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S33138 [*N*-[4-[2-[(3*aS*,9*bR*)-8-cyano-1,3*a*,4,9*b*-tetrahydro[1]-benzopyrano[3,4-c]pyrrol-2(3*H*)-yl)-ethyl]phenylacetamide], A Preferential Dopamine D_3 versus D_2 Receptor Antagonist and Potential Antipsychotic Agent: I. Receptor-Binding Profile and Functional Actions at G-Protein-Coupled Receptors

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ABSTRACT

The novel, potential antipsychotic, S33138 (*N*-[4-[2-[(3*aS*,9*bR*)-8-cyano-1,3*a*,4,9*b*-tetrahydro[1]benzopyrano[3,4-c]pyrrol-2(3*H*)-yl)-ethyl]phenylacetamide), displayed ~25-fold higher affinity at human (h) dopamine D_3 versus hD_{2L} (long isoform) and hD_{2S} (short isoform) receptors (pK_i values, 8.7, 7.1, and 7.3, respectively). Conversely, haloperidol, clozapine, olanzapine, and risperidone displayed similar affinities for hD_3, hD_{2L}, and hD_{2S} sites. In guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]-GTPγS) filtration assays, S33138 showed potent, pure, and competitive antagonist properties at hD_3 receptors, displaying pK_B and pA_2 values of 8.9 and 8.7, respectively. Higher concentrations were required to block hD_{2L} and hD_{2S} receptors. Preferential antagonist properties of S33138 at hD_3 versus hD_{2L} receptors were underpinned in antibody capture/scintillation proximity assays (SPAs) of $G\alpha_{i3}$ recruitment and in measures of extracellular-regulated kinase phosphorylation. In addition, in cells cotransfected with hD_3 and hD_{2L} receptors that assemble into heterodimers, S33138 blocked (pK_B, 8.5) the inhibitory influence of quinpirole upon forskolin-stimulated cAMP formation. S33138 had low affinity for hD_4 receptors (<5.0) but revealed weak antagonist activity at hD_1 receptors (Gαs/SPA, pK_B, 6.3) and hD_5 sites (adenylyl cyclase, 6.5). Modest antagonist properties were also seen at human serotonin $(5\text{-HT})_{2A}$ receptors ($G\alpha_q$/SPA, pK_B, 6.8, and inositol formation, 6.9) and at 5-HT_7 receptors (adenylyl cyclase, pK_B, 7.1). In addition, S33138 antagonized $h\alpha_{2C}$ adrenoceptors ([^{35}S]GTPγS, 7.2; $G\alpha_{i3}$/SPA, 6.9; $G\alpha_o$/SPA, 7.3, and extracellular-regulated-kinase, 7.1) but not $h\alpha_{2A}$ or $h\alpha_{2B}$ adrenoceptors (<5.0). Finally, in contrast to haloperidol, clozapine, olanzapine, and risperidone, S33138 displayed negligible affinities for multiple subtypes of α_1-adrenoceptor, muscarinic, and histamine receptor. In conclusion, S33138 possesses a distinctive receptor-binding profile and behaves, in contrast to clinically available antipsychotics, as a preferential antagonist at hD_3 versus hD_2 receptors.

Schizophrenia is a complex, progressive, and debilitating disorder of early onset that afflicts approximately 1% of the population. It is characterized by disorganized thought and a constellation of symptoms generally classified as positive (delusions and hallucinations), negative (social withdrawal, blunted affect, and mutism), and cognitive (deficits in attention, working and verbal memory, social cognition, and exec-

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ABBREVIATIONS: EPS, extrapyramidal motor symptom; AR, adrenoceptor; H_1, histamine; DA, dopamine; S33138 *N*-[4-[2-[(3*aS*,9*bR*)-8-cyano-1,3*a*,4,9*b*-tetrahydro[1]benzopyrano[3,4-c]pyrrol-2(3*H*)-yl)-ethyl]phenylacetamide; h, human; MDL100,907, *R*(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol; SB269,970, (*R*)-1-{2-[1-(3-hydroxy benzensulfonyl) pyrrolidin-2-yl] ethyl}-4-methylpiperidine; RX821,002, 2-(2,3-dihydro-2-methoxy-1,4-benzodioxan-2-yl)-4,5-dihydro-1*H*-imidazoline; SCH23390, *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; CHO, chinese hamster ovary; [^{35}S]GTPγS, guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate; SPA, scintillation proximity assay; ERK, extracellular-regulated kinase; MAP, mitogen-activated protein; AC, adenylyl cyclase; Ro 20-1724, 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone; NA, noradrenaline; 5-HT, serotonin; IP, inositol phosphate; CGP-12177, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one; SB414,796, *trans*-3-(2-(4-((3-(3-(5-methyl-1,2,4-oxadiazolyl))-phenyl)carboxamido)cyclohexyl)ethyl)-7-methylsulfonyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; A437,203, 2-{3-4-(2-*tert*-butyl-6-trifluoromethyl-pyrimidin-4-yl)-piperazin-1-yl]propyl-sulfanyl}-3*H*-pyrimidin-4-one.

utive function) (Kapur and Mamo, 2003; Meltzer, 2004; Lieberman et al., 2005).

Conventional neuroleptics like haloperidol, which behaves principally as a dopaminergic antagonist, moderate positive symptoms. However, some 20 to 30% of patients are resistant, and efficacy against negative and cognitive symptoms is limited. Furthermore, there is only a modest therapeutic window to doses provoking extrapyramidal motor symptoms (EPSs), and long-term utilization may be associated with the development of irreversible tardive dyskinesia (Dean and Scarr, 2004; Lieberman et al., 2005; Margolese et al., 2005). The "atypical" antipsychotic, clozapine, which potently interacts with several classes of monoaminergic receptor, is active in certain neuroleptic-refractory patients, does not elicit EPS, and is more effective than haloperidol against negative symptoms (Dean and Scarr, 2004; Meltzer, 2004; Lieberman et al., 2005). However, its advantages are offset by the risk of agranulocytosis. Furthermore, clozapine elicits sedation, obesity, cardiovascular-autonomic, and metabolic side effects via actions at α_1-adrenoceptors (ARs), histamine (H_1) receptors, and muscarinic receptors (Cunningham-Owens, 1996; Millan et al., 2000a; Pacher and Kecskemeti, 2004). A number of other multireceptorial antipsychotics have been introduced with similar, although not identical, broad-based receptor-binding profiles: notably, olanzapine and risperidone (Millan et al., 2000a; Dean and Scarr, 2004; Lieberman et al., 2005; McCue et al., 2006). However, despite their undeniable utility, they do not fully reproduce the clinical benefits of clozapine, and therapeutic indexes to doses eliciting undesirable actions are limited; in particular, EPS for risperidone and obesity for olanzapine (Dean and Scarr, 2004; Lieberman et al., 2005; Margolese et al., 2005). Moreover, although the more recently launched partial dopaminergic agonist, aripiprazole, is well tolerated, its efficacy is not superior to that of other agents (Abi-Dargham and Laruelle, 2005; Burstein et al., 2005; McCue et al., 2006; Urban et al., 2007b). Clearly, there remains a need for innovative drugs possessing improved efficacy and favorable side-effect profiles.

It remains to be clinically demonstrated that antipsychotics that act independently of dopaminergic mechanisms are robustly and consistently effective, and several authorities have recently reasserted the importance of dopamine D_2 receptors in the management of schizophrenia (Kapur and Mamo, 2003; McGowan et al., 2004; Abi-Dargham and Laruelle, 2005). Nonetheless, no clinically employed antipsychotic differentiates D_2 from D_3 receptors (Vanhauwe et al., 2000; Dean and Scarr, 2004; Burstein et al., 2005; Joyce and Millan, 2005), so their respective importance remains uncertain. Interestingly, several arguments support current interest in D_3 receptors as a target for the potentially improved treatment of schizophrenia (Joyce and Millan, 2005; Boeckler and Gmeiner, 2006; Sokoloff et al., 2006).

First, although the density of D_3 versus D_2 receptors in rodent central nervous system is modest, they are better represented in primates and in man (Joyce, 2001; Sokoloff et al., 2006). In all species, in contrast to the enrichment of D_2 sites in striatal regions, D_3 receptors are concentrated in the nucleus accumbens and corticolimbic structures implicated in the control of mood, cognition, and the pathogenesis of schizophrenia (Joyce, 2001; Joyce and Millan, 2005; Sokoloff et al., 2006). Second, the density of D_3, but not D_2, receptors was elevated in (off-treatment) psychotic patients (Gurevich et al., 1997). Similar increases were seen in individuals chronically exposed to cocaine, which is known to aggravate and precipitate psychotic states (Richtand et al., 2001; Heidbreder et al., 2005). Third, the S9G polymorphism of D_3 receptors [which more potently binds dopamine (DA)] has been correlated to a higher risk for schizophrenia. Although this association was not seen in all patient populations, studies of the interaction between D_3 receptors and other genes support a relationship between this D_3 receptor polymorphism and vulnerability to schizophrenia (Dubertret et al., 1998; Jonsson et al., 2003; Joyce and Millan, 2005). Fourth, long-term administration of D_3 receptor antagonists reduces the spontaneous activity of mesolimbic but not nigrostriatal dopaminergic pathways (Ashby et al., 2000). Fifth, selective blockade of D_3 versus D_2 receptors improves cognitive performance and enhances frontocortical cholinergic transmission (Laszy et al., 2005; Millan et al., 2007a). Furthermore, consistent with an improvement in negative symptoms, pharmacological or genetic deletion of D_3 receptors enhances social interaction in rodents (Millan, 2003). Finally, gene knockout and pharmacological studies in rodents and primates have shown that antagonism of D_3 receptors favors motor function and counters its disruption by D_2 receptor blockade (Millan et al., 2004b; Joyce and Millan, 2005; Sokoloff et al., 2006).

Clinical evidence that selective blockade of D_3 receptors moderates the core symptoms of schizophrenia is awaited. Nonetheless, the above observations suggest that antipsychotics displaying a relative preference for D_3 over D_2 sites may conserve activity against positive symptoms, display strengthened efficacy against cognitive (and negative) symptoms and have a low EPS potential (Joyce and Millan, 2005). On this basis, we selected the benzopyranopyrrolidine derivative, S33138 (Fig. 1) (Dubuffet et al., 1999), for clinical evaluation. In the accompanying article (Millan et al., 2007), we characterize the actions of S33138 in a broad range of experimental models in vivo. The present article comprises a foundation for these studies in evaluating the interactions of S33138 with cloned, dopamine hD_3, hD_2, and hD_{2S} receptors and with several other classes of G-protein-coupled receptor.

