopram by reverse dialysis. (C) Reversal by systemic DU-125530 administration of the effects produced by local application of 8-OH-DPAT on mPFC. (D) Augmentation by DU-125530 of the paroxetine-induced increase in mPFC extracellular 5-HT levels. Arrows mark systemic injections. Doses are given in mg·kg^{-1}. Horizontal bars indicate local perfusion by inverse microdialysis. See text for statistical analysis.

respectively) differed between groups. Current episode duration ranged from 1 to 6 months for 63.6% of patients receiving fluoxetine + DU-125530 and for 54.5% of those receiving fluoxetine + placebo. Treatments were generally well tolerated with no differences in the incidence of adverse events between the two groups (32% for DU-125530, 16% for placebo, $\chi^2 = 1.75$; $P = 0.16$). Regarding sexual dysfunction, one patient treated with DU-125530 reported anorgasmia. Five patients were withdrawn from the clinical trial because of side effects and two due to patient's decision. Repeated-measures ANOVA for blood pressure did not show a significant main effect of time × group ($F_{7,196} = 0.5$, $P = 0.8$) nor a group effect ($F_{1,27} = 1.3$, $P = 0.3$). Heart rate showed a similar non-significant time × group effect ($F_{7,182} = 1.1$, $P = 0.4$) and no group effect ($F_{1,26} = 0.1$, $P = 0.8$). These results indicated that vital signs were stable during the study and with no significant differences between groups. Plasma concentration of fluoxetine at days 14 and 42 did not differ between groups. At day 14, fluoxetine mean values were 57.5 ng·mL^{-1} (SD = 31.7) in the fluoxetine + DU-125530 group and 66.4 ng·mL^{-1} (SD = 31.9) in the fluoxetine + placebo group ($t = -0.9$, $P = 0.4$). At day 42, fluoxetine values were 86.1 ng mL^{-1} (SD = 46.3) in the fluoxetine + DU-125530 group and 119 ng·mL^{-1} (SD = 76.3) in the fluoxetine + placebo group ($t = -1.7$, $P = 0.1$).

Regarding the main analysis with HDRS scores, repeated-measures ANOVA showed a significant effect of time ($F_{7,280} = 96.6$; $P < 0.001$) but not of the group ($P = 0.9$) nor of time × group interaction ($P = 0.6$). Figure 8A shows the temporal evolution of cumulative percentages of sustained responses for both groups. A tendency towards higher sustained responses in the fluoxetine + DU-125530 group was seen at 3–4 weeks, but it did not reach statistical significance.

The response rate of patients receiving DU-125530 + fluoxetine was similar to that of patients receiving fluoxetine + placebo. The survival analysis (Figure 8B) confirmed the absence of significant differences between the two treatment arms, being mean survival times until first response 44 days for DU-125530 and 37 days for placebo (log-rank, $\chi^2 = 0.3$, $P = 0.6$).

We performed an additional analysis with the OC ($n = 21$ for DU-125530; and $n = 20$ for placebo), which gave essentially the same results (group effect: $F_{1,39} = 0.1$, $P = 0.8$; time × group effect: $F_{7,273} = 0.5$, $P = 0.8$).

Discussion and conclusions

The present study shows that DU-125530 is a high-affinity and silent 5-HT$_{1A}$ receptor antagonist in rodent brain that prevents and reverses the actions of 5-HT and 5-HT$_{1A}$ receptor agonists (8-OH-DPAT) at pre- and post-synaptic 5-HT$_{1A}$ receptors. It binds to rat and human 5-HT$_{1A}$ receptors with low nM affinity, and it antagonizes the actions of the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT and SSRIs (fluoxetine and paroxetine) in

