S33084, a Novel, Potent, Selective, and Competitive Antagonist at Dopamine D3-Receptors: I. Receptorial, Electrophysiological and Neurochemical Profile Compared with GR218,231 and L741,626

MARK J. MILLAN, ALAIN GOBERT, ADRIAN NEWMAN-TANCREDI, FRANÇOISE LEJEUNE, DIDIER CUSSAC, JEAN-MICHEL RIVET, VALÉRIE AUDINOT, THIERRY DUBUFFET, and GILBERT LAVIELLE


Accepted for publication February 21, 2000

ABSTRACT

The benzopyranopyrrole S33084 displayed pronounced affinity (pKᵢ = 9.6) for cloned human hD₃-receptors, and >100-fold lower affinity for hD₂ and all other receptors (>30) examined. S33084 concentration dependently, potently, and competitively (pKᵢ = 9.7) antagonized dopamine (DA)-induced [³²P]guanosine-5'-O-(3-thio)triphosphate (GTPγS) binding at hD₃-receptors. It also concentration dependently abolished stimulation by DA of hD₃-receptor-coupled mitogen-activated protein kinase. Administered alone, S33084 did not modify dialysate levels of DA in the frontal cortex, nucleus accumbens, or striatum of freely moving rats, nor the firing rate of ventrotegmental dopaminergic cell bodies. Furthermore, it had minimal effect on DA turnover in mesocortical, mesolimbic, and nigrostriatal projection regions. However, S33084 dose dependently blocked the suppressive influence of the preferential D₃-agonist PD128,907 on frontocortical release of DA. Furthermore, it likewise antagonized the inhibitory influence of PD128,907 on the electrical activity of ventrotegmental dopaminergic neurons. Although less potent than S33084, GR218,231 likewise behaved as a selective hD₃- versus hD₂-receptor antagonist and its neurochemical and electrophysiological profiles were similar. In contrast, L741,626 was a preferential antagonist at hD₂ versus hD₃ sites. In vivo, on administration alone, L741,626 increased frontocortical, mesolimbic, and (more potently) striatal DA release, enhanced the firing rate of dopaminergic perikarya, and accelerated cerebral DA synthesis. It also blocked the actions of PD128,907. In conclusion, S33084 is a novel, potent, selective, and competitive antagonist at hD₃-receptors. Although GR218,231 behaves similarly, L741,626 is a preferential D₂-receptor antagonist. DA D₃- but not D₂-(auto) receptors tonically inhibit ascending dopaminergic pathways, although the latter may contribute to phasic suppression of DA release in frontal cortex.

Received for publication October 25, 1999.

ABBREVIATIONS: DA, dopamine; L741,626, 4-(4-chlorophenyl)-1-(1H-indol-3-ylmethyl)piperidin-4-ol; 7-OH-DPAT, 7-hydroxy-2-dipropylaminotetralin; 5-HT, 5-hydroxytryptamine (serotonin); MAP, mitogen-activated protein; GR218,231, 2(R,S)-(di-n-propylamino)-6-(4-methoxyphenylsulfonyl methyl)-1,2,3,4-tetrahydro-3-phenylbenzopyrano[3,4-cpyrrole-2-y]-butyl-(4-phenyl) benzamide; GTPγS, guanosine-5'-O-(3-thio)triphosphate; CHO, Chinese hamster ovary; ERK, extracellular signal receptor-activated kinase; VTA, ventrotentorial area; NA, noradrenaline; 5-HTP, 5-hydroxytryptophan; DOPAC, dihydroxyphenylalanine; FCX, frontal cortex; PD128,907, (+)-4-(4R,10β)-3,4,4a,10b-tetrahydro-4-propyl-2H,5H-[1]benzopyrano[4,3-b]-1,4-oxazin-9-ol; U99194, (6,6-dimethoxy-2-ylidipropylamino)-4-(4-acetyl-N-[4-[2-methoxyphenyl]-piperazin-1-yl]-butyl)-biphenyl-4-carboxamide; S14297, (+)-(7-N-N-dipropylamino)-5,6,7,8-tetrahydro-naphtha[2,3-b]dihydro-2,3-furan; NSD1015, m-hydroxybenzylhydrazine.
Nevertheless, the arylpiperazine derivative L741,626 [4-(4-chlorophenyl)-1-(1-indol-3-ylmethyl)piperidin-4-ol], was developed to identification of selective D2 antagonists. With regard to the last strategy, the agonist bromocriptine shows a modest preference for D2-receptors, activation of which contributes to its antiparkinsonian properties (Newman-Tancredi et al., 1997; Perachon et al., 1999). DA D2-receptor blockade is implicated in extrapyramidal side effects of neuroleptics, such as haloperidol, which displays a modest preference for D3-receptors, activation of which contributes to its antiparkinsonian properties (Newman-Tancredi et al., 1997; Perachon et al., 1999). Interestingly, most of the better-known agonists possess higher affinity for cloned hD3- versus hD2-receptors (Millan et al., 1998; Audinot et al., 1998). DA [125I]iodosulpride (0.1) 7.5 7.2 8.4

