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(−)*S* amisulpride binds with high affinity to cloned dopamine D_3 and D_2 receptors

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Abstract

Amisulpride is a substituted benzamide antipsychotic with nanomolar affinity and high selectivity for dopamine D_2 and dopamine D_3 receptors. The interaction of racemic (+/−)*RS* amisulpride and its two enantiomers (+)*R* and (−)*S* with dopamine D_2 and dopamine D_3 receptors subtypes were compared with that of haloperidol. Binding studies were performed using either [^{3}H]spiperone or [^{3}H]nemonapride in baculovirus/*Spodoptera frugiperda* insect (Sf-9) cell system expressing either the human dopamine recombinant D_2long (hD_{2L}) or the rat dopamine recombinant D_3 (rD_3) receptors. K_i values at dopamine rD_3 receptors were similar regardless of the radioligand used, whereas at hD_{2L} receptors values were higher using [^{3}H]spiperone than [^{3}H]nemonapride. However, the rank order of compound potency against radiolabeled spiperone or nemonapride both at dopamine hD_{2L} and at dopamine rD_3 receptors was similar. (−)*S* amisulpride displaced [^{3}H]spiperone or [^{3}H]nemonapride binding from both dopamine hD_{2L} or dopamine rD_3 receptors, being twofold more potent than the racemic form and 38–19-fold more potent than (+)*R* enantiomer. Both racemic and the (−)*S* enantiomer exhibited 2–4 ([^{3}H]spiperone)- and 3–4 ([^{3}H]nemonapride)-fold higher affinity than haloperidol for dopamine rD_3 receptor, respectively. The (+)*R* enantiomer has weaker affinity with respect to haloperidol for both dopamine hD_{2L} and dopamine rD_3 receptors. Our results show that (−)*S* amisulpride is the active enantiomer of amisulpride, showing high affinity for dopamine D_3 and dopamine D_2 receptors.

Keywords: Amisulpride; Dopamine receptor; Benzamide

1. Introduction

Amisulpride, an atypical antipsychotic belonging to the class of substituted benzamides, has been shown to be effective in treating positive as well as negative symptoms in schizophrenia, to have less propensity than conventional neuroleptics to induce extrapyramidal side effects, and at low doses (i.e. 50–100 mg/die), to possess antidepressant properties (Coukell et al., 1996). The singular profile of other atypical antipsychotics such as clozapine, olanzapine and risperidone has been explained through their interaction with non-dopaminergic receptors, such as histamine H_1, muscarinic, 5-HT_2 receptor and α-adrenoceptor, which are thought to either enhance the antipsychotic action or to reduce extrapyramidal side effects (Leysen et al., 1998). Since amisulpride selectively blocks dopamine D_2 and dopamine D_3 receptors with no affinity for any other known receptor, it has been proposed that its atypical profile may depend on a preferential blockade of mesolimbic rather than nigrostriatal dopaminergic transmission. Alternatively, it may be due to a preferential blockade of dopamine D_2 autoreceptors rather than postsynaptic dopamine D_2 receptors and, finally, on a preferential blockade of dopamine D_3 receptors in the limbic areas (Schoemaker et al., 1997). The role of dopamine D_3 receptors as a potential target in the therapeutic action of antipsychotics is of particular interest due to their preferential localization on dopaminoceptive cells in the limbic areas, such as the nucleus accumbens (Sokoloff et al., 1990). In view of the high affinity of amisulpride for these receptors, the latter have been indicated as a feasible candidate to suit its atypical profile. Amisulpride, used in both preclinical and clinical studies and in clinical practice, is a racemic mixture of two enantiomers, (−)*S* and (+)*R* amisulpride, recently separated in the laboratories of Sanofi ~ Synthélabo (Bagneux, France). With the aim of better defining the atypical profile of amisulpride, we studied the interaction of the racemic

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compound and its two enantiomers with dopamine D_2 and dopamine D_3 receptors subtypes in comparison with the traditional neuroleptic haloperidol. The study was carried out using a baculovirus/*Spodoptera frugiperda* (Sf-9) insect cell system, expressing only one or another subtype of the receptor: human dopamine recombinant D_2 long (hD_{2L}) or rat dopamine recombinant dopamine D_3 receptor(rD_3) receptors. The radioactive antagonists [^{3}H]spiperone and [^{3}H]nemonapride were used to assess the binding characteristics of dopamine hD_{2L} and dopamine rD_3 receptors expressed in Sf-9 cells.