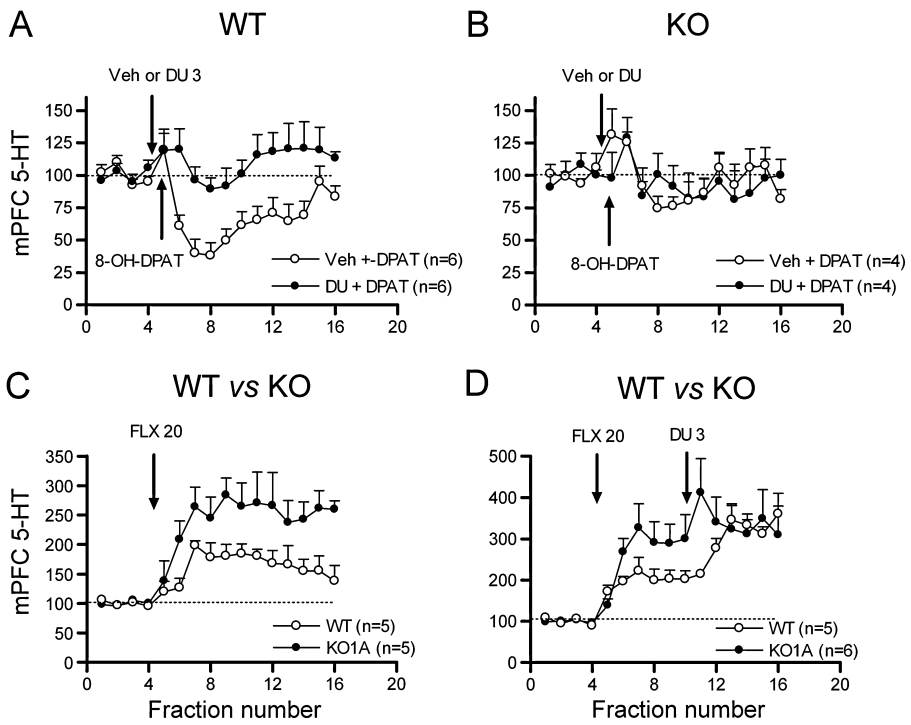

Figure 6

In vivo microdialysis experiments in wild type (WT) and 5-HT$_{1A}$ receptor knock out (KO1A) mice showing the effects of DU-125530. The extracellular 5-HT concentration (shown as percentages of baseline; set to 100, dotted line) in mPFC was used in all instances. (A) Prevention by DU-125530 of the reduction in 5-HT output induced by systemic 8-OH-DPAT administration in WT mice. (B) Lack of effects of systemic administration of 8-OH-DPAT and DU-125530 in KO mice. (C) Comparison of the effects of systemic injections of fluoxetine (FLX) in WT versus KO mice. (D) Augmentation of the effects of fluoxetine by DU-125530 in WT mice but not in KO mice. Note that the treatment of WT with fluoxetine + DU-125530 increases extracellular 5-HT concentration to the same extent than fluoxetine alone in KO mice. Arrows mark systemic injections. Doses are given in mg·kg^{-1}. See text for statistical analysis.

electrophysiological and microdialysis experimental paradigms. As a consequence, DU-125530 augments the elevation in forebrain extracellular 5-HT concentration produced by SSRIs by preventing the 5-HT$_{1A}$ autoreceptor-mediated negative feedback evoked by these agents (Artigas *et al.*, 1996). Despite these excellent pharmacological properties, DU-125530 did not accelerate nor enhance the antidepressant action of fluoxetine. To our knowledge, this is the first study testing the 5-HT$_{1A}$ receptor augmentation hypothesis (Artigas, 1993; Artigas *et al.*, 1996) with a selective 5-HT$_{1A}$ receptor antagonist and consequently will affect antidepressant drug design.