<table>
<thead>
<tr>
<th>Receptor Species Tissue</th>
<th>Radioligand</th>
<th>S33084</th>
<th>GR218,231</th>
<th>L741,626</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 Human CHO cell line</td>
<td>[125I]iodosulpride (0.2)</td>
<td>9.6</td>
<td>9.0</td>
<td>7.2</td>
</tr>
<tr>
<td>D2 Human CHO cell line</td>
<td>[125I]iodosulpride (0.1)</td>
<td>7.5</td>
<td>7.2</td>
<td>8.4</td>
</tr>
<tr>
<td>D3 Rat Striatum</td>
<td>[3H]raclopride (2.0)</td>
<td>7.3</td>
<td>7.1</td>
<td>8.2</td>
</tr>
<tr>
<td>D3 Human CHO cell line</td>
<td>[3H]sperometrine (0.4)</td>
<td>5.7</td>
<td>5.0</td>
<td>6.5</td>
</tr>
<tr>
<td>D4 Rat Striatum</td>
<td>[3H]SCH23390 (0.2)</td>
<td>&lt;6.0</td>
<td>&lt;6.0</td>
<td>6.1</td>
</tr>
<tr>
<td>D5 Human L cell line</td>
<td>[3H]SCH23390 (0.3)</td>
<td>6.3</td>
<td>&lt;6.0</td>
<td>6.2</td>
</tr>
<tr>
<td>D5 Human GH4 cell line</td>
<td>[3H]SCH23390 (0.3)</td>
<td>5.9</td>
<td>&lt;6.0</td>
<td>6.2</td>
</tr>
<tr>
<td>5-HT1A Rat Hippocampus</td>
<td>[3H]8-OH-2-DPAT (0.4)</td>
<td>6.9</td>
<td>6.3</td>
<td>5.4</td>
</tr>
<tr>
<td>5-HT1B Human CHO cell line</td>
<td>[3H]8-OH-2-DPAT (0.4)</td>
<td>&lt;6.0</td>
<td>6.8</td>
<td>&lt;6.0</td>
</tr>
<tr>
<td>5-HT2B Guinea Pig Striatum</td>
<td>[3H]HOIC (2.0)</td>
<td>&lt;6.0</td>
<td>&lt;6.0</td>
<td>&lt;6.0</td>
</tr>
<tr>
<td>5-HT2A Human CHO cell line</td>
<td>[3H]GR125,743 (1.0)</td>
<td>6.1</td>
<td>&lt;6.0</td>
<td>&lt;6.0</td>
</tr>
<tr>
<td>5-HT2A Human CHO cell line</td>
<td>[3H]GR125,743 (1.0)</td>
<td>6.9</td>
<td>&lt;6.0</td>
<td>&lt;6.0</td>
</tr>
<tr>
<td>5-HT2A Rat Frontal Cortex</td>
<td>[3H]ketanserin (0.5)</td>
<td>&lt;6.0</td>
<td>&lt;6.0</td>
<td>&lt;6.0</td>
</tr>
<tr>
<td>5-HT2A Human CHO cell line</td>
<td>[3H]ketanserin (0.5)</td>
<td>6.0</td>
<td>&lt;6.0</td>
<td>6.5</td>
</tr>
<tr>
<td>5-HT2C Pig Choroid plexus</td>
<td>[3H]mesulergine (1.0)</td>
<td>6.5</td>
<td>&lt;6.0</td>
<td>6.2</td>
</tr>
<tr>
<td>5-HT2C Human CHO cell line</td>
<td>[3H]mesulergine (1.0)</td>
<td>6.7</td>
<td>&lt;6.0</td>
<td>6.4</td>
</tr>
<tr>
<td>5-HT1 Human NIE-115</td>
<td>[3H]BRL43694 (1.0)</td>
<td>&lt;6.0</td>
<td>&lt;6.0</td>
<td>&lt;6.0</td>
</tr>
<tr>
<td>a1 Rat Frontal cortex</td>
<td>[3H]prazosin (0.2)</td>
<td>6.9</td>
<td>&lt;6.0</td>
<td>6.6</td>
</tr>
<tr>
<td>a2A Rat Cortex</td>
<td>[3H]Rx821002 (0.4)</td>
<td>&lt;6.0</td>
<td>6.4</td>
<td>3.9</td>
</tr>
<tr>
<td>h3 Human CHO cell line</td>
<td>[3H]NMS* (0.15)</td>
<td>6.4</td>
<td>&lt;6.0</td>
<td>&lt;6.0</td>
</tr>
<tr>
<td>a1 Guinea pig Brain</td>
<td>[3H]pentazocine (2.0)</td>
<td>&lt;6.0</td>
<td>6.8</td>
<td>6.8</td>
</tr>
</tbody>
</table>

* NMS, N-methyl-scopolamine.
Moreover, although GR103,691 is a selective (60-fold) D3- versus D2-receptor antagonist, it possesses significant affinity for 5-hydroxytryptamine (serotonin; 5-HT)1A and α1-adrenergic receptors, and shows poor bioavailability (Murray et al., 1995; Audinot et al., 1998). Compared with the above-mentioned characteristics, the aminotetralin antagonist S14297 displays substantial potency and selectivity for hD3- versus hD2-receptors, as well as satisfactory bioavailability (Gobert et al., 1995; Millan et al., 1995; Audinot et al., 1998). However, S14297 showed partial agonist activity in stimulating hD3-receptor-coupled mitogen-activated protein (MAP) kinase (Cussac et al., 1999). Furthermore, the only modest preference of S14297 for D3- versus muscarinic receptors compromises its use as an experimental tool (Millan et al., 1995).

Clearly, there remains a need for improved, selective antagonists at dopamine D₃-receptors. Characterization of structure-activity relationships in a series of benzopyrano[3,4-c]pyrroles identified the cyano-substituted, diphenyl derivative S33084 as a potent D₃-receptor ligand (Dubuffet et al., 1999; Cussac et al., 2000a,b; Fig. 1). A principle objective of this study was, thus, with various cellular paradigms, to characterize the interaction of S33084 at dopamine D₃-compared with D₂-receptors. In this respect, its actions were compared with those of the D₂ antagonist L741,626 (vide supra) and GR218,231 (Murray et al., 1996), a novel hD₃-receptor antagonist (Newman-Tancredi et al., 1999). A complementary aim of this study was to exploit S33084, GR218,231, L741,626, and a combined neurochemical and electrophysiological approach for a characterization of the potential role of D₃- compared with D₂-receptors in the modulation of cerebral dopaminergic transmission.

### Materials and Methods

**Animals.** In vivo studies used, in line with our extensive previous studies of the modulation of dopaminergic transmission (Gobert et al., 1995, 1996; Lejeune and Millan, 1995; Millan et al., 1995), male Wistar rats weighing 250 to 325 g (Ifa-Credo, L'Arbresle, France). They were maintained in sawdust-lined cages with unrestricted access to food and water. The laboratory temperature was held at 21 ± 1°C and humidity was controlled at 60 ± 5%. There was a 12-h light/dark cycle, with lights on from 7:30 AM to 7:30 PM. Before experimentation, all animals were adapted for at least 1 week to laboratory conditions.