Materials and Methods

Drug Evaluation, Salts, and Sources. S33138 was fully characterized in all experimental procedures. In addition, its binding profile at hD_3, hD_{2L}, and hD_{2S} receptors and at sites underlying metabolic, cardiovascular, and autonomic side effects was compared with those of the clinically established antipsychotics, haloperidol,

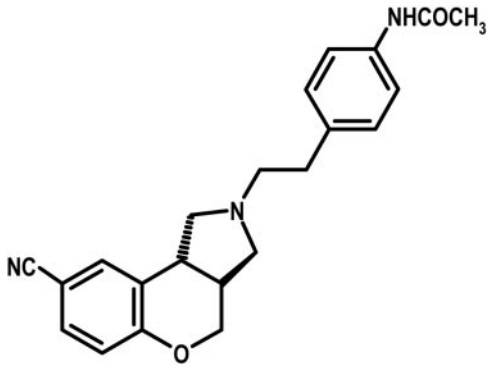

Fig. 1. Chemical structure of S33138.

clozapine, olanzapine, and risperidone. The majority of protocols employed have been extensively characterized in previous publications from this laboratory (see citations below). However, for those protocols that we have not, as yet, documented in detail, EC_{50}s for endogenous agonists are given together with pK_B values for prototypical antagonists evaluated in parallel as "internal" validators. In this regard, haloperidol was used as an internal reference agent for hD_3, hD_{2L}, and hD_{2S} receptors. Efficacies of S33138 were determined at all subtypes of dopaminergic receptor, with the exception of D_4 receptors, where its affinity (<5.0) was too low for study, and at all sites for which its affinities were within 2 "logs" (100-fold) of its affinity at hD_3 receptors. In all studies, results are based on at least three independent determinations performed in triplicate. S33138 and MDL100,907 were synthesized by G. Lavielle (Servier); SB269,970, olanzapine base, and risperidone base were synthesized by J.-L. Péglion (Servier); clozapine base, RX821,002, dopamine HCl, SCH23390, (−)quinpirole diHCl, forskolin base, noradrenaline ditartrate, serotonin creatinine sulfate, and haloperidol base were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France); [^{3}H]spiperone and [^{125}I]iodosulpride were purchased from GE Healthcare Europe (Orsay, France); and methiothepin maleate was purchased from Hoffman-LaRoche (Basel, Switzerland).

Evaluation of the Affinities of S33138 at Dopamine hD_3, hD_{2L}, and D_{2S} Receptors and at Other Classes of Binding Site. All protocols used for determination of the affinities of S33138 at multiple classes of native and cloned receptor have been documented previously (Millan et al., 2000a,b, 2004a; Newman-Tancredi et al., 2002; for summaries, see tables). Isotherms were analyzed by nonlinear regression using PRISM (GraphPad Software, San Diego, CA) to generate IC values. They were transformed into K_i values according to Cheng-Prussof: $K_i = IC_{50}/(1 + L/K_d)$, where L is the radioligand concentration, and K_d is the dissociation constant.

Antagonist Properties of S33138 at hD_3, hD_{2L}, and hD_{2S} Receptors: Guanosine-5′-*O*-(3-[^{35}S]thio)-Triphosphate Binding. The procedures used for determination of the functional actions of S33138 at Chinese hamster ovary (CHO) cell line-expressed hD_3, hD_{2L}, and hD_{2S} receptors (15, 2.2, and 1.6 pmol/mg protein, respectively) by guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) (PerkinElmer Life and Analytical Sciences, Boston, MA) binding have been described in detail elsewhere (Newman-Tancredi et al., 1999; Millan et al., 2000b, 2004a). In brief, [^{35}S]GTPγS was employed at a concentration of 1.0 nM for hD_3 receptors and at a concentration of 0.1 nM for hD_{2L} and hD_{2S} receptors. In each case, the pH was 7.4, the temperature was 22°C, and the incubation period was 40 min. The buffer comprised HEPES (20 mM), NaCl (150 mM for hD_3 receptors and l00 mM for hD_{2L} and hD_{2S} receptors), GDP (3 μM), and $MgCl_2$ (3 mM for D_3 receptors and 10 mM for hD_{2L} and hD_{2S} receptors). For evaluation of the actions of S33138 alone, membranes were incubated with incremental concentrations for 15 min before the addition of [^{35}S]GTPγS. For evaluation of the antagonist properties of S33138, interaction studies were undertaken with a fixed concentration of DA (1 μM for hD_3 sites, 10 μM for hD_{2L} sites, and 3 μM for hD_{2S} sites). In addition, the concentration-response curve for DA-induced [^{35}S]GTPγS binding at hD_3 receptors was examined in the presence of incremental, fixed concentrations of S33138 and the pA_2 value derived by Schild analysis. Experiments were terminated by rapid filtration through Unifilter-96 GF/B filters using a filter mate harvester (PerkinElmer Life and Analytical Sciences). The radioactivity retained on the filters was quantified using a TopCount microplate scintillation counter (PerkinElmer Life and Analytical Sciences). K_B values for inhibition of DA-stimulated [^{35}S]GTPγS binding by S33138 were calculated according to Cheng-Prusoff: $K_B = IC_{50}/(1 + (agonist/EC_{50}))$, where IC_{50} is the IC_{50} of S3318, agonist is the concentration of DA, and EC_{50} is the EC_{50} of DA alone.

Antagonist Properties of S33138 at hD_3 and hD_{2L} Receptors: Antibody-Capture/Scintillation Proximity Assay Studies of Coupling to $G\alpha_{i3}$. The influence of S33138 upon DA-induced activation of $G\alpha_{i3}$ subunits coupled to CHO-transfected hD_3 and hD_{2L} receptors was determined essentially as described previously (Millan et al., 2004a). In brief, [^{35}S]GTPγS binding was performed, as outlined above for filtration protocols, in 96-well optiplates (PerkinElmer Life and Analytical Sciences). After incubation, 20 μl of Nonidet P-40 (Roche Diagnostics, Mannheim, Germany, 0.27% final concentration) was added to each well, and plates were incubated for 30 min. Then, 10 μl of mouse anti-$G\alpha_{i3}$ monoclonal antibody (SA-281; Biomol, San Diego, CA) was added, and incubation was continued for 30 min. Scintillation proximity assay (SPA) beads coated with secondary, anti-mouse antibodies (GE Healthcare, Les Ulis, France) were added in a volume of 50 μl, and plates were incubated for 3 h with gentle agitation. They were then centrifuged (10 min, 1300*g*), and radioactivity was determined on a Top Count microplate scintillation counter (PerkinElmer Life and Analytical Sciences). Membranes were incubated with S33138 alone or with S33138 plus DA (1 μM for hD_3 and 10 μM for hD_{2L} receptors) for 15 min before addition of [^{35}S]GTPγS. K_B values were calculated according to the Cheng-Prussoff equation (see above).

Antagonist Properties of S33138 at hD_3 and hD_{2L} Receptors: Immunoblot Studies of Coupling to Extracellular-Regulated Kinase. Extracellular-regulated kinase [ERK; also known as mitogen-activated protein (MAP) kinase] phosphorylation was quantified as previously (Cussac et al., 1999; Millan et al., 2000b, 2004a). In brief, CHO cells expressing hD_3 or hD_{2L} receptors were grown in 24-well plates until 90% confluent and then starved overnight in serum-free medium. S33138 was diluted in the medium and added to each well at the desired concentration. For antagonist studies, cells were preincubated with S33138 for 20 min, then exposed to DA (0.1 μM for hD_3 and hD_{2L} receptors in each case) for 5 min. After incubation, 0.25 ml of Laemmli sample buffer containing 200 mM dithiothreitol was added. Whole-cell lysates were boiled for 3 min at 95°C, and 14 μl of cell extracts was loaded onto 15-well, 10% polyacrylamide gels. Phosphorylated forms of $pp42^{MAP\text{-}KINASE}$ (ERK 2) and $pp44^{MAP\text{-}KINASE}$ (ERK 1) were revealed using a monoclonal antibody (NanoTools, Denzlingen, Germany) followed by chemiluminescence detection with horseradish peroxidase coupled to the secondary antibody (GE Healthcare). Immunoblots shown are from representative experiments. Autoradiograms were analyzed, and phosphorylated ERK1/2 was quantified by computerized densitometry using AIS software (Imaging Research Inc., ON, Canada). Isotherms were analyzed by nonlinear regression with PRISM. K_B values were calculated according to the Cheng-Prusoff equation (see above).

Antagonist Actions of S33138 at hD_3, hD_{2L}, hD_3/hD_{2L}, and $hD_{3i3(D2)}$ Receptors: Adenylyl Cyclase Assays. The methods used have been detailed previously (Maggio et al., 2003; Novi et al., 2007). In brief, COS-7 cells, grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, were seeded at a density of 5×10^5 per 100-mm dish. Twenty-four hours later, they were transiently cotransfected by the DEAE-dextran chloroquine method with plasmid DNA encoding adenylyl cyclase (AC)-V and hD_3 receptors or with chimeric AC-V/VI plus hD_3 receptors, hD_{2L} receptors, hD_3 and hD_{2L} receptors, or chimeric $hD_{3i3(D2)}$ receptors. In this chimeric construct, kindly provided by Dr. Daniel Lévesque, the hD_2 receptor sequence, corresponding to amino acids 327 to 338 (KTMSRRKLSQQK) of the C-terminal portion of the "i3" loop, was introduced into the hD_3 receptor, replacing amino acids 312 to 323. This substitution changes neither ligand recognition profile nor cell surface expression compared with wild-type hD_3 receptors (Novi et al., 2007). Twenty-four hours after transfection, cells were trypsinized, recultured in 24-well plates, and, after an additional 24 h, the cells were assayed for AC activity. In brief, the cells were incubated for 2 h with 0.25 ml/well fresh growth medium containing 5 μCi/ml [^{3}H]adenine. This medium was replaced with 0.5 ml/well Dulbecco's modified Eagle's medium containing 20 mM HEPES, pH 7.4, 0.1 mg of bovine serum albumin, and the phosphodiesterase inhibitors, 1-methyl-3-isobutylxanthine (0.5 mM) and RO 20-1724 (0.5 mM). AC activity

was stimulated by addition of 1 μM forskolin in the presence or absence of S33138 and the D_2/D_3 receptor agonist, quinpirole, at concentrations indicated in the legend to Fig. 4. After 10 min of incubation at 30°C, the medium was removed, and the reaction was terminated by the addition of perchloric acid containing 0.1 mM unlabeled cAMP, followed by neutralization with KOH. The amount of [^{3}H]cAMP formed was determined by a two-step column separation procedure, and disintegrations per minute values were expressed with respect to the protein content of each sample; the average value (mean ± S.E.M.) was 56.7 ± 4.2 μg. K_B values were calculated according to the Cheng-Prussoff equation (see above).