Preclinical studies

Overall, the preclinical data support that DU-125530 interacts with 5-HT$_{1A}$ autoreceptors and post-synaptic 5-HT$_{1A}$ receptors in a manner similar to that of the prototypical antagonist WAY-100635 (Forster *et al.*, 1995; Fletcher *et al.*, 1996), which – unlike DU-125530 – is not available for human use. Indeed, DU-125530 displaced the agonist (^{3}H-8-OH-DPAT) and antagonist (^{3}H-WAY-100635) binding to rat and human 5-HT$_{1A}$ receptors with nM affinity and blocked the effects of exogenous (8-OH-DPAT) and endogenous (5-HT) 5-HT$_{1A}$ receptor agonists on (i) 5-HT neurone activity and (ii) 5-HT release, in rats and mice, as previously observed with WAY-100635 using the same experimental paradigms (Romero and Artigas, 1997; Casanovas *et al.*, 1999; Celada *et al.*, 2001; Romero *et al.*, 2003; Lladó-Pelfort *et al.*, 2011). Moreover, DU-125530 augmented the increase in extracellular 5-HT induced by SSRI to an extent comparable with that produced by WAY-100635 (Romero and Artigas, 1997; Hervas *et al.*, 1998). Interestingly, the dose used in the present preclinical experiments appears to fully occupy 5-HT$_{1A}$ receptors, as (i) no further antagonism was produced by WAY-100635 when it was used after DU-125530, and (ii) the 5-HT increase induced by fluoxetine + DU-125530 on extracellular 5-HT in WT mice was identical to that produced by fluoxetine alone in 5-HT$_{1A}$R KO mice.

The ability of DU-125530 to antagonize post-synaptic 5-HT$_{1A}$ receptors is shown by the following: (i) pilot electrophysiological experiments [reversal of 8-OH-DPAT-induced effects on mPFC pyramidal neurones, an effect depending on post-synaptic 5-HT$_{1A}$ receptor activation (Lladó-Pelfort *et al.*, 2011)]; and (ii) microdialysis experiments in which the systemic administration of DU-125530 significantly reversed the reduction in 5-HT release evoked by the activation of mPFC 5-HT$_{1A}$ receptors by local 8-OH-DPAT administration, as previously observed with WAY-100635 (Celada *et al.*, 2001). Indeed, the direct activation of pyramidal 5-HT$_{1A}$ receptors in mPFC neurones attenuates the excitatory input onto DR 5-HT

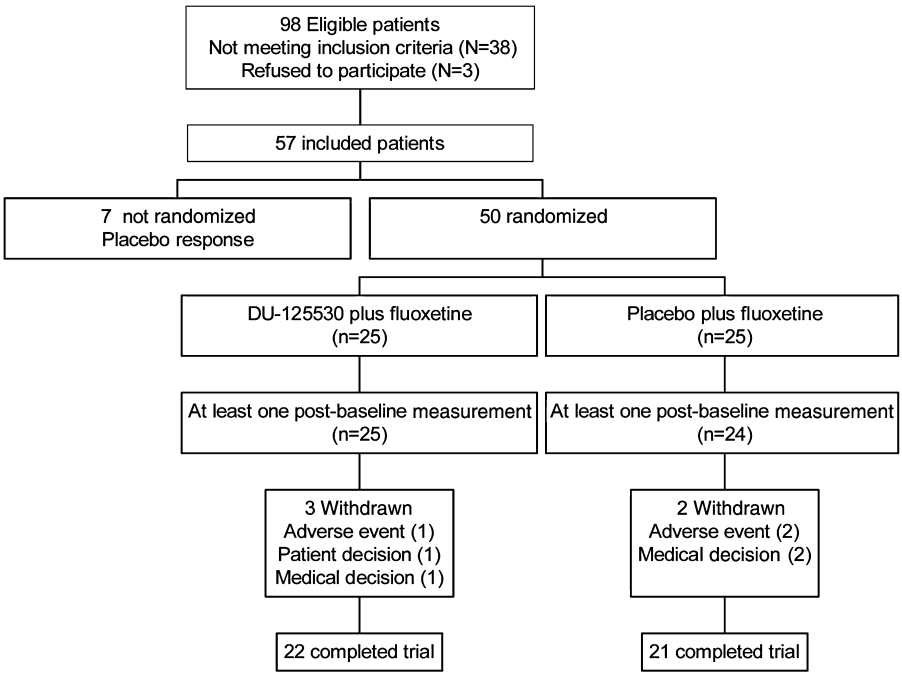

Figure 7

Flow diagram of subject progress through the phases of a randomized trial.