**Determination of Drug Affinities.** Procedures used for the determination of drug affinities at multiple native and cloned human dopaminergic, serotonergic, and adrenergic receptors, and at other binding sites, have been described in detail (Millan et al., 1998). They are summarized in Table 1. Isotherms were subjected to nonlinear regression analysis with PRISM (GraphPad, San Diego, CA) to yield IC₅₀ values. These values were subsequently transformed into \( K_i \) values according to the Cheng-Prusoff equation: \( K_i = \frac{IC_{50}}{1 + \frac{L}{K_d}} \), where \( L \) corresponds to the radioligand concentration and \( K_d \) to its dissociation constant (Cheng and Prusoff, 1973).

### Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>( pK_i ) hD₃</th>
<th>( pK_i ) hD₂</th>
<th>Ratio D₂:D₃</th>
<th>( pK_i ) hD₃</th>
<th>( pK_i ) hD₂</th>
<th>Ratio D₂:D₃</th>
<th>( pK_b ) hD₃</th>
<th>( pK_b ) hD₂</th>
<th>Ratio D₂:D₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>S33084</td>
<td>9.59 ± 0.10</td>
<td>7.54 ± 0.20</td>
<td>120</td>
<td>9.40 ± 0.09</td>
<td>7.28 ± 0.17</td>
<td>125</td>
<td>9.61 ± 0.11</td>
<td>7.75 ± 0.05</td>
<td>66</td>
</tr>
<tr>
<td>GR218,231</td>
<td>8.96 ± 0.18</td>
<td>7.18 ± 0.03</td>
<td>60</td>
<td>8.87 ± 0.03</td>
<td>6.86 ± 0.10</td>
<td>100</td>
<td>9.02 ± 0.05</td>
<td>7.27 ± 0.15</td>
<td>65</td>
</tr>
<tr>
<td>L741,626</td>
<td>7.22 ± 0.12</td>
<td>8.39 ± 0.07</td>
<td>0.07</td>
<td>7.06 ± 0.01</td>
<td>8.18 ± 0.06</td>
<td>0.06</td>
<td>7.38 ± 0.08</td>
<td>8.74 ± 0.01</td>
<td>0.04</td>
</tr>
</tbody>
</table>

**Antagonist Properties at hD₃- and hD₂-Receptors:**

---

**Fig. 2.** Interaction of S33084, GR218,231, and L741,626 at hD₃-compared with hD₂-receptors expressed in CHO cells. Isotherms were obtained in competition experiments with \( ^{[35S]} \)Guanosine-5′-O-(3-thio)triphosphate (GTP₇S) Binding.
The protocol used to quantify the binding of \( ^{35}S \)GTP·S (1000 Ci/mmol; NEN, Les Ulis, France) at hD2- and hD3-receptors has been described in detail in Newman-Tancredi et al. (1999). The buffer composition was as follows: HEPES (20 mM), NaCl (100 mM), GDP (3 \( \mu \)M), and MgSO\(_4\) (3 mM). Incubations were performed at 22°C and pH 7.4 for 60 min. Drug actions were evaluated both alone and in the presence of a fixed concentration of DA (3 and 1 \( \mu \)M, respectively). Agonist efficacy (alone) was expressed relative to that of a maximally effective concentration of DA (10 \( \mu \)M, defined as 100%). For antagonist studies, concentration-response curves of the blocking properties of drugs versus DA were analyzed in the presence of incremental concentrations of S33084, and \( p_A^2 \) values were derived by Schild analysis.

Activation of MAP kinase at hD3-receptors. As described in Cussac et al. (1999), Chinese hamster ovary (CHO) cells transfected with hD2-receptors were cultivated in six-well plates until 90% confluent, washed, and incubated overnight in serum-free medium. Drugs were diluted in this medium and added to cells to yield the desired, final concentration. After a 5-min preincubation with the test drug, cells were exposed to DA (1 \( \mu \)M) for 5 min. Subsequently, 0.25 ml/well of Laemmli sample buffer (containing 200 mM dithiothreitol) was added. Whole-cell lysates were boiled at 95°C for 3 min. Thereafter, 14 \( \mu l \) of the cell extract was loaded onto 15-well, 10% polyacrylamide gels and “fully” activated MAP kinase was detected by use of a monoclonal antibody specifically directed against phosphorylated [extracellular signal receptor-activated kinase 2 (ERK2) pp42MAPK and pp44MAPK (ERK1)] forms on both threonine and tyrosine residues (NanoTools, Denzlingen, Germany). This was followed by enhanced chemiluminescence detection with horseradish peroxidase as a secondary antibody (Amersham, Les Ulis, France).

Modulation of Electrical Activity of Dopaminergic Neurons in Anesthetized Rats. The procedure used for evaluation of drug actions on the electrical activity of dopaminergic perikarya localized in the ventrotegmental area (VTA) has been described in detail in Lejeune and Millan (1995). Rats were anesthetized with chloral hydrate (400 mg/kg i.p.) and, after placement in a stereotaxic apparatus, a tungsten microelectrode was lowered into the VTA: coordinates AP, 5.5 from bregma; L, 0.7; and H, -7.8/5.5 from dura. Dopaminergic neurons were identified according to their waveform (Lejeune and Millan, 1995) and baseline activity was monitored for 5 min. The influence of S33084, GR218,231, and L741,626 alone on firing rate was evaluated on their administration in cumulative doses at intervals of 3 to 5 min and drug effects were evaluated for 60 s at their time of peak action. For examination of their antagonist actions, a single dose of the antagonist was administered 1 min after PD128,907 (0.005 mg/kg i.v.) and drug effects were evaluated 2 to 3 min after antagonist injection. Spike 2 software (CED, Cambridge, England) was used to accomplish data acquisition. The data are expressed as percentage of change from preinjection, basal values (defined as 0%). They were analyzed by ANOVA followed by Newman-Keuls test.