Antagonist Properties of S33138 at hD_1 Receptors: SPA Studies of Coupling to Gα_s. Activation of hD_1 receptor-coupled Gα_s-protein was determined essentially as previously (Cussac et al., 2004). In brief, membranes of L cells expressing hD_1 receptors (11 pmol/mg protein) were preincubated with S33138 or assay buffer containing 20 mM HEPES (pH 7.4, 1 μM GDP, 50 mM $MgCl_2$, and 100 mM NaCl). The reaction was started by adding DA (1.0 μM) and [^{35}S]GTPγS (~0.3 nM) to 96-well optiplates (PerkinElmer Life and Analytical Sciences) for 60 min at room temperature. At the end of incubation, 20 μl of Nonidet P-40 (0.27% final concentration) was added to each well, and plates were incubated with gentle agitation for 30 min. Polyclonal antibodies against G$\alpha_{s/olf}$ (C18; Santa Cruz Biotechnology Inc., Santa Cruz, CA) were added to each well in a volume of 10 μl before 30 min of additional incubation. After addition of SPA beads coated with secondary antibodies (50 μl; GE Healthcare) and incubation for 3 h, plates were centrifuged (10 min, 1300*g*), and radioactivity was detected on a TopCount microplate scintillation counter (PerkinElmer Life and Analytical Sciences). K_B values were calculated according to the Cheng-Prussoff equation (see above).

Antagonist Properties of S33138 at hD_5 Receptors: Fluorescence Quantification of cAMP Production. GH4 cells stably expressing human D_5 receptors (0.34 pmol/mg protein) were resuspended in Hanks' balanced salt solution buffer containing 20 mM HEPES/NaOH, pH 7.4, 138 mM NaCl, 5.3 mM KCl, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 1.25 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, and 0.1% glucose and preincubated for 30 min at 37°C with 0.5 mM 3-isobutyl-1-methylxanthine. Cells were distributed at a density of 1.10^4 cells/well in microplates and preincubated for 10 min at room temperature with S33138, the D_5 receptor antagonist, SCH23390, or buffer (control). In antagonist studies, DA (30 nM) was added, and the mixture was incubated for 20 min at room temperature. The fluorescence acceptor (XL665-labeled cAMP) and the fluorescence donor (anti-cAMP antibody labeled with europium cryptate) were then added. After 60 min at room temperature, fluorescence transfer was measured at λ_{ex} = 337 nm, λ_{em} = 620 nm, and λ_{em} = 665 nm using a "high time-resolved fluorescence" microplate reader (RUBYstar; BMG, Offenburg, Germany). The production of cAMP was determined by dividing the signal measured at 665 nm by that measured at 620 nm (ratio). The results were expressed as percentage inhibition of the level of cAMP induced by 30 nM DA. K_B values were calculated according to the Cheng-Prussoff equation (see above).

Antagonist Properties of S33138 at hα_{2C}-ARs: [^{35}S]GTPγS Binding. [^{35}S]GTPγS binding was determined as documented in detail elsewhere (Audinot et al., 2002; Newman-Tancredi et al., 2002). In brief, membranes of CHO cells expressing hα_{2C}-AR (1.9 pmol/mg protein) were incubated for 60 min at 22°C with noradrenaline (NA) and [^{35}S]GTPγS at a concentration of 0.1 nM in a buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 3 μM GDP, and 3 mM $MgSO_4$. Antagonist properties of S33138 and the selective α_2-AR antagonist, RX821,002, were evaluated against a fixed concentration of NA (10 μM). Incubations were terminated by rapid filtration through Whatman GF/B filters using a Filter Harvester (PerkinElmer Life and Analytical Sciences). Radioactivity retained on the filters was quantified by liquid scintillation counting. K_B values were calculated according to the Cheng-Prussoff equation (see above).

Antagonist Properties of S33138 at hα_{2C}-ARs: SPA Studies of Coupling to Gα_{i3} and Gα_o. The coupling of hα_{2C}-ARs to Gα_{i3} was evaluated using an SPA procedure essentially as described outlined above for hD_3 receptors. The protocol used for Gα_o was the same as for Gα_{i3}. In brief, membranes were incubated on 96-well plates with NA (10 μM) and [^{35}S]GTPγS (0.2 nM) for 1 h at 22°C following preincubation with S33138, RX821,002, or assay buffer for 30 min. The buffer contained 20 mM HEPES, pH 7.4, 0.3 μM GDP, 3 mM $MgCl_2$, and 150 mM NaCl. The reaction was stopped by solubilizing membranes with detergent, Nonidet P-40 (0.27% final concentration). After gentle agitation for 30 min, 10 μl of mouse monoclonal anti-Gα_{i3} antibodies (see above) or anti-Gα_o antibodies (SA-280; Biomol) were then added, and plates were incubated for a further 1 h. At the end of the incubation period, SPA beads coated with anti-mouse secondary antibodies (GE Healthcare) were added. They were incubated with gentle agitation overnight before counting radioactivity on a TopCount microplate scintillation counter. Nonspecific binding was defined with 10 μM GTPγS. K_B values were calculated according to the Cheng-Prussoff equation (see above).

Antagonist Properties of S33138 at hα_{2C}-ARs: ERK Phosphorylation. The procedure used was essentially that outlined above for hD_3 receptors. CHO cells expressing hα_{2C}-ARs were grown in six-well plates until confluent. Cells were then washed twice with serum-free medium and incubated overnight in this medium. Cells were preincubated for 20 min with S33138, RX821,002, or buffer and then stimulated with NA (1 μM) for 5 min. Phosphorylated ERK 1/2 was measured in cell extracts using a monoclonal antibody against phosphorylated $pp42^{MAP\text{-}Kinase}$ (ERK 2) and $pp44^{MAP\text{-}Kinase}$ (ERK 1) (NanoTools). Autoradiograms were analyzed, and K_B values were calculated according to the Cheng-Prusoff equation (see above).

Antagonist Properties of S33138 at h5-HT_{1D} Receptors: [^{35}S]GTPγS Binding. Membranes of CHO cells expressing h5HT_{1D} receptors (1.6 pmol/mg protein) were incubated for 30 min at 22°C with 5-HT and [^{35}S]GTPγS at a concentration of 0.1 nM in a buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 3 μM GDP, and 3 mM $MgCl_2$. Incubations were terminated by rapid filtration through Whatman GF/B filters using a Filter Harvester (PerkinElmer Life and Analytical Sciences). Radioactivity retained on the filters was quantified by liquid scintillation counting. Antagonist properties of S33138 and the 5-HT_{1D} receptor antagonist, methiothepin, were evaluated against a fixed concentration of 5-HT (10 nM). K_B values were calculated according to the Cheng-Prussoff equation (see above).

Antagonist Properties at h5-HT_{2A} Receptors: SPA Studies of Coupling to Gα_q. Efficacy at h5-HT_{2A} receptors was determined by Gα_q activation in CHO cells expressing h5-HT_{2A} receptors (4.4 pmol/mg protein) employing an SPA protocol previously used for study of Gα_q-coupled h5-HT_{2C} receptors (Cussac et al., 2002). Cells were preincubated with S33138, the selective 5-HT_{2A} receptor antagonist, MDL100,987, or buffer for 30 min at 22°C. They were then incubated for 1 h with 5-HT (at a concentration of 100 nM in antagonist studies) and [^{35}S]GTPγS (0.3 μM). K_B values were calculated according to the Cheng-Prussoff equation (see above).

Antagonist Properties at h5-HT_{2A} Receptors: Studies of Inositol Phosphate Production. Determination of the accumulation of inositol phosphate (IP) in CHO cells expressing h5-HT_{2A} receptors was performed in 96-well plates (0.25×10^6 cells/well) after overnight labeling with [^{3}H]myoinositol (0.5 μCi/well). Stimulation by 5-HT (0.3 μM) was conducted for 30 min in a medium containing 10 mM LiCl and S33138, MDL100,907, or buffer. The reaction was stopped by adding formic acid (0.1 M). Supernatants were recovered, and IPs were purified by ion-exchange chromatography using DOWEX AG1-X8 resin (Bio-Rad, Hercules, CA) in 96-well filter plates (Millipore, Bedford, MA). The total radioactivity remaining in the membrane fraction was counted after treatment of the cells by a mixture of 10% Triton X-100 and 0.1 N NaOH. Radioactivity was quantified using a TopCount microplate scintillation counter (PerkinElmer Life and Analytical Sciences). Data are expressed (per-

centage) as (amount of total IP produced/amount of radioactivity remaining in membranes) × 100. K_B values were calculated according to the Cheng-Prussoff equation (see above).

Antagonist Properties of S33138 at h5-HT_7 Receptors: Fluorescence Quantification of cAMP Production. Actions of S33138 were determined in CHO cells stably expressing human 5-HT_7 receptors (0.25 pmol/mg protein) (1.10^4 cells/well in microplates) as described above for hD_5 receptors. Cells were preincubated for 10 min at room temperature with S33138, the selective 5-HT_7 receptor antagonist, SB269,970, or buffer. In antagonist studies, 5-HT (100 nM) was added, and incubations were performed for 20 min at room temperature. Fluorescence was quantified as described above for hD_5 receptors. The results are expressed as percent inhibition of the level of cAMP induced by 5-HT. K_B values were calculated according to the Cheng-Prussoff equation (see above).