Table 4

Demographic and clinical variables of the two treatment groups

Variables	DU-125530 (n = 25)	Placebo (n = 25)	χ^2/t	P
Gender (% females)	83.3	84	0	n.s.
Age	42.1 (11.5)	42.5 (13.9)	0.9	n.s.
Family psychiatric history (% present)	64	36	3.7	n.s.
No previous depressive episode (%)	41.7	54.2	0.1	n.s.
Age at first depressive episode	34 (14.3)	38.2 (15.5)	0.9	n.s.
Number of depressive episodes (including current episode)	2.6 (2.8)	1.7 (1.1)	1.4	n.s.
Concomitant treatment (% patients taking)			2.4	n.s.
No treatment	25	35.7		
Benzodiazepines	56.2	28.6		
Hypnotic	12.5	21.4		
Benzodiazepines plus hypnotic	6.2	14.3		
HDRS				
Pre	24.7 (3.7)	25.6 (4.4)	0.7	n.s.
Post	13 (9.6)	11.1 (6.8)	0.7	n.s.
MADRS				
Pre	31.3 (4.5)	32.6 (5.1)	0.9	n.s.
Post	14.8 (12)	14 (10.3)	0.2	n.s.
CGI				
Pre	4.7 (0.6)	4.7 (0.6)	0.3	n.s.
Post	2.5 (1.3)	2 (1)	1	n.s.

DU-125530 = patients treated with fluoxetine + DU-125530; Placebo = patients treated with fluoxetine + placebo. Dta are shown as means (with SD). n.s., non-significant; pre, pretreatment; post, post treatment.

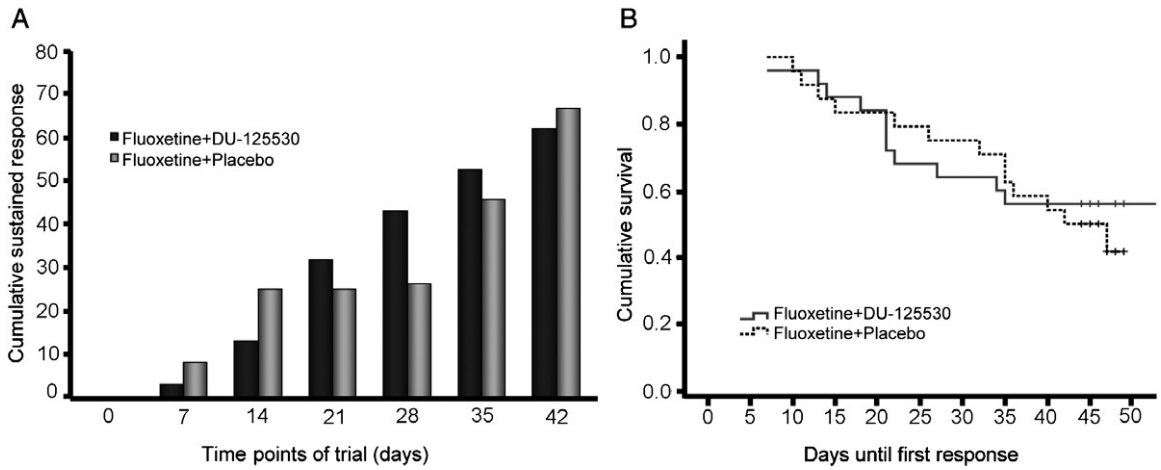

Figure 8

(A) Bar graph showing the cumulative percentages of patients with sustained response throughout the trial period. Repeated-measures ANOVA showed a significant effect of the treatment but not of the group or treatment × group interaction. (B) Kaplan–Meier survival analyses of days until response.

neurones (Celada *et al.*, 2001) and evokes a subsequent reduction of forebrain 5-HT release. The data show that the systemic administration of DU-125530 antagonized this effect, showing a clear antagonist action at post-synaptic 5-HT$_{1A}$ receptors.