Modulation of Cerebral Synthesis of DA, Noradrenaline (NA), and 5-HT. The modulation of cerebral synthesis of DA, NA, and 5-HT was evaluated as described in Millan et al. (1998). DA and 5-HT synthesis was determined in the striatum (rich in DA but not NA) and NA and 5-HT synthesis was evaluated in the hippocampus (rich in NA but not DA). Drug actions were measured 60 min after administration and 30 min after injection of the decarboxylase inhibitor NSD1015 (100 mg/kg s.c.). HPLC analysis followed by electrochemical detection was used for determination of tissue levels of L-dopa and 5-hydroxytryptophan (5-HTP) as described in Millan et al. (1998). Levels of L-dopa and 5-HTP were expressed relative to those of vehicle values (defined as 0%). Data were analyzed by ANOVA followed by Dunnett’s test.

Modulation of Cerebral DA Turnover. As described in Millan et al. (1998), the ratio of levels of the DA metabolite dihydroxyphenylalaninecarboxylic acid (DOPAC) to those of DA was character-
Binding Profile of S33084 at Dopaminergic Receptors (Tables 1 and 2; Fig. 2). At cloned hD3-receptors, S33084 was used for determination of levels of DopAC and DA as described in Millan et al. (1998). DopAC/DA ratios were expressed relative to vehicle values (defined as 0%). Data were analyzed by ANOVA followed by Dunnett's test.

Results

Binding Profile of S33084, GR218,231, and L741,626 at Dopaminergic Receptors (Tables 1 and 2; Fig. 2). In analogy to S33084, GR218,231 exhibited a pronounced preference for hD3 versus hD2-receptors labeled by either [125I]iodosulpride (60-fold selectivity) or [3H]spiperone (100-fold) (Table 2; Fig. 2). Its absolute affinity at hD3 sites was some 5-fold lower than that of S33084. The affinity of GR218,231 for native, rat striatal D3-receptors was 80-fold inferior to its affinity at hD3-receptors (Table 1). Furthermore, GR218,231 displayed weak (1000-fold lower) affinity for hD2-, hD1-, and hD4-versus hD3-receptors (Table 1). L741,626 also displayed an opposite pattern of affinity at hD3- and hD2-receptors compared with S33084 and GR218,231. Its affinity at hD3 sites labeled by [125I]iodosulpride and [3H]spiperone was, thus, modest (Table 2; Fig. 2). However, it showed more pronounced (~15-fold in each case) affinity for hD2- versus hD3-receptors labeled by [125I]iodosulpride and [3H]spiperone (Table 2). L741,626 also manifested marked affinity for native, rat striatal D3 sites labeled by [3H]raclopride (Table 1). The affinity of L741,626 for hD2-receptors was modest, ~70-fold lower than for hD3-receptors. Furthermore, L741,626 showed modest affinity for hD1- and hD5-receptors versus hD3-receptors (Table 1).

Binding Profile of S33084, GR218,231, and L741,626 to Nondopaminergic Receptors (Table 1). At diverse, native and cloned 5-HT receptor subtypes indicated in Table 1, the affinity of S33084 was at least 500-fold lower than at hD3-receptors. It also displayed >1000-fold lower affinity at 5-HT reuptake sites compared with hD3-receptors (data not shown). GR218,231 also showed marked (>100-fold) selectivity for hD3 versus various 5-HT receptor types (Table 1). Furthermore, the affinity of L741,626 for the various 5-HT receptor types indicated in Table 1 was >200-fold lower than for hD3-receptors. S33084 and GR218,231 also displayed considerably (>500-fold) lower affinity for a1- and a2A-adrenergic receptors (Table 1) and NA reuptake sites (data not shown) compared with hD3 sites. Similarly, the affinity of L741,626 for a2A-adrenergic receptors (Table 1) and NA uptake sites (data not shown) was low, whereas it displayed 60-fold lower affinity for a1-adrenergic receptors compared with hD3-receptors (Table 1). Compared with hD3-receptors, S33084 showed >1000-fold lower affinity for cloned human, muscarinic (M1) receptors and a1-receptors (Table 1), as well as histamine (H1) γ-amino butyric acid, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and N-methyl-D-aspartate receptors and several other sites indicated in the legend to Table 1.

Antagonist Properties of hD3- versus hD2-Receptors: [35S]GTPγS Binding (Fig. 3). At cloned hD3- and hD2-receptors transfected into CHO cells, DA concentration de-
Fig. 5. Influence of S33084, GR218,231, and L741,626 on extracellular levels of DA compared with 5-HT and NA in the FCX of freely moving rats. Dialysate levels are expressed as a percentage of basal, preinjection values that were defined as 0%. These values were 1.2 ± 0.1, 0.8 ± 0.1, and 1.4 ± 0.2 pg/20 μl of dialysate for DA, 5-HT, and NA, respectively. Data are mean ± S.E. (n ≥ 5 per value). ANOVA with dose as between-factor and with time as within-factor was performed over 20 to 180 min. DA: influence of S33084, $F_{3,23} = 1.0$, $P > .05$; influence of GR218,231, $F_{1,13} = 0.1$, $P > .05$; and influence of L741,626, $F_{3,22} = 20.8$, $P < .01$. 5-HT: influence of S33084, $F_{3,23} = 0.4$, $P > .05$; influence of GR218,231, $F_{1,13} = 0.3$, $P > .05$; and influence of L741,626, $F_{3,22} = 0.2$, $P > .05$. NA: influence of S33084, $F_{3,22} = 0.8$, $P > .05$; influence of GR218,231, $F_{1,12} = 0.1$, $P > .05$; and influence of L741,626, $F_{3,21} = 1.1$, $P > .05$. Asterisks indicate significance (*$P < .05$) of differences to respective vehicle values in Dunnett’s test.
pendently and markedly (1.5- and 2.2-fold, respectively) stimulated \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding with pEC\text{\textsubscript{50}} values of 8.16 ± 0.10 and 6.45 ± 0.05, respectively (data not shown; Newman-Tancredi et al., 1999). In contrast, S33084, GR218,231, and L741,626 all failed to modify \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding when applied alone. S33084 potently and concentration dependently suppressed stimulation of \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding at hD\text{3}-receptors with a pK\text{b} of 9.61 ± 0.11. It was, however, considerably less potent in blocking the action of DA at hD\text{2} sites displaying a pK\text{b} of 7.75 ± 0.05. In confirmation of our previous study (Newman-Tancredi et al., 1999), a similar pattern of data was acquired for GR218,231, which displayed pK\text{b} values of 9.02 ± 0.05 and 7.27 ± 0.15 at hD\text{2} and hD\text{3}-sites, respectively. In contrast, L741,626 more potently blocked the action of DA at hD\text{2} than at hD\text{3}-receptors with pK\text{b} values of 8.74 ± 0.01 and 7.38 ± 0.08, respectively. In the presence of incremental concentrations of S33084, the concentration-response curve for stimulation by DA of hD\text{3}-receptors was displaced in parallel to the right without any loss of maximal stimulation, indicative of competitive antagonist activity. Furthermore, these data generated a linear Schild plot with a slope of 1.04 ± 0.09 (r = 0.96), yielding a pA\text{2} value of 9.69. This value is very similar to the pK\text{i} of S33084 at hD\text{3}-receptors (9.6; Table 1).