Results

Binding Profile of S33138 at Multiple Subtypes of Dopamine Receptor (Table 1; Fig. 2). S33138 concentration-dependently and potently displaced the binding of [^{3}H]spiperone to hD_3 receptors (Fig. 2A). S33138 similarly competed with [^{3}H]spiperone at hD_{2L} receptors (long isoform) and hD_{2S} receptors (short isoform), but its affinity was markedly (approximately 25-fold) lower compared with hD_3 receptors (Fig. 2A). A similar pattern of data was acquired with [^{125}I]iodosulpride (used at 0.2 nM for hD_3 receptors and 0.1 nM for hD_{2L} receptors); this radioligand yielded affinities (pK_i values) of S33138 of 8.66 ± 0.09 and 7.43 ± 0.02 at hD_3 and hD_{2L} receptors, respectively. The affinity of S33138 for native, rat striatal D_2 receptors labeled by [^{3}H]spiperone was close to that seen at recombinant hD_2 receptors (Table 1). In contrast to hD_3 sites, the affinity of S33138 for cloned hD_4 receptors was low, although it displayed modest affinity for hD_1 and hD_5 receptors. S33138 displayed low affinity for native, rat, and cloned, human DA reuptake sites (pK_i values, <5.0 and 4.91 ± 0.03, respectively).

Binding Profile of S33138 at Nondopaminergic Receptors (Table 1). S33138 displayed significant affinity for h5-HT_7 receptors as well as for h5-HT_{2A} receptors, although its affinity for native, rat 5-HT_{2A} receptors was slightly lower than for h5-HT_{2A} sites. Modest affinity of S33138 also was seen at h5-HT_{1D} and, less markedly, at h5-HT_{1A}, h5-HT_{1B}, h5-HT_{2B}, and h5-HT_{2C} receptors. The affinity of S33138 was, however, negligible at h5-HT_3, h5-HT_4, h5-HT_{5A}, and h5-HT_6 receptors. S33138 also displayed low affinity for cloned, human 5-HT reuptake sites ([lt]5.0). The affinity of S33138 for multiple subtypes of hα_1-ARs was modest, and it manifested

TABLE 1

Affinities of S33138 at multiple classes of dopamine receptor, 5-HT receptor, and AR

For procedural details, see Millan et al. (2000a,b, 2004a) and Newman-Tancredi et al. (2002). Data are means ± S.E.M. of pK_i values, based on three to four determinations, each performed in triplicate. S33138 displayed negligible (pK_i < 5.0) affinities for the following sites: histamine H_2; opiate μ, δ, and κ; adenosine (A_1, A_2 adenosine); angiotensin I; benzodiazepine; bradykinin B_2; calcitonin gene-related peptide; cannabinoid (CB_1, CB); cholecystokinin (A, B); choline uptake; endothelin (A, B); GABA (A, B); glutamate (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, *N*-methyl-D-aspartate); imidazoline (I_2); melatonin (MT_1, MT_2); neurokinin (NK_1, NK_2); neuropeptide Y (Y_1, Y_5); and nicotinic, prostanoid (thromboxane A_2/PGH_2), σ (1, 2), estrogen, progesterone, and testosterone receptors. It also showed low affinity (pK_i < 5.0) for Ca^{2+} channels (diltiazem site), Na^+ channels (batrachotoxin site), and K^+ channels (ATP, Ca^{2+} and voltage dependent) and for a variety of enzymes, including acetylcholinesterase, adenyl cyclase, cyclooxygenase 1, guanylyl cyclase, 5-lipoxygenase, monoamine oxydase A and B, nitric-oxide synthase, phospholipase A_2, phospholipase C, phosphodiesterase I and III, protein kinase C, and sodium-potassium ATPase.

Receptor	Species	Tissue	Radioligand	pK_i
			nM	
D_3	Human	CHO cell line	[^{3}H]Spiperone (0.5)	8.68 ± 0.01
D_{2L}	Human	CHO cell line	[^{3}H]Spiperone (0.5)	7.13 ± 0.03
D_{2S}	Human	CHO cell line	[^{3}H]Spiperone (0.5)	7.26 ± 0.08
D_2	Rat	Striatum	[^{3}H]Spiperone (0.2)	7.12 ± 0.02
D_4	Human	CHO cell line	[^{3}H]Spiperone (0.4)	<5
D_1	Rat	Striatum	[^{3}H]SCH23390 (0.2)	5.60 ± 0.1
D_1	Human	L cell line	[^{3}H]SCH23390 (0.3)	6.22 ± 0.03
D_5	Human	GH4 cell line	[^{3}H]SCH23390 (0.3)	6.36 ± 0.05
5-HT_{1A}	Human	CHO cell line	[^{3}H]8-Hydroxy-2-dipropylaminotetralin (0.4)	6.15 ± 0.05
5-HT_{1A}	Human	CHO cell line	[^{3}H]WAY100,635 (0.4)	<5
5-HT_{1B}	Human	CHO cell line	[^{3}H]GR125,743 (1.0)	5.88 ± 0.09
5-HT_{1D}	Human	CHO cell line	[^{3}H]GR125,743 (1.0)	6.89 ± 0.04
5-HT_{2A}	Rat	Frontal cortex	[^{3}H]Ketanserin (0.5)	6.69 ± 0.05
5-HT_{2A}	Human	CHO cell line	[^{3}H]Ketanserin (0.5)	7.03 ± 0.02
5-HT_{2B}	Human	CHO cell line	[^{3}H]Mesulergine (1.0)	6.39 ± 0.07
5-HT_{2C}	Human	CHO cell line	[^{3}H]Mesulergine (1.0)	5.97 ± 0.01
5-HT_3	Human	NIE-115 cell line	[^{3}H]BRL43,694 (1.0)	<5
5-HT_4	Human	CHO cell line	[^{3}H]GR113,808 (0.1)	<5
5-HT_{5A}	Human	Human embryonic kidney 293 cell line	[^{3}H]LSD (1.0)	<5
5-HT_6	Human	Human embryonic kidney 293 cell line	[^{3}H]LSD (2.0)	5.26 ± 0.23
5-HT_7	Human	CHO cell line	[^{3}H]LSD (4.0)	7.43 ± 0.03
α_{2A}-AR	Rat	Cortex	[^{3}H]RX821,002 (0.4)	<5
α_{2A}-AR	Human	CHO cell line	[^{3}H]RX821,002 (0.8)	<5
α_{2B}-AR	Human	CHO cell line	[^{3}H]RX821,002 (4.0)	<5
α_{2C}-AR	Human	CHO cell line	[^{3}H]RX821,002 (0.6)	7.11 ± 0.15
α_1-AR	Rat	Frontal cortex	[^{3}H]Prazosin (0.25)	6.02 ± 0.05
α_{1A}-AR	Human	CHO cell line	[^{3}H]Prazosin (0.3)	6.10 ± 0.13
α_{1B}-AR	Human	CHO cell line	[^{3}H]Prazosin (0.3)	5.73 ± 0.06
α_{1C}-AR	Human	CHO cell line	[^{3}H]Prazosin (0.3)	6.12 ± 0.10
β_1-AR	Human	Sf9 cell line	[^{3}H]CGP-12177 (0.15)	<5
β_2-AR	Human	Sf9 cell line	[^{3}H]CGP-12177 (0.15)	<5

LSD, *d*-lysergic acid diethylamide; WAY100,635, ((*N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-*N*-(2-pyridinyl)cyclo-hexanecarboxamide)fumarate; GR125,743, *N*-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl)benzamide; BRL43,694, *N*-(9-methyl-9-azabicyclo[3.3.1.]non-3-yl-1-methyl-1*H*-indazol-3-carboxamide hydrochloride; GR113,808, 1-methyl-1*H*-indole-3-carboxylic acid 1-[2-(methylsulfonamido)ethyl]piperidin-4-ylmethyl ester.