2. Materials and methods

Racemic amisulpride (+/−)*RS*, (−)*S* and (+)*R* amisulpride enantiomers were supplied by Sanofi ~ Synthélabo (Bagneux, France). Haloperidol, (+)-butaclamol hydrochloride were purchased from Tocris (London, UK). [^{3}H]Spiperone (96 Ci/mmol) and [^{3}H]nemonapride (85 Ci/mmol) were obtained from Amersham Life Science, Buckinghamshire, UK, and from New England Nuclear (NEN), Boston, MA, respectively. Sf-9 membranes expressing either dopamine hD_{2L} or dopamine rD_3 receptors were purchased from New England Nuclear. [^{3}H]Spiperone binding for both hD_{2L} and dopamine rD_3 receptors was carried out according to a modified version of the protocol provided by supplier of Sf-9 membranes. [^{3}H] Nemonapride binding was performed by a modification of the method previously described by Elmhurst et al. (2000) and Javitch et al. (1994) for dopamine hD_{2L} and dopamine rD_3, respectively. Briefly, on the day of the experiment, the frozen membranes (Sf-9-hD_{2L} or Sf-9-rD_3) were thawed on ice, homogenized with an Ultra-turrax and suspended in appropriate binding buffer to a final concentration of 10 and 5 μg protein/tube for [^{3}H]spiperone and [^{3}H]nemonapride binding, respectively. The protein concentration present on the reaction tube was determined to contain not enough receptor protein to significantly deplete the ligand (less than 10% of the added radioligand was bound). When using [^{3}H]spiperone or [^{3}H]nemonapride, the buffer used in binding assay was (in mM): 50 Tris–HCl, pH 7.4, 5 $MgCl_2$, 1 EDTA, 5 KCl, 1.5 $CaCl_2$, 120 NaCl, or 50 Tris–HCl, pH 7.4, 5 $MgCl_2$, 5 EDTA, 5 KCl, 1.5 $CaCl_2$, 120 NaCl, for the dopamine hD_{2L} and dopamine rD_3 receptors, respectively. [^{3}H]Spiperone binding assays were performed in duplicate in a total volume of 0.540 ml at 22 °C for 60 min, or at 27 °C for 60 min for dopamine hD_{2L} and dopamine rD_3 receptors, respectively. Non-specific binding was estimated in the presence of 10 μM haloperidol for both dopamine hD_{2L} and dopamine rD_3 receptors. [^{3}H]Nemonapride binding was assayed in duplicate in a volume of 2 ml at 22 °C for 90 or 120 min for dopamine hD_{2L} and dopamine rD_3 receptors, respectively. (+)-Butaclamol (1 μM) was used to define non-specific binding. The reaction was terminated by rapid filtration through Whatman GF/C filters presoaked in 0.3% polyethyleneimine