**Antagonist Properties at hD\text{3}-Receptors: MAP Kinase Activation (Fig. 4).** In CHO cells transfected with hD\text{3}-receptors, DA activated (phosphorylated) both ERK1 and ERK2 species of MAP kinase (Cussac et al., 1999). In contrast to DA, S33084 failed to induce either ERK2 (Fig. 4) or ERK1 (data not shown) forms of MAP kinase. Furthermore, after pretreatment of cells for 5 min with S33084, the induction of both forms of MAP kinase by DA (1 \text{\mu}M) was concentration dependently abolished (Fig. 4; data not shown). This action was expressed specifically inasmuch as the induction of MAP kinase by fibroblast growth factor (20 ng/ml) was not modified by S33084 (10 \text{\mu}M; data not shown). At a single concentration, GR218,231 (1 \text{\mu}M) likewise abolished the action of DA without itself inducing MAP kinase.
a complementary, redundant role in the tonic control of DA release. In this case, release of DA via D₃-receptor blockade might immediately lead to engagement of colocalized D₃-autoreceptors, thereby masking its actions. Therefore, the influence of their concomitant blockade was examined by coadministration of S33084 and L741,626. However, as shown in Fig. 7, even in the presence of L741,626 to block D₂-receptors, S33084 failed to elevate FCX levels of DA. Similarly, the facilitatory influence of L741,626 on DA release was not significantly enhanced after pretreatment with S33084. These data do not, thus, reveal any complementary role of D₃ with D₂-autoreceptors in the tonic control of frontocortical DA release.

**Influence on Suppression by PD128,907 of Dialysate Levels of DA in FCX (Fig. 8).** PD128,907 markedly reduced dialysate levels of DA in FCX (Fig. 8) without significantly modifying those of 5-HT or NA (data not shown). This action of PD128,907 was dose dependently attenuated by both S33084 and GR218,231. Similarly, L741,626 dose dependently blocked the action of PD128,907. The ID₅₀ values (95% CL) for blocking the action of PD128,907 were 0.97 (0.43–2.2), 0.95 (0.44–2.07), and 0.53 (0.29–0.95) mg/kg s.c. for S33084, GR218,231, and L741,626, respectively.

**Influence on Inhibition of Dopaminergic Neuron Firing by PD128,907 (Figs. 9 and 10).** The electrical activity of VTA-localized dopaminergic neurons was markedly inhibited by PD128,907 at a dose of 0.005 mg/kg i.v. This action of PD128,907 was dose dependently and significantly reduced by S33084 (Fig. 9), but S33084 did not itself modify firing rate (data not shown). Likewise, administered alone, GR218,231 did not modify the activity of dopaminergic neurons (data not shown) but, in its presence, the inhibitory influence of GR218,231 was dose dependently abrogated (Fig. 9). L741,626 elicited a dose-dependent and marked acceleration in the firing rate of dopaminergic neurons on administration alone (Fig. 10). The action of PD128,907 was dose dependently reversed by L741,626. The ID₅₀ values (95% CL) for blocking the action of PD128,907 were 0.57 (0.19–1.71), 0.36 (0.13–0.81), and 0.27 (0.09–0.80) mg/kg i.v. for S33084, GR218,231, and L741,626, respectively.

**Influence on Cerebral Turnover and Synthesis of DA, 5-HT, and NA in Dialysates of Freely Moving Rats (Figs. 5 and 6).** Over a broad range of doses, S33084 failed to modify basal levels of DA, NA, or 5-HT simultaneously determined in single dialysate samples of the FCX of freely moving rats (Fig. 5). At a dose of 2.5 mg/kg s.c., S33084 did not modify basal levels of DA in the nucleus accumbens or striatum (Fig. 6). Levels of 5-HT were likewise unaffected (data not shown). Similarly, GR218,231 did not influence levels of these monoamines in any structure examined (Figs. 5 and 6). In contrast to S33084 and GR218,231, L741,626 dose dependently elevated levels of DA in the FCX (Fig. 5). It also dose dependently (0.16–10.0 mg/kg s.c.) elevated levels of DA in both the accumbens and, more potently, the striatum (Fig. 6). These actions were specific inasmuch as levels of 5-HT and/or NA were not significantly altered in the same dialysis samples (Fig. 5; data not shown).