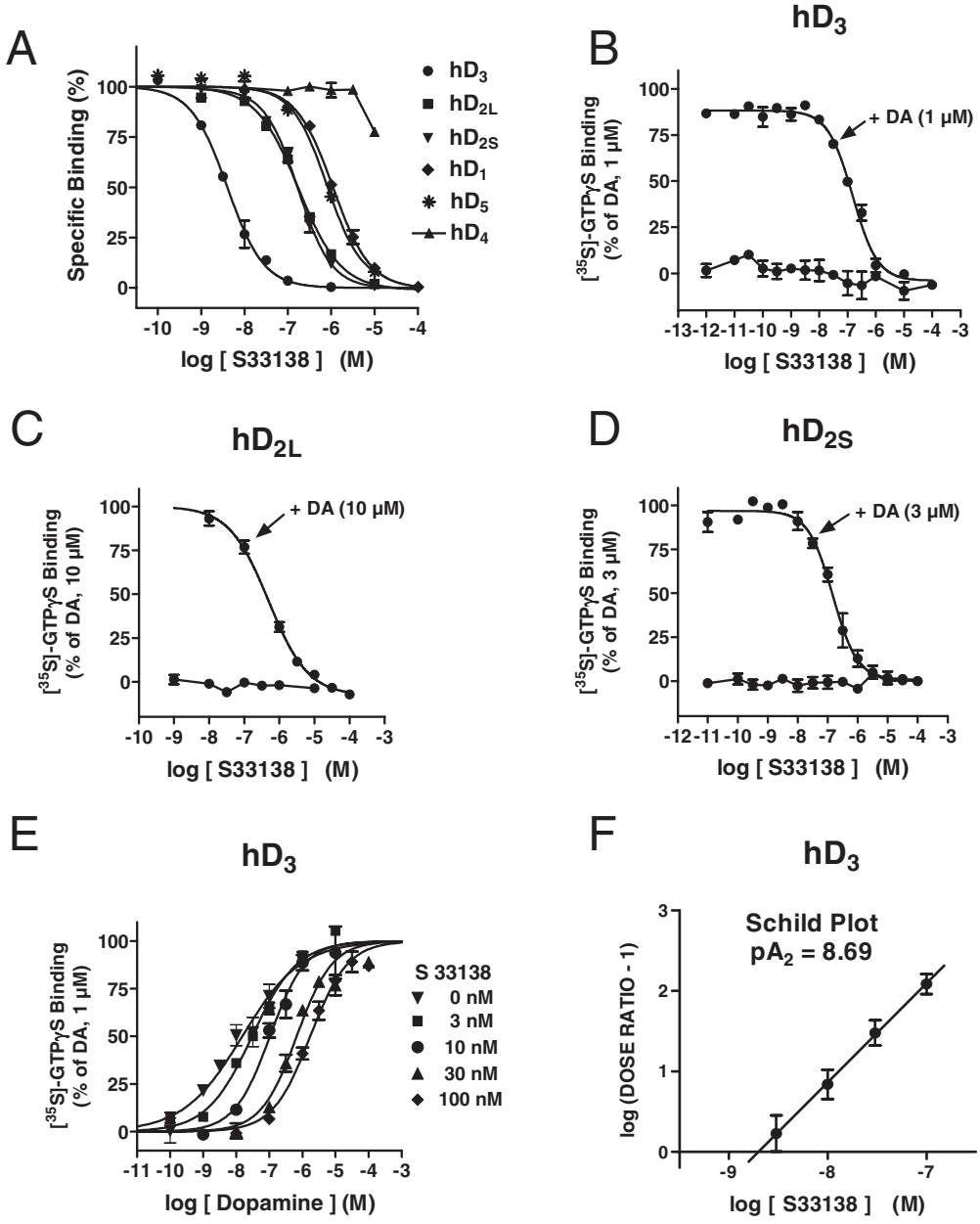

Fig. 2. Antagonism by S33138 of hD_3, hD_{2L}, and hD_{2S} receptor-coupled G-protein stimulation as determined by "total" [^{35}S]GTPγS binding. A, competition isotherms for binding of S33138 to hD_3 receptors compared with other dopamine receptor subtypes. B to D, concentration-dependent blockade by S33138 of the actions of DA (1, 10, and 3 μM, respectively) at hD_3, hD_{2L}, and hD_{2S} receptors. E, dextral displacement of the concentration-response to DA at hD_3 receptors in the presence of incremental (fixed) concentrations of S33138. F, Schild transformation of data in E. Data are representative of three to four independent experiments, each of which was performed in triplicate.

negligible affinity for hα_{2A}- and hα_{2B}-ARs. Conversely, S33138 showed greater affinity for hα_{2C}-ARs. The affinity of S33138 for hβ_1 and hβ_2-ARs was negligible, and it also showed low affinity for cloned, human NA reuptake sites (pK_i, 5.34 ± 0.14). S33138 showed negligible (<5.0) affinity for numerous other sites listed in the legend to Table 1.

Low Affinity of S33138 as Compared with Prototypical Antipsychotics for Receptors Underlying Metabolic, Cardiovascular, and Autonomic Side Effects (Table 2). Compared with its high affinity for hD_3 receptors, S33138 displayed only low affinities for h5-HT_{2C} receptors, hα_{1A}-ARs, hα_{1B}-ARs, hα_{1C}-ARs, and hH_1 receptors. It displayed negligible affinity for hM_1, hM_2, hM_3, and hM_4 receptors and weak affinity (K_i, 525 ± 11, n = 3) for hM_5 receptors. The affinity of haloperidol for h5-HT_{2C} compared with hD_3 receptors was low, although it showed marked affinities at α_{1A}-ARs, hα_{1B}-ARs, and hα_{1C}-ARs. It only weakly recognized hH_1 sites and multiple subtypes of muscarinic receptor. Compared with hD_3 receptors, clozapine manifested high affinity for h5-HT_{2C} receptors, for each subtype of hα_1-AR, and for hH_1 receptors. Its affinities at hM_1, hM_3, and hM_4 receptors were also high, and it displayed modest affinity for hM_2 receptors. Olanzapine revealed high affinities for h5-HT_{2C} receptors, for each subtype of hα_1-AR, for hH_1 receptors, and for all subtypes of muscarinic receptor, except hM_3, for which its affinity was modest. The affinities of risperidone for h5-HT_{2C} receptors, hα_1-AR subtypes, and hH_1 receptors were high, but, in contrast to clozapine and olanzapine, it only weakly interacted with muscarinic receptors.

Antagonist Properties of S33138 at hD_3 versus hD_{2L} and hD_{2S} Receptors: Filtration Assays of [^{35}S]GTPγS Binding (Fig. 2; Table 3). In line with previous studies (Newman-Tancredi et al., 1999; Millan et al., 2000b, 2004a), DA concentration-dependently (approximately 2-fold) stimulated [^{35}S]GTPγS binding at hD_3, hD_{2L}, and hD_{2S} receptors, actions blocked by haloperidol with pK_B values of 8.51 ±

TABLE 2

Interaction of S33138 and other antipsychotics at hD_3, hD_{2L}, and hD_{2S} receptors compared with sites underlying metabolic, cardiovascular, and autonomic side effects

All studies were performed in CHO cell lines. Drug affinities at hD_3, hD_{2L}, and hD_{2S} sites were determined as summarized in Table 1. Radioligands (concentrations) for the other sites were as follows: h5-HT_{2C} receptors ([^{3}H]mesulergine, 0.3 nM); hα_{1A}-, hα_{1B}-, and hα_{1C}-ARs ([^{3}H]prazosin, 0.3 nM); hH_1 receptors ([^{3}H]pyrilamine, 2.0 nM); hM_1 receptors ([^{3}H]pirenzepine, 2.0 nM); hM_2 receptors ([^{3}H]AF-DX384, 2.0 nM); and hM_3 and hM_4 receptors ([^{3}H]4-diphenylacetoxy-*N*-methylpiperidine, 0.2 nM). Data for S33138 at hα_1-AR subtypes and h5-HT_{2C} receptors are transformed from pK_i values in Table 1 and are included for the sake of completeness. Data are means ± S.E.M. based on three to four determinations, each performed in triplicate.

Drug	K_I					
	hD_3	hD_{2L}	hD_{2S}	hα_{1A}-AR	hα_{1B}-AR	hα_{1C}-AR
	nM					
S33138	2.1 ± 0.1	74.1 ± 1.5	54.9 ± 9.1	794 ± 102	1862 ± 26	758 ± 94
Haloperidol	4.1 ± 1.1	1.3 ± 0.1	1.4 ± 0.4	6.7 ± 1.3	1.8 ± 0.5	18 ± 3
Clozapine	562.3 ± 16.5	177.8 ± 4.0	166.0 ± 13.4	1.3 ± 0.1	1.1 ± 0.1	2.1 ± 0.2
Olanzapine	75.8 ± 3.1	46.8 ± 2.6	54.9 ± 1.4	20 ± 8	6.5 ± 0.4	26 ± 5
Risperidone	17.8 ± 7.1	9.7 ± 4.3	10.2 ± 4.2	0.56 ± 0.05	0.47 ± 0.05	1.4 ± 0.2

Drug	K_I					
	h5-HT_{2C}	hH_1	hM_1	hM_2	hM_3	hM_4
	nM					
S33138	1080 ± 25	>5000	>5000	>5000	>5000	>5000
Haloperidol	512 ± 62	551 ± 16	>5000	>5000	>5000	>5000
Clozapine	5.4 ± 0.7	1.9 ± 0.02	3.9 ± 0.1	146 ± 12	45 ± 5	18 ± 2
Olanzapine	7.6 ± 3.1	1.1 ± 0.2	8.2 ± 0.9	24 ± 6	107 ± 20	20 ± 1
Risperidone	6.7 ± 0.2	17 ± 1.1	>5000	2200 ± 200	>5000	580 ± 60

AF-DX384, 5,11-dihydro-11-{[(2-[2-[(dipropylamino)methyl]-1-piperidinyl}ethyl)amino]carbonyl}-6*H*-pyrido[2,3-*b*](1,4)-benzodiazepine-6-one methane sulfonate.

TABLE 3

Summary of antagonist properties of S33138 at hD_3, hD_{2L}, and hD_{2S} receptors as determined by blockade of DA-stimulated [^{35}S]GTPγS binding, Gα_{i3} recruitment, and extracellular-regulated-kinase (ERK) phosphorylation

Data (pK_i and pK_B values) are means ± S.E.M. of three to four independent experiments.

hD_3						
[^{3}H]Spiperone	[^{35}S]GTPγS		SPA (Gα_{i3})		ERK1/2	
pK_i	pK_B	% MOI	pK_B	% MOI	pK_B	% MOI
8.68 ± 0.01	8.85 ± 0.10	97 ± 3	8.24 ± 0.07	95 ± 7	9.08 ± 0.02	104 ± 4

hD_{2L}							hD_{2S}		
[^{3}H]Spiperone	[^{35}S]GTPγS		SPA (Gα_{i3})		ERK1/2		[^{3}H]Spiperone	[^{35}S]GTPγS	
pK_i	pK_B	% MOI	pK_B	% MOI	pK_B	% MOI	pK_i	pK_B	% MOI
7.13 ± 0.03	7.82 ± 0.03	104 ± 8	7.76 ± 0.14	83 ± 12	8.03 ± 0.02	101 ± 2	7.26 ± 0.08	7.79 ± 0.06	96 ± 5

MOI, maximal observed inhibition.

0.06, 9.49 ± 0.16, and 8.90 ± 0.16, respectively. S33138 did not modify [^{35}S]GTPγS binding when applied alone. Indeed, it potently suppressed the stimulation of [^{35}S]GTPγS binding at hD_3 receptors by DA with a pK_B of 8.85 ± 0.10; this value corresponds well to its affinity (8.68) at these sites. Furthermore, in the presence of incremental concentrations of S33138 (3–100 nM), the concentration-response curve for DA-induced [^{35}S]GTPγS binding at hD_3 receptors was shifted in parallel to the right with no loss of maximal effect (Fig. 2E). Schild analysis (Fig. 2F) yielded a slope not significantly different to unity (1.18, $r = 0.88$) and a pA_2 (8.69) very close to its pK_B and pK_i values at hD_3 sites. These observations indicate that S33138 behaves as a competitive and reversible antagonist at hD_3 receptors. S33138 likewise suppressed DA-induced [^{35}S]GTPγS binding at hD_{2L} and hD_{2S} receptors, indicating that it is also an antagonist at these sites (Fig. 2, C and D). However, pK_B values of 7.82 ± 0.03 and 7.79 ± 0.06 at hD_{2L} and hD_{2S} receptors, respectively, were lower than for hD_3 sites.

Antagonist Properties of S33138 at hD_3 versus hD_{2L} Receptors: SPA Studies of Coupling to Gα_{i3} (Table 3). At hD_3 and hD_{2L} receptors, in line with previous work (Millan et al., 2004a), DA enhanced [^{35}S]GTPγS binding to Gα_{i3} by approximately 2-fold; its actions were blocked by haloperidol, with pK_B values of 8.25 ± 0.01 and 9.18 ± 0.26, respectively. In contrast, S33138 alone was without activity. At hD_3 receptors, S33138 concentration-dependently suppressed DA-induced stimulation of Gα_{i3} with a pK_B of 8.24 ± 0.07. S33138 also behaved as an antagonist at hD_{2L} receptor-coupled Gα_{i3} in blocking its activation by DA with a pK_B of 7.76 ± 0.14.