Despite its *in vitro* affinity for α_1-adrenoceptors (~10 times lower than for 5-HT$_{1A}$ receptors), DU-125530 did not reduce 5-HT release by itself, as expected from blockade of raphe α_1-adrenoceptors (Vandermaelen and Aghajanian, 1983; Bortolozzi and Artigas, 2003). Moreover, no cardiovascular side effects were observed in patients treated with fluoxetine + DU-125530. Both observations allow us to discount a significant occupancy of α_1-adrenoceptors at the doses used. Likewise, DU-125530 shows nM affinity for dopamine D$_2$ receptors (Table 1). However, none of the observed preclinical effects of the compound can be attributed to interaction with such D$_2$ receptors. Likewise, no side effects derived from D$_2$ receptor blockade (e.g. extrapyramidal symptoms) were observed in patients treated with fluoxetine + DU-125530.

Thus, the present preclinical results indicate that:

- DU-125530 displays equal nM affinity at pre- and post-synaptic 5-HT$_{1A}$ receptors.
- DU-125530 is a silent pre- and post-synaptic 5-HT$_{1A}$ receptor antagonist in rodent brain.
- DU-125530 cancels the 5-HT$_{1A}$ receptor-mediated negative feedback induced by SSRIs, thereby augmenting their increase of extracellular 5-HT concentration.

Clinical trial

The present clinical trial was conducted to examine whether the augmentation of 5-hydroxytryptaminergic function that resulted from the blockade of 5-HT$_{1A}$ autoreceptors was translated into an increased speed or efficacy of the antidepressant fluoxetine. To this end, the trial design was identical to that used previously to examine the augmenting action of pindolol (Perez *et al.*, 1997). Due to the lack of selective 5-HT$_{1A}$ receptor antagonists available for clinical use, pindolol was used in past studies testing the 5-HT$_{1A}$ receptor augmentation strategy (Artigas, 1993; Artigas *et al.*, 1994; 1996; Perez *et al.*, 1997; Bordet *et al.*, 1998; Zanardi *et al.*, 1998; Ballesteros and Callado, 2004; Portella *et al.*, 2011). However, the addition of DU-125530 to fluoxetine treatment did not enhance nor accelerate its antidepressant action in a population of depressive patients with clinical characteristics similar to those included in previous studies (Perez *et al.*, 1997; 1999). This difference cannot be attributed to pharmacokinetic factors as fluoxetine plasma levels were similar to those previously reported in the fluoxetine + pindolol study (Perez *et al.*, 2001) and did not differ between treatment arms.

However, several remarkable differences exist between pindolol and DU-125530. PET scan studies have revealed a preferential occupancy of pre-synaptic versus post-synaptic 5-HT$_{1A}$ receptors by pindolol, using ^{11}C-WAY-100635 as a ligand (Artigas *et al.*, 2001; Martinez *et al.*, 2001). However, DU-125530 shows a comparable occupancy of pre- and post-synaptic 5-HT$_{1A}$ receptors using the same ligand (Rabiner *et al.*, 2002). Thus, the occupancy of pre- and post-synaptic 5-HT$_{1A}$ receptors by the dose of DU-125530 used herein (20 mg·day^{-1}) is 50–60% in most individuals tested (Rabiner *et al.*, 2002). In contrast, the pindolol dose used in most clinical studies (7.5 mg·day^{-1}) (Martinez *et al.*, 2001) produced an occupancy of 40% pre-synaptic and 18% post-synaptic 5-HT$_{1A}$ receptors. These PET scan studies are paralleled by electrophysiological (Romero *et al.*, 1996) and histological (Castro *et al.*, 2000; Serrats *et al.*, 2004) studies showing a preferential affinity of pindolol for pre- versus post-synaptic 5-HT$_{1A}$ receptors. Hence, pindolol antagonized the 5-HT$_{1A}$ autoreceptor-mediated inhibition of 5-hydroxytryptaminergic cell firing produced by SSRIs (Romero *et al.*, 1996), but not the activation of hippocampal 5-HT$_{1A}$ receptors induced by 5-HT and 5-HT$_{1A}$ receptor agonists (Romero *et al.*, 1996; Tada *et al.*, 1999). In agreement, G-protein activation studies indicated a significantly higher potency of pindolol for 5-HT$_{1A}$ autoreceptors than for post-

synaptic 5-HT$_{1A}$ receptors in the hippocampus and entorhinal cortex in rat, guinea pig and human brain (Serrats *et al.*, 2004). Likewise, pindolol showed a greater affinity for pre- than for post-synaptic 5-HT$_{1A}$ receptors in human brain (Castro *et al.*, 2000). A second difference between pindolol and DU-125530 lies in the partial agonist character of pindolol (Newman-Tancredi *et al.*, 1998). Pindolol may increase cortical catecholamine release via activation of mPFC 5-HT$_{1A}$ receptors when administered alone (see Artigas *et al.*, 2001). However, it appears unlikely that this property can be relevant in a pharmacological situation dominated by the excess 5-HT – and therefore high 5-HT$_{1A}$ receptor activation – produced by SSRIs.