**Effect of Coadministration of S33084 and L741,626 (Fig. 7).** It is possible that D₂ and D₃-receptors might fulfill

---

**Fig. 7.** Influence of sequential administration of S33084 and L741,626 on DA release in FCX. Dialysate levels are expressed as a percentage of basal, preinjection values that were defined as 0%. Data are mean ± S.E. (n = 5 per value). ANOVA with dose as between-factor and with time as within-factor was performed over 40 to 200 min. A, L741,626 followed by S33084 versus L741,626 and vehicle, F₃,₈₄ = 1.1, P > .05. B, S33084 followed by L741,626 versus vehicle and L741,626, F₅,₆₈ = 0.1, P > .05. (data not shown). In view of its low affinity for hD₃-receptors, L741,626 was not evaluated in this protocol.

**Influence on Basal Levels of DA, 5-HT, and NA in Dialysates of Freely Moving Rats (Figs. 5 and 6).** Over a broad range of doses, S33084 failed to modify basal levels of DA, NA, or 5-HT simultaneously determined in single dialysate samples of the FCX of freely moving rats (Fig. 5). At a dose of 2.5 mg/kg s.c., S33084 did not modify basal levels of DA in the nucleus accumbens or striatum (Fig. 6). Levels of 5-HT were likewise unaffected (data not shown). Similarly, GR218,231 did not influence levels of these monoamines in any structure examined (Figs. 5 and 6). In contrast to S33084 and GR218,231, L741,626 dose dependently elevated levels of DA in the FCX (Fig. 5). It also dose dependently (0.16–10.0 mg/kg s.c.) elevated levels of DA in both the accumbens and, more potently, the striatum (Fig. 6). These actions were specific inasmuch as levels of 5-HT and/or NA were not significantly altered in the same dialysis samples (Fig. 5; data not shown).

**Effect of Coadministration of S33084 and L741,626 (Fig. 7).** It is possible that D₂ and D₃-receptors might fulfill

---

**Receptor Profile.** The pronounced affinity and selectivity of S33084 at hD₃ versus D₂-receptors is underpinned by
studies of [3H]S33084 that binds with high affinity ($K_d = 9.7$) to hD$_3$-receptors (Cussac et al., 2000a). S33084 likewise displays high affinity and (>100-fold) selectivity for cloned and native, rat D$_3$- versus D$_2$-receptors (Cussac et al., 2000b). These observations, and the marked (>100-fold) selectivity of S33084 versus all (>30) receptors examined, underline its utility for exploration of the functional role of D$_3$-receptors. Indeed, S33084 is substantially more selective than other D$_3$ antagonists characterized in vivo (Wustrow and Wise, 1997; Audinot et al., 1998). Furthermore, although several selective antagonists at hD$_3$- versus hD$_2$-receptors were recently documented, their functional actions in vivo remain to be examined (Boyfield et al., 1997; Yuan et al., 1998; Austin et al., 1999). GR218,231 was originally characterized with two different cell lines (Murray et al., 1996) and, with a common CHO expression system, the present investigation confirms its marked selectivity for hD$_3$- versus hD$_2$-receptors. Indeed, S33084 is substantially more selective than other D$_3$ antagonists characterized in vivo (Wustrow and Wise, 1997; Audinot et al., 1998). Furthermore, although several selective antagonists at hD$_3$- versus hD$_2$-receptors were recently documented, their functional actions in vivo remain to be examined (Boyfield et al., 1997; Yuan et al., 1998; Austin et al., 1999). GR218,231 was originally characterized with two different cell lines (Murray et al., 1996) and, with a common CHO expression system, the present investigation confirms its marked selectivity for hD$_3$- versus hD$_2$-receptors. Similarly, we extend previous studies demonstrating the preference of L741,626 for hD$_2$- versus hD$_3$-receptors (Kulagowski et al., 1996).

**Antagonist Properties at hD$_3$- versus hD$_2$-Receptors.** Activation of hD$_3$-receptors enhances [$^35$S]GTP$_\gamma$S binding to G-proteins (Missale et al., 1998; Newman-Tancredi et al., 1999; Vanhauwe et al., 1999), and this response was potently suppressed by S33084, demonstrating antagonist properties at hD$_3$ sites. GR218,231 behaved similarly. Confirming their selectivity, only markedly higher concentrations blocked [$^35$S]GTP$_\gamma$S binding at hD$_2$-receptors. Furthermore, S33084 displaced the DA stimulation-response curve for induction of [$^35$S]GTP$_\gamma$S binding at hD$_3$-receptors without compromising its maximal effect, yielding a $pA_2$ (9.7) close to its $pK_i$ (9.6), and demonstrating competitive interaction with hD$_3$ sites. In contrast to S33084, L741,626 more potently suppressed hD$_2$- versus hD$_3$-receptor-mediated [$^35$S]GTP$_\gamma$S binding, revealing its opposite preference for hD$_2$ sites. Downstream of G-protein coupling, hD$_3$-receptors activate MAP kinase (Cussac et al., 1999) and S33084 and GR218,231 both abolished DA-stimulated MAP kinase. Inasmuch as this parameter is highly sensitive, this finding underpins “pure” antagonist properties of S33084 and GR218,231 at hD$_3$-receptors, an interpretation supported by in vivo studies (Results; Millan et al., 2000).

**In Vivo Actions at D$_3$- versus D$_2$-Receptors.** Although the above-mentioned observations demonstrate the striking selectivity of S33084 (and GR218,231) for hD$_3$- versus hD$_2$-receptors in vitro, and a marked preference of L741,626 for hD$_2$- versus hD$_3$-receptors, the question arises concerning...
their actions in vivo. Drug selectivity may best be established in vivo by determination of active dose ranges in well-defined models reflecting activity at specific receptors. However, although functional models of activity at D2 sites are available (see below; Millan et al., 2000), no functional response in vivo has, as yet, been unambiguously attributed to D3-receptors. This difficulty, common to all studies of D3-receptor function, encourages caution in the interpretation of actions of even highly selective ligands, such as S33084. Nevertheless, it is reasonable to make the following inferences. First, in accordance with their 100-fold higher affinities versus L741,626 at D3-receptors, S33084 and GR218,231 should be 100-fold more potent than L741,626 in models exclusively mediated by D3-receptors. Second, as discussed in Bristow et al. (1997) and Millan et al. (1998) for selective D3-receptor antagonists, one may exploit residual (“surrogate”) actions of S33084 and GR218,231 at D2-receptors for an estimation of doses at which they should selectively block D2-receptors. Thus, based on weak actions of S33084 and GR218,231 in (certain) models involving D2-receptors (Millan et al., 2000), any effects in models reflecting only D3-receptor activation should be seen at ~100-fold lower doses or ~0.1 mg/kg s.c. These estimations provide a framework for cautious interpretation of the actions of S33084.