Antagonist Properties of S33138 at hD_3 versus hD_{2L} Receptors: Immunoblot Studies of ERK Phosphorylation (Fig. 3; Table 3). By analogy to previous studies (Cussac et al., 1999; Millan et al., 2004a), in CHO cells transfected with hD_3 or hD_{2L} receptors, DA triggered a transient yet marked activation (phosphorylation) of ERK 1/2 (MAP kinase). In distinction, S33138 did not increase levels of the phosphorylated forms of ERK. Furthermore, S33138 concentration-dependently abolished induction of ERK 1/2 at hD_3 receptors with a pK_B of 9.08 ± 0.02 (Fig. 3A). S33138 simi-

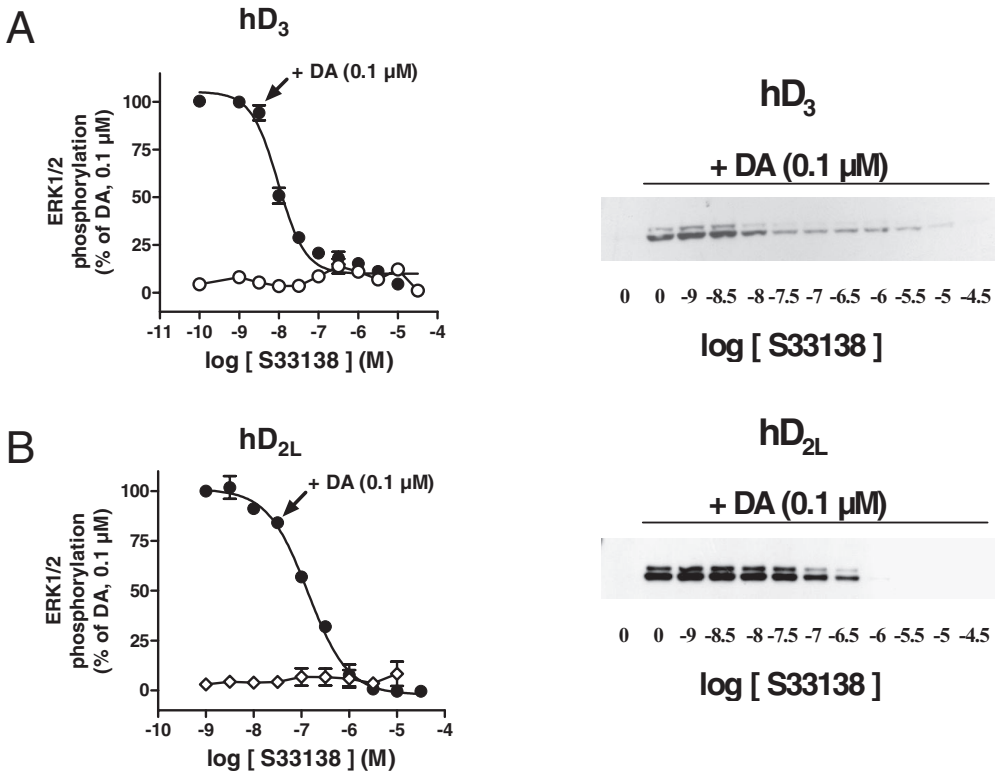

Fig. 3. Antagonism by S33138 of hD_3 and hD_{2L} receptor-coupled extracellular-regulated-kinase phosphorylation as determined by immunoblot assays. Concentration-dependent antagonist properties of S33138 at hD_3 receptors (A) and at hD_{2L} receptors (B) against DA (0.1 μM). Immunoblots are shown to the right of the S33138 concentration-response curves. Data are representative of three to four independent experiments, each of which was performed in triplicate.

larly behaved as an antagonist of DA-stimulated ERK 1/2 phosphorylation at hD_{2L} receptors with a pK_B of 8.03 ± 0.02 (Fig. 3B).

Antagonist Properties of S33138 at hD_3 Receptors, hD_{2L} Receptors, Cotransfected hD_3 and hD_{2L} Receptors, and $hD_{3i3(D2)}$ Receptors: Enzymatic Studies of AC Activity (Fig. 4). In COS-7 cells transfected with a chimeric AC-V/VI, forskolin elicited a marked (approximately 6-fold) increase in cAMP accumulation. This AC isoform is insensitive to D_3 receptors, and, confirming previous work (Maggio et al., 2003; Novi et al., 2007), quinpirole did not modify the influence of forskolin in cells cotransfected with hD_3 receptors. On the other hand, quinpirole (EC_{50}, 1.2 nM) robustly and potently suppressed forskolin-induced cAMP accumulation in cells cotransfected with AC-V/VI and hD_{2L} receptors. With similar potency, it reduced the activity of AC-V/VI in cells cotransfected with both hD_{2L} and hD_3 receptors (EC_{50}, 0.97 nM). S33138 alone did not modify the effects of forskolin in hD_3, hD_{2L}, or hD_3/hD_{2L} cotransfected cells. However, it concentration-dependently abolished the inhibitory influence of quinpirole both in cells expressing hD_{2L} receptors (pK_B, 7.57 ± 0.12) and, more potently, in cells cotransfected with hD_3 and hD_{2L} receptors (pK_B, 8.69 ± 0.14) (Fig. 4A). In COS-7 cells transfected with AC-V/VI and chimeric $hD_{3i3(D2)}$ receptors, where a 12-amino acid sequence of the third intracellular loop was substituted by the corresponding sequence of hD_{2L} receptors to enhance coupling efficacy to AC-V/VI, quinpirole (EC_{50}, 1.01 nM) markedly suppressed cAMP formation. Its action was blocked by S33138 with pK_B of 8.71 ± 0.16 (Fig. 4B). In contrast to AC-V/VI, the AC-V isoform is responsive to D_3 receptors, although effects of agonists are less robust than at hD_{2L} receptors (Robinson and Caron, 1997; Maggio et al., 2003). Quinpirole significantly reduced the influence of forskolin upon cAMP accumulation in cells transfected with hD_3 receptors and AC-V; this effect was reversed by S33138, which was inactive alone (Fig. 4C).

Antagonist Properties of S33138 at hD_1 and hD_5 Receptors. In L cells transfected with hD_1 receptors, DA (EC_{50}, 11.5 nM) elicited an approximately 2-fold increase in [^{35}S]GTPγS binding to $G\alpha_s$ as quantified using an SPA protocol. In contrast, S33138 was ineffective and concentration-dependently eliminated the action of DA with a pK_B of 6.21 ± 0.11. In CHO cells transfected with hD_5 receptors, DA elevated cAMP accumulation with an EC_{50} of 41.7 nM. This action of DA was blocked by the D_5 receptor antagonist, SCH23390, with a pK_B of 8.82 ± 0.08. It was also concentration-dependently abrogated by S33138 (pK_B, 6.46 ± 0.16), which was inactive alone. S33138 then behaves as an antagonist of hD_1 and hD_5 receptors with modest potency.

Antagonist Properties of S33138 at $h\alpha_{2C}$-ARs (Fig. 5). Corroborating previous studies at CHO-transfected $h\alpha_{2C}$-ARs (Audinot et al., 2002; Newman-Tancredi et al., 2002), NA elicited an approximately 2-fold enhancement in [^{35}S]GTPγS binding, whereas S33138 was without effect. The action of NA (10 μM) was abolished by S33138 with a pK_B of 7.23 ± 0.15. In SPA assays, NA robustly (approximately 1.5-fold in each case) enhanced the binding of [^{35}S]-GTPγS to $G\alpha_{i3}$ (EC_{50}, 513 nM) and $G\alpha_o$ (EC_{50}, 145 nM), actions blocked by the prototypical α_2-AR antagonist, RX821,002, with pK_B values of 9.04 ± 0.09 and 8.57 ± 0.05, respectively. S33138, which was inactive alone, also abolished the effects of NA with pK_B values of 6.85 ± 0.07 and 7.29 ± 0.14 (Fig. 5, A and B), respectively. Likewise, in CHO cells transfected with $h\alpha_{2C}$-ARs, NA (EC_{50}, 27.7 nM) elicited a robust increase in ERK 1/2 phosphorylation. This effect of NA (1 μM) was blocked by RX821,002 (pK_B, 8.32 ± 0.10) and dose-dependently abolished by S33138 (pK_B, 7.11 ± 0.15) (Fig. 5C). In line with its weak affinity for $h\alpha_{2A}$- and $h\alpha_{2B}$-