The inability of DU-125530 to accelerate or augment the antidepressant action of fluoxetine is likely to be attributable to its simultaneous blockade of pre- and post-synaptic 5-HT$_{1A}$ receptors, given the enhanced post-synaptic 5-HT$_{1A}$ receptor activation produced by several antidepressant drug classes in rodents (Haddjeri *et al.*, 1998; Blier and Ward, 2003). The present data support that this process may also occur in human brain. Thus, while 5-HT$_{1A}$ autoreceptor blockade augments pre-synaptic 5-HT function by preventing the negative feedback at pre-synaptic (raphe) level, the simultaneous blockade of post-synaptic 5-HT$_{1A}$ receptors in corticolimbic areas may cancel this effect. Moreover, the present results indicate that other 5-HT receptors (e.g. 5-HT$_4$) (Lucas, 2009) are involved in the antidepressant action of fluoxetine, because the extensive blockade of post-synaptic 5-HT$_{1A}$ receptors did not cancel the clinical effect of fluoxetine, as it would be expected if post-synaptic 5-HT$_{1A}$ receptors were the only mediators of its antidepressant action.

Limitations of the study

The wide range of techniques and methodologies used in preclinical studies to characterize the action of DU-125530 in rodent brain support a full antagonist action of this agent at pre- and post-synaptic receptors with a low level of uncertainty. In any case, we carried out a reduced number of experiments to examine the action of DU-125530 at post-synaptic 5-HT$_{1A}$ receptors using electrophysiology. However, microdialysis data are fully supportive of such an antagonist action of post-synaptic 5-HT$_{1A}$ receptors. In the clinical trial, the main limitation of the study is that only one dose of DU-125530 was used, based on PET scan data (Rabiner *et al.*, 2002). Given the antidepressant properties of post-synaptic 5-HT$_{1A}$ receptor activation in animal models (see above), it is unknown whether a lower DU-125530 dose, leading to a less post-synaptic 5-HT$_{1A}$ receptor occupancy would have augmented the antidepressant effects of fluoxetine. Trial design does not appear to be a limitation, as we used the same one than in a previous trial (Perez *et al.*, 1997), which was able to detect significant differences between two similar arms (fluoxetine + placebo vs. fluoxetine + pindolol).

In summary, the present study shows that DU-125530 is an excellent antagonist of pre- and post-synaptic 5-HT$_{1A}$ receptors. Despite this, its addition to fluoxetine did not accelerate nor enhance its antidepressant properties in patients with major depression. These results show that simultaneous blockade of pre- and post-synaptic 5-HT$_{1A}$ receptors does not improve the antidepressant actions of SSRI, indicating that post-synaptic 5-HT$_{1A}$ receptor activation is required to achieve an enhancement of the antidepressant effects of SSRIs, a conclusion relevant to antidepressant drug design.

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Conflicts of interest

EA has received consulting and educational honoraria from several pharmaceutical companies including Eli Lilly, Sanofi-Aventis, Lundbeck and Pfizer, and he has participated as main local investigator in clinical trials from Eli Lilly, Bristol-Myers and Sanofi-Aventis and also as national coordinator of clinical trials from Servier and Lundbeck. VP has received educational honoraria from the following pharmaceutical companies: Sanofi-Aventis, Lundbeck, Pfizer and Eli Lilly. FA has received consulting or educational honoraria from Boehringer-Ingelheim, Eli Lilly, Lundbeck and Pierre Fabre. The rest of authors declare no conflicts of interest related directly or indirectly to this work.

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