**Fig. 9.** Influence of S33084 and GR218,231 on the inhibition by PD128,907 of electrical activity of dopaminergic neurons in the VTA. Data are mean ± S.E. (n = 5 per value). Top and left, blockade by S33084 of the actions of PD128,907 (0.005 mg/kg i.v.). ANOVA, \( F_{3,24} = 7.0, P < .01 \). Top and right, representative recording of the influence of S33084 (0.25 mg/kg i.v.) on the inhibitory action of PD128,907 (0.005 mg/kg i.v.). Bottom and left, blockade by GR218,231 of the actions of PD128,907 (0.005 mg/kg i.v.). ANOVA, \( F_{4,26} = 10.7, P < .01 \). Bottom and right, representative recording of the influence of GR218,231 (0.25 mg/kg i.v.) on the inhibitory action of PD128,907 (0.005 mg/kg i.v.). Asterisks indicate significance of difference to respective vehicle values. *P < .05.

**Autoreceptor Modulation of Dopaminergic Transmission.** Dopaminergic neurons are tonically inhibited by dendritic and terminal autoreceptors, operating in interaction with DA transporters (Gobert et al., 1995; Koeltzow et al., 1998; L’hirondel et al., 1998; Dickinson et al., 1999). Dopaminergic neurons display a high density of D2-receptors, whereas D3-(auto)receptors are present in only low concentrations (Levant, 1997; Tepper et al., 1997; Suzuki et al., 1998). Nevertheless, transduction mechanisms potentially permitting an inhibitory role of D3 sites have been identified (Werner et al., 1996; Kuzhikandathil and Oxford, 1999; Liu et al., 1999).

**Tonic Control of Cerebral DA Synthesis.** The observation that S33084 and GR218,231 do not modify cerebral DA synthesis supports studies of D3-receptor-deficient mice (Koeltzow et al., 1998; Jung et al., 1999) in suggesting that D3-receptors do not play a major role in the modulation of DA turnover. Nevertheless, antisense probes neutralizing D3-receptors increased nucleus accumbens DA synthesis (Nissbrandt et al., 1995) and, in knockout mice lacking D2- and D3-receptors, Jung et al. (1999) observed a more pronounced increase in striatal DA turnover than in D2-receptor-deficient counterparts. These observations suggest that D3-autoreceptors might play a minor role, complementary to that of D2-
autoreceptors, in modulation of DA synthesis (Gobert et al., 1995). Correlation analyses with (nonselective) dopaminergic antagonists suggested a role of D₂ sites in the tonic control of cerebral DA synthesis (Gobert et al., 1995; Gainetdinov et al., 1996), and a major, tonic, inhibitory influence of D₂-autoreceptors was revealed herein with L741,626. This finding is important because D₂-receptor-deficient mice display no consistent alterations in levels of DA or tyrosine hydroxylase, presumably due to compensatory mechanisms, including enhanced translation of D₃-receptor mRNA and alterations in DA clearance via the DA transporter (Kelly et al., 1998; Dickinson et al., 1999; Jung et al., 1999).

Tonic Control of Mesolimbic and Striatal DA Release. Neither S33084 nor GR218,231 elevated resting extracellular levels of DA in nucleus accumbens or striatum, suggesting that D₃-autoreceptors do not play a prominent role in tonic control of DA release herein. Furthermore, although transgenic mice lacking D₃-receptors, and rats treated with D₃-receptor antisense, showed enhanced spontaneous DA release in the accumbens (Ekman et al., 1998), Koeltzow et al. (1998) proposed that this effect reflects a short-loop, feedback control of mesolimbic DA release via postsynaptic D₃ sites. In contrast to S33084 and GR218,231, L741,626 markedly enhanced dialysis levels of DA, demonstrating that D₂-autoreceptors tonically regulate limbic and striatal DA release. Previous studies of the interaction of L741,626 with dopaminergic agonists (Bowery et al., 1996) likewise suggested a role of D₂-autoreceptors in phasic control of striatal DA release. Interestingly, no alterations in basal, striatal DA release were observed in D₂-receptor-deficient mice, presumably due to compensatory mechanisms (L’hirondel et al., 1998; Dickinson et al., 1999). Thus, the present facilitatory influence of L741,626 on DA release and synthesis underlines the continuing importance of pharmacological tools for elucidation of potential roles of D₂- (and D₃-) receptors.