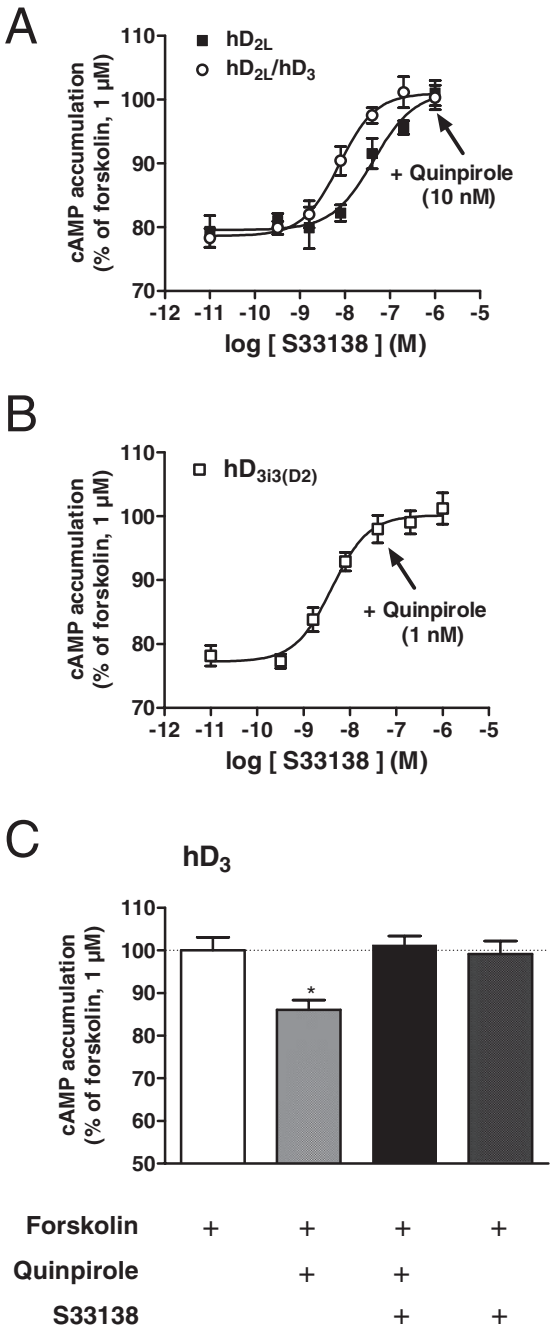

Fig. 4. Antagonism by S33138 of quinpirole-induced inhibition of adenylyl cyclase activity in CHO cells transfected with hD_{2L} and/or hD_3 receptors or with $hD_{3i3(D2)}$ receptors. A, concentration-dependent blockade by S33138 of the inhibitory influence of the D_3/D_2 receptor agonist, quinpirole (10 nM), upon forskolin-stimulated cAMP formation in CHO cells transfected either with hD_{2L} receptors or with hD_{2L} receptors and hD_3 receptors. In both cases, cells were also transfected with a chimeric adenylate cyclase (V/VI) resistant to hD_3 receptors. B, concentration-dependent blockade by S33138 of the inhibitory influence of quinpirole (1 nM) upon forskolin-stimulated cAMP formation in CHO cells transfected with chimeric $hD_{3i3(D2)}$ receptors that couple more robustly than their wild-type counterparts to AC-V/VI. C, blockade by a fixed concentration of S33138 (1 μM) of the inhibitory influence of quinpirole (1 μM) upon forskolin (1 μM)-stimulated cAMP accumulation in CHO cells transfected with hD_3 receptors together with adenylate cyclase-V. Data are representative of three to four independent experiments, each of which was performed in triplicate. *, $P < 0.01$ versus forskolin alone.

ARs, S33138 showed no antagonist properties (up to 10 μM) in [^{35}S]GTPγS binding studies of these sites (data not shown).

Antagonist Properties of S33138 at 5-HT Receptor Subtypes (Fig. 5). Serotonin (EC_{50}, 1.3 nm) evoked an approximately 1.3-fold increase in [^{35}S]GTPγS binding at h5-HT_{1D} receptors. The antagonist, methiothepin, antagonized the action of 5-HT with a pK_B of 7.55 ± 0.07. S33138 also blocked the influence of 5-HT upon [^{35}S]GTPγS binding at h5-HT_{1D} receptors with a pK_B of 6.79 ± 0.18. It was inactive alone at these sites. At h5-HT_{2A} receptors, an SPA assay revealed activation by 5-HT (EC_{50}, 27.8 nM) of [^{35}S]GTPγS binding to Gα_q with a maximal 2-fold increase versus basal values. The effect of 5-HT (0.3 μM) was abolished by the prototypical 5-HT_{2A} antagonist, MDL100,907, with a pK_B of 9.26 ± 0.20. Activation of Gα_q coupled to h5-HT_{2A} receptors was also abolished by S33138 with a pK_B of 6.86 ± 0.02 (Fig. 5D). S33138 did not influence Gα_q alone. Serotonin (EC_{50}, 31.9 nM) also activated phospholipase C coupled to h5-HT_{2A} receptors as reflected in a marked increase in IP formation. This action of 5-HT (0.3 μM) was abolished by MDL100,907 with a pK_B of 8.9 ± 0.1. It was also blocked by S33138 with a pK_B of 6.79 ± 0.07 (Fig. 5E). At h5-HT_7 receptors, 5-HT enhanced the activity of AC with an EC_{50} of 30.0 nM. The 5-HT_7 antagonist, SB269,970, blocked the action of 5-HT with a pK_B of 8.85 ± 0.12. S33138 was inactive alone and blocked this action of 5-HT with a pK_B of 7.06 ± 0.25 (Fig. 5F).

Discussion

Preferential Antagonism by S33138 of hD_3 versus hD_{2L} and hD_{2S} Receptors. The higher affinity of S33138 for hD_3 versus hD_{2L} and hD_{2S} receptors distinguishes it from haloperidol, clozapine, olanzapine, risperidone, and other clinically available antipsychotics displaying similar affinities for hD_3, hD_{2L}, and hD_{2S} sites (Table 2) (Millan et al., 2000a; Vanhauwe et al., 2000; Burstein et al., 2005). Furthermore, the marked D_3 versus D_2 receptor preference of S33138 likewise differentiates it from aripiprazole and bifeprunox, which behave as partial agonists at hD_3 and hD_2 receptors (Shapiro et al., 2003; Burstein et al., 2005; Novi et al., 2007; Urban et al., 2007b). Although the preference of S33138 for hD_3 versus hD_2 sites is less pronounced than the 100-fold selectivity of antagonists like S33084, SB414,796, and A437,203 (Millan et al., 2000b; Reavill et al., 2000; Joyce and Millan, 2005; Boeckler and Gmeiner, 2006), it was selected for clinical development precisely on this basis. That is, rather than "absolute" selectivity, preferential D_3 versus D_2 receptor blockade should allow for antipsychotic activity against positive and cognitive-negative symptoms in the relative absence of extrapyramidal dysfunction (Joyce and Millan, 2005). This preferential interaction of S33138 with D_3 versus D_2 receptors is supported by in vivo findings reported in the accompanying article (Millan et al., 2007b).

hD_3 receptors couple via pertussis-sensitive G-proteins to AC and ERK 1/2 (MAP kinases) (Ahlgren-Beckendorf and Levant, 2004; Beom et al., 2004; Neve et al., 2004; Sokoloff et al., 2006). Activation of hD_3 receptors by DA led to enhanced [^{35}S]GTPγS binding (Newman-Tancredi et al., 1999, 2002; Vanhauwe et al., 2000), an effect potently and reversibly blocked by S33138, indicating competitive antagonist properties. Moreover, using an SPA procedure coupled to a highly selective antibody (Millan et al., 2004a; Neve et al., 2004), the activation of Gα_{i3} by DA was also shown to be antagonized by S33138. Coupling of hD_3 receptors to AC is variable and

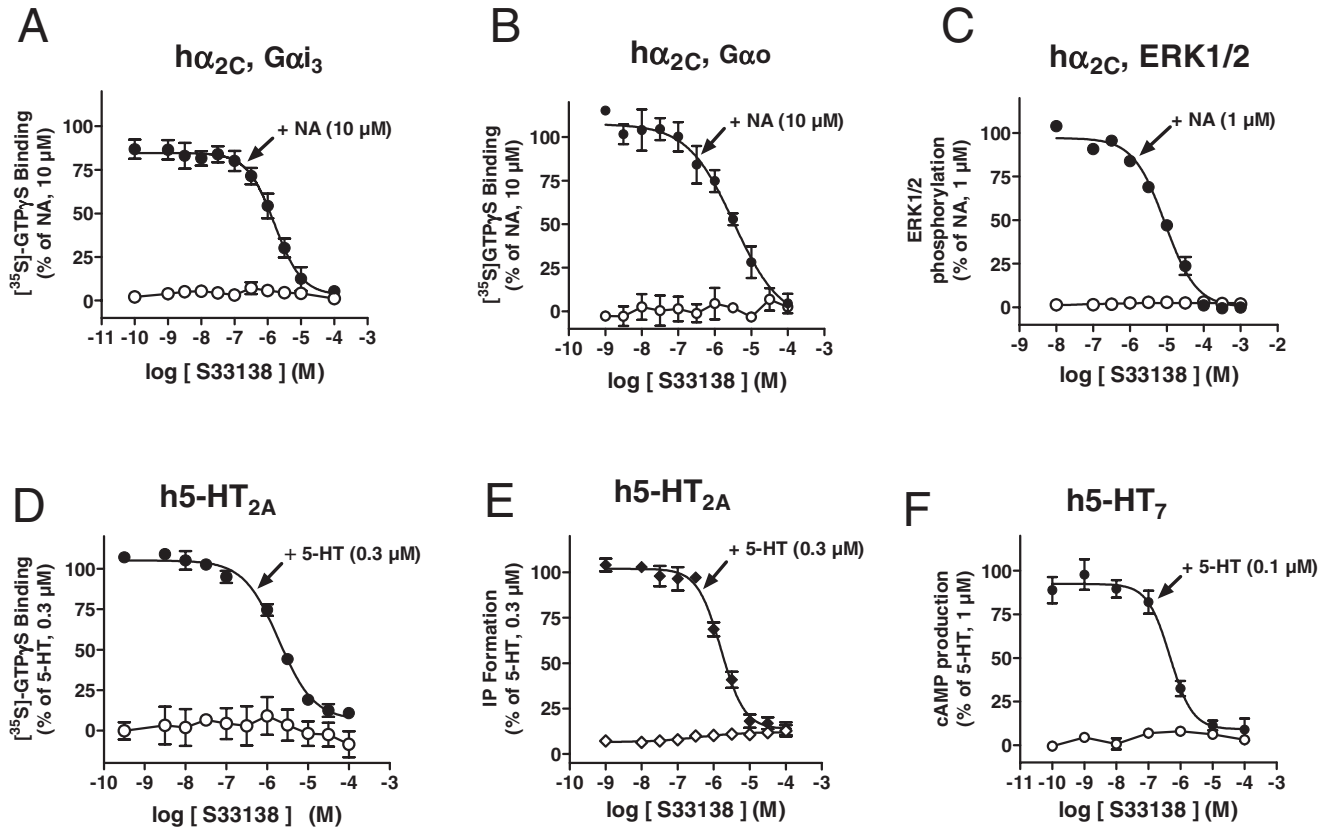

Fig. 5. Antagonist properties of S33138 at $h\alpha_{2C}$-adrenoceptors, h5-HT_{2A} receptors, and h5-HT_7 receptors. Concentration-dependent blockade by S33138 of $h\alpha_{2C}$-adrenoceptors as measured by NA-induced activation of $G\alpha_{i3}$ (A), $G\alpha_o$ (B), and ERK phosphorylation (C). D, concentration-dependent blockade by S33138 of the stimulation of h5-HT_{2A} receptor-coupled $G\alpha_q$ by 5-HT (0.3 μM). E, concentration-dependent blockade by S33138 of the stimulation of h5-HT_{2A} receptor-coupled IP production by 5-HT (0.3 μM). F, concentration-dependent blockade by S33138 of the stimulation of h5-HT_7 receptor-coupled adenylyl cyclase by 5-HT (0.1 μM). Data are representative of three to four independent experiments, each of which was performed in triplicate.