Tonic and Phasic Control of Mesocortical DA Release. The lack of influence of S33084 and GR218,231 versus L741,6216 on resting frontocortical levels of DA suggest that D₂-receptors do not, in contrast to their D₂ counterparts, tonically regulate DA release in FCX. It is unlikely that this inactivity of S33084 and GR218,231 reflects release of DA onto colocalized D₂-autoreceptors because, in the presence of L741,626, S33084 still failed to increase frontocortical DA levels. However, in analogy to S14297 (Gobert et al., 1995), S33084 and GR218,231 dose dependently blocked the inhibitory influence of PD128,907 on DA levels. There are several possible interpretations. First, there may be a selective implication of D₃-receptors in the phasic control of frontocortical DA release. This is, however, unlikely because active doses of S33084 and GR218,231 were superior to those estimated as D₃-receptor selective (vide supra). Furthermore, L741,626 was active at doses similar to those of S33084 and GR218,231. Nevertheless, D₃-autoreceptor isoforms (splice variants or post-translationally modified) may differ from...
postsynaptic populations (Jung et al., 1999), and affinities of S33084 and GR218,231 versus L741,626 for (terminal) D3-autoreceptors controlling DA release in rat FCX may differ from hD3-receptors characterized in CHO cells. Second, actions of S33084 and GR218,231 might reflect blockade of D2-receptors. However, this can be largely ruled out because 1) doses of S33084 and GR218,231 inhibiting PD128,907 were not significantly greater than those of L741,626; 2) in contrast to L741,626, basal DA release in FCX was not affected by S33084 or GR218,231; and 3) over this dose range, S33084 and GR218,231 do not block other D2-receptor-mediated responses (Millan et al., 2000). A third possibility is that actions of S33084 and GR218,231 reflect physical and/or functional interactions between D2- and D3-autoreceptors either at the recognition site or intracellular level (Missale et al., 1998). And last, regarding a possible role of both D2- and D3-autoreceptors, previous studies provided evidence for two distinct classes of dopaminergic autoreceptor modulating cerebral DA release (Pizzi et al., 1993; Patel et al., 1994). Thus, the present data with L741,626 demonstrate a role of D2-autoreceptors in the phasic control of DA release in FCX, but further study is required to characterize the effect of S33084 and the potential implication of D3-receptors.

**Electrical Activity of VTA Dopaminergic Perikarya.**

The lack of modification by S33084 and GR218,231 of the electrical activity of VTA dopaminergic perikarya concurs with a lack of change in D3-receptor-deficient mice and rats treated with D3 antisense probes (Tepper et al., 1997; Koeltzow et al., 1998) in indicating that D3-receptors do not tonically control the electrical activity of dopaminergic perikarya. In contrast, firing rate was accelerated by L741,626, revealing the tonic inhibitory role of D2-autoreceptors. For phasic actions, in analogy to S14297 (Lejeune and Millan, 1995), S33084 and GR218,231 attenuated the inhibitory influence of PD128,907 on VTA neurons, raising the possibility of a phasic role of D3 sites. The high doses of S33084 and GR218,231 required suggest, however, caution in the interpretation of these actions (see above). Nevertheless, antisense probes to D2- and D3-receptors additively attenuated the inhibitory influence of apomorphine on the electrical activity of VTA dopaminergic perikarya.
activity of substantia nigra dopaminergic neurons (Tepper et al., 1997), suggesting a cojoint role of D3 and D2 sites. For the phasic role of D2-autoreceptors, L741,626 blocked the inhibitory influence of PD128,907 on VTA-localized dopaminergic neurons herein and it prevented the inhibitory influence of PD128,907 at VTA- and substantia nigra-localized dopaminergic neurons in vitro (Bowery et al., 1996). Furthermore, the inhibitory influence of DA on dopaminergic perikarya is absent in D2-receptor-deficient mice (Mercuri et al., 1997), whereas PD128,907 is equipotent in inhibiting VTA- and substantia nigra-localized dopaminergic neurons in wild-type versus D2-receptor knockout mice (Koeltzow et al., 1998).

**Potential Inverse Agonist Actions.** One possible explanation for a lack of intrinsic influence of S33084 and GR218,231 on dopaminergic transmission might be that they behave as “neutral antagonists” rather than “inverse agonists” at D3-(auto)receptors. However, evidence for constitutive activity at hD3 sites is contradictory (Griffon et al., 1997; Malmberg et al., 1998; Cussac et al., 1999; Newman-Tancredi et al., 1999; Vanhauwe et al., 1999) so this seems unlikely. For the “tonic” role of D2-receptors, if “inverse agonist” properties (Nilsson et al., 1996; Hall and Strange, 1997) were required to enhance dopaminergic transmission, this could explain 1) the lack of influence of D2 antisense probes and null mutations for D2-receptors on dopaminergic pathways (vide supra), and 2) increases in dopaminergic transmission of contrasting magnitude (proportional to “negative efficacy”) by various “antagonists” (Gobert et al., 1995). However, inverse agonist properties of L741,626 have not, to date, been demonstrated and unambiguous proof of the significance of inverse agonist actions at D2 sites in vivo is needed (Millan et al., 1999). The present interpretation that L741,626 enhances dopaminergic transmission by interrupting tonic activity at D2-autoreceptors seems, then, more parsimonious.

**Serotonergic and Adrenergic Transmission.** Although fragmentary data suggest that dopaminergic mechanisms modulate adrenergic and serotonergic transmission (Rossetti et al., 1989; Suzuki et al., 1998; Adell and Artigas, 1999), S33084, GR218,231, L741,626, and PD128,907 failed to modify release and turnover of 5-HT and NA herein. Alterations in 5-HT and NA turnover have likewise not been reported in D3- or D2-receptor-deficient mice (Koeltzow et al., 1998; Dickinson et al., 1999). D2- and D3-receptors do not, thus, exert a pronounced influence on serotonergic or adrenergic pathways.
Conclusions

In conclusion, S33084 is a potent, selective, and competitive antagonist at D₃-receptors which, together with GR218,231 and L741,626, should be of considerable use for exploration of their pathophysiological significance. Based on actions of these ligands, D₂- but not D₃-autoreceptors fulfill a major role in the tonic inhibition of dopaminergic transmission. Nevertheless, the influence of S33084 on dopaminergic pathways, and the possibility that D₂(auto)receptors contribute to the phasic modulation of DA release, justify further examination. In this regard, although this study focused on the FCX, it would be of interest to examine a potential role of D₃-receptors in structures such as the Isles of Calleja or subregions of the nucleus accumbens that are enriched in D₃-receptors (Levant, 1997).

Acknowledgments

We thank C. Langaney for secretarial assistance and C. Chaput, L. Cistarelli, C. Melon, V. Paseau, and M. Touzard for technical assistance.

References


Send reprint requests to: Dr. Mark J. Millan, Institut de Recherches Servier, Centre de Recherches de Croissy, Psychopharmacology Department, 125 Chemin de Ronde, 78290 - Croissy-sur-Seine, France.