isoform-dependent (Griffon et al., 1997; Robinson and Caron, 1997; Hall and Strange, 1999; Neve et al., 2004). Nonetheless, in COS-7 cells transfected with hD_3 receptors, quinpirole inhibited forskolin-stimulated AC-V activity, and this action was abolished by S33138. Furthermore, S33138 blocked the inhibition of cAMP formation by quinpirole in cells transfected with a chimeric $hD_{3i3(D2)}$ receptor displaying enhanced coupling efficacy to AC-V/VI (Novi et al., 2007), yet identical ligand recognition profiles and cell surface expression levels versus wild-type hD_3 receptors (Novi et al., 2007). D_3 receptors recruit the ERK pathway by pertussis toxin-sensitive $G\alpha_i$ (including $G\alpha_{i3}$) proteins and an array of intracellular cascades involving both α and β subunits, a "phosphoinoside-3 kinase," and an atypical protein kinase C (Cussac et al., 1999; Newman-Tancredi et al., 1999; Beom et al., 2004; Neve et al., 2004). Correspondingly, in CHO cells bearing hD_3 receptors, DA stimulated ERK 1/2 phosphorylation, an effect abolished by S33138. The lack of effect of S33138 alone is important because ERK 1/2 activation is highly sensitive even to weak partial agonist actions; for example, aripiprazole and bifeprunox (M. J. Millan and C. Mannoury la Cour, unpublished observation; Cussac et al., 1999; Shapiro et al., 2003; Burstein et al., 2005; Urban et al., 2007b). In certain cell lines, D_3 receptors couple to G_o, $G_{q/11}$, G_z, and (via pertussis-insensitive pathways) phospholipase D (Everett and Senogles, 2004; Neve et al., 2004). Thus, it would be of interest to extend the present work to other intracellular cascades.

D_2 receptors show subtle differences to hD_3 sites as concerns their interactions with signaling pathways (Hall and Strange, 1999; Vanhauwe et al., 1999'02; Ahlgren-Beckendorf and Levant, 2004; Beom et al., 2004; Neve et al., 2004). Nonetheless, like their D_3 counterparts, both hD_{2S} and hD_{2L} receptors (which are principally localized presynaptically and postsynaptically to dopaminergic pathways, respectively) couple via $G_{o/i}$ to AC and ERK 1/2. Across a broad range of measures, [^{35}S]GTPγS binding to hD_{2L} and hD_{2S} receptors (Vanhauwe et al., 2000; Newman-Tancredi et al., 2002), as well as hD_{2L} receptor coupling to $G\alpha_{i3}$ (Millan et al., 2004a; Lane et al., 2007), AC (O'Hara et al., 1996; Hall and Strange, 1999), and ERK 1/2 (Beom et al., 2004; Neve et al., 2004), S33138 behaved as a pure antagonist at both hD_{2L} and hD_{2S} receptors with lower potency than at hD_3 receptors. Recent work on "agonist-directed" trafficking and "protean" agonism at hD_{2L} sites emphasizes that drug efficacy can vary as a function of the intracellular pathway (Neve et al., 2004; Lane et al., 2007; Urban et al., 2007a). Thus, it would be interesting to examine the influence of S33138 upon other D_2 receptor-coupled transduction cascades.

Antagonism by S33138 of Cotransfected hD_3 and hD_{2L} Receptors. D_3 and D_2 receptors are partially colocalized in dopaminergic pathways and postsynaptic neurones (Surmeier et al., 1992; Joyce and Millan, 2005), and they assemble into

functional D_3/D_{2L} heterodimers in vitro (Maggio et al., 2003; Novi et al., 2007). Heterodimers display functional characteristics distinct from monomers, so it is of significance that S33138 abolished the inhibitory influence of quinpirole upon AC in cells expressing both hD_3 and hD_{2L} receptors. Furthermore, S33138 was more potent in cells expressing hD_3 and hD_2 sites than in cells expressing hD_2 receptors alone. This finding resembles the altered potencies of other drugs at D_3/D_2 heterodimers compared with constituent monomers (Lee et al., 2003; Maggio et al., 2003; Novi et al., 2007). The underlying reasons remain to be defined. Nonetheless, proof that functional heterodimers form in the brain is awaited. Thus, relevance of the actions of S33138 (and other antipsychotics) at D_3/D_2 heterodimers to the control of psychotic states remains to be clarified (Lee et al., 2003; Maggio et al., 2003).

Antagonism by S33138 of hD_1 and hD_5 Receptors. Dopamine D_1 and (closely related) D_5 receptors couple positively to AC via G_s (Cai et al., 1999; Neve et al., 2004). As quantified by SPA coupled to a specific anti-$G\alpha_s$ antibody (Cussac et al., 2004), recruitment of G_s by hD_1 receptors was blocked by S33138, indicating mild antagonist properties at these sites. Blockade of mesolimbic D_1 receptors may participate in the antipsychotic properties of certain antipsychotics, and equilibrated antagonism of striatal D_1 and D_2 receptors contributes to the low EPS potential of clozapine (Josselyn et al., 1997; Tauscher et al., 2004). However, in vivo studies suggest that blockade of D_1 sites is unlikely to play a major role in the therapeutic actions of S33138 (Millan et al., 2007). Antagonist properties of S33138 at hD_5 receptors were also expressed at concentrations well below those blocking D_3 receptors. Furthermore, although clozapine likewise antagonizes D_5 receptors (Neve et al., 2004), any potential advantage in the management of schizophrenia remains conjectural.

Antagonist Actions of S33138 at $h\alpha_{2C}$-ARs. An intriguing finding differentiating S33138 from other antipsychotics was its interaction with $h\alpha_{2C}$-ARs despite low affinities for their $h\alpha_{2A}$ and $h\alpha_{2B}$ counterparts. All $h\alpha_2$-AR subtypes couple via G_i/G_o to downstream pathways converging on ERK 1/2 (Hieble et al., 1995), and, by analogy to other α_2-AR antagonists, S33138 abolished activation of $h\alpha_{2C}$-ARs in procedures of G-protein activation, recruitment of $G\alpha_{i3}$ and $G\alpha_o$, and ERK 1/2 phosphorylation (Alblas et al., 1993; Jasper et al., 1998; Audinot et al., 2002). Few ligands possessing a marked preference for α_{2C}- versus α_{2A}/α_{2B}-ARs have been described previously (Hieble et al., 1995), so S33138 may serve as a useful template for construction of chemically novel selective α_{2C}-AR antagonists. Exploration of the significance of α_{2C}-AR blockade to the functional profile of S33138 would be of interest because activation of α_{2C}-ARs in frontal cortex and hippocampus may compromise cognitive performance (Marcus et al., 2005; Soto-Moyano et al., 2005). Moreover, antagonism of corticolimbic and striatal α_{2C}-ARs may contribute to the atypical profile of clozapine, including its low EPS potential (Millan et al., 2001'02; Kalkman and Loetscher, 2003; Svensson, 2003).

Antagonist Actions of S33138 at h5-HT_{2A} and h5-HT_7 Receptors. S33138 behaved as a pure antagonist at h5-HT_{2A} receptors in protocols quantifying the activation of $G\alpha_q$ and the turnover of IP (Cussac et al., 2002; Kurrasch-Orbaugh et al., 2003). This observation is of potential importance because the more marked affinity of atypical antipsychotics for 5-HT_{2A} versus D_2 receptors has been related to improved control of negative symptoms and a low propensity to elicit EPS (Meltzer et al., 2003; Werkman et al., 2006). Activation of 5-HT_7 receptors engages AC via recruitment of G_s (Thomas and Hagan, 2004), and modest antagonist actions of S33138 at h5-HT_7 sites were seen in a protocol of cAMP formation. Blockade of 5-HT_7 receptors by S33138 mimics clozapine and several other antipsychotics (Roth et al., 1994; Thomas and Hagan, 2004). 5-HT_7 receptors are enriched in the cortex, hippocampus, striatum, and suprachiasmatic nucleus, and their blockade may favorably influence mood, sleep, and circadian rhythms, which are perturbed in schizophrenic patients (Thomas and Hagan, 2004; Monti and Monti, 2005).

Low Affinity of S33138 for hH_1, Human Muscarinic, and $h\alpha_1$-AR Receptors. The weak affinity of S33138 for H_1 receptors suggests a low sedative potential and a low risk of obesity compared with antipsychotics that potently block these sites (Table 2) (Cunningham-Owens, 1996; Kroeze et al., 2003; Kim et al., 2007). A low risk of sedation is consistent with the weak affinity of S33138 for α_1-ARs, which also suggests a low risk of orthostatic hypotension and cardiovascular perturbation (Pacher and Kecskemeti, 2004; Lieberman et al., 2005). Moreover, S33138 did not interact with hM_1-hM_4 muscarinic receptors, suggesting that it should not provoke autonomic side effects such as troubled vision and gastrointestinal discomfort (Cunningham-Owens, 1996). Importantly, moreover, S33138 should not compromise cognitive function by blocking postsynaptic populations of muscarinic and H_1 receptors (Lieberman et al., 2005).

Conclusions

The novel benzopyranopyrrolidine derivative, S33138, behaves as a preferential antagonist of hD_3 versus hD_{2L} and hD_{2S} receptors. It also displays modest antagonist properties at $h\alpha_{2C}$-ARs, h5-HT_{2A} receptors, and h5-HT_7 receptors, actions likewise of potential relevance to the treatment of schizophrenia. This distinctive receptor-binding profile of S33138 was corroborated by in vivo studies in rodents and primates (Millan et al., 2007), underscoring its innovative profile for the improved management of psychotic states. Conceivably, low doses may suffice for maintenance therapy, whereas during acute exacerbation of psychosis, doses can be increased to more markedly antagonize D_2 receptors. This remains to be seen. In any case, ongoing (phase II) clinical studies should soon shed light on the long-standing question of how antipsychotics that preferentially block D_3 versus D_2 receptors compare with clinically established agents acting with similar affinity at these sites.

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