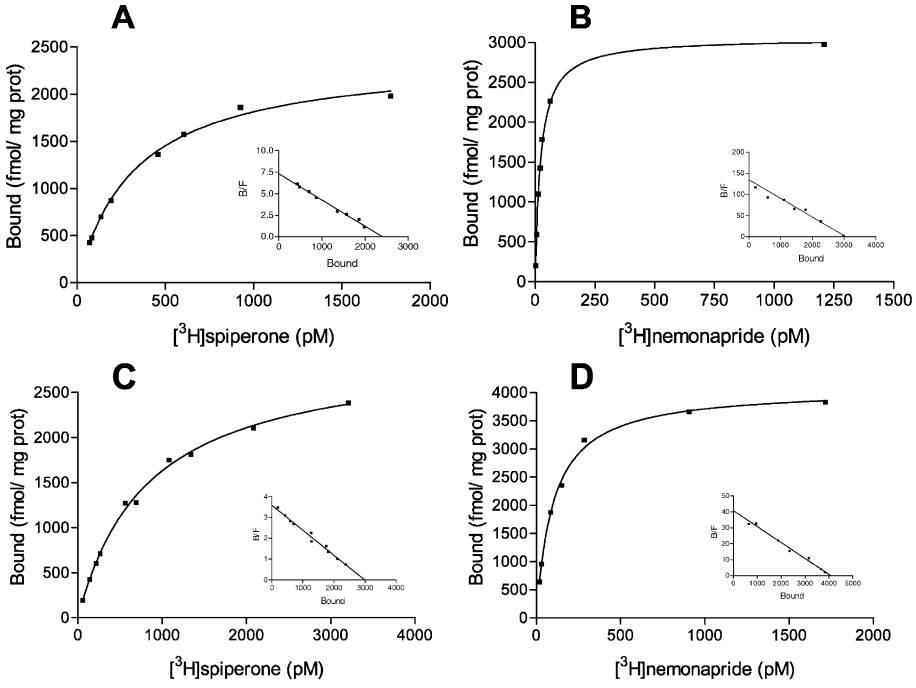

Fig. 1. Radioligand binding isotherm using [^{3}H]spiperone or [^{3}H]nemonapride binding at dopamine hD_{2L} (panel A and B) and at dopamine rD_3 receptors (panel C and D) expressed in Sf-9 cells. Experiments were performed as described in the text. Results are representative of data from three independent experiments. Scatchard transformations of the data are shown in the inset.

Table 1
K_d and B_{max} values of [^{3}H]nemonapride and [^{3}H]spiperone binding to Sf-9 cell membranes expressing dopamine hD_{2L} or dopamine rD_3 receptors

Receptor subtype	[^{3}H]Nemonapride		[^{3}H]Spiperone	
	K_d (pM)	B_{max} (pmol/mg prot.)	K_d (pM)	B_{max} (pmol/mg prot.)
hD_{2L}	38 ± 0.004	3.00 ± 0.1	270 ± 0.04	2.45 ± 0.06[a]
rD_3	120 ± 0.010	3.28 ± 0.4	770 ± 0.05	2.82 ± 0.08

The results are means ± S.E.M. from three independent experiments. Statistical significance was determined by Student's *t*-test.
[a] $P<0.01$ with respect to hD_{2L} B_{max} using [^{3}H]Nemonapride.

using a Brandell 96-sample harvester (Gaithersburg, MD, USA). Filters were rinsed twice with 5 ml of ice-cold 50 mM Tris–HCl buffer, pH 7.4. The filter bound radioactivity was measured in a liquid scintillation counter (Tricarb 2100, Packard, Meridien, USA) with 3 ml of scin- tillation fluid (Ultima Gold MV, Packard). For radioligand concentration-binding isotherms [^{3}H]spiperone (96 Ci/mmol) was used at 8–10 concentrations in the range of 5–1500 pM for dopamine hD_{2L} and 50–4000 pM for dopamine rD_3 receptors, while [^{3}H]nemonapride (85 Ci/mmol) was used at seven concentrations in the range of 2.5–1250 and 50–1600 pM for dopamine hD_{2L} and dopamine rD_3 receptors, respectively. In competition binding experiments, serial dilutions ranging from 10^{-10}–10^{-5} M of the unlabeled compounds: haloperidol, (+/−)*RS* amisulpiride, the (+)*R* and (−)*S* enantiomers were incubated either with [^{3}H]spiperone or [^{3}H]nemonapride for both dopamine D_{2L} and dopamine rD_3 receptors. Radioligand concentrations used in the assays were approximately equivalent to their K_d.

Protein determination was performed by means of Bradford (1976) protein assay using bovine serum albumin (BSA) as a standard according to the protocol of the supplier (Bio-Rad, Milan, Italy).

Radioligand concentration-binding isotherms were calculated using computerized nonlinear regression analysis of a rectangular hyperbola (Graph Pad Prism Program, San Diego, CA, USA).

Data from radioligand inhibition experiments were analyzed by nonlinear regression analysis of a Sigmoid Curve using Graph Pad Prism program. IC_{50} values were derived from the calculated curves and converted to K_i values as described previously (Cheng and Prusoff, 1973).

3. Results

Fig. 1 shows representative saturation binding curves of [^{3}H]spiperone and [^{3}H]nemonapride to dopamine hD_{2L} (A–B) and dopamine rD_3 receptors (C–D). Scatchard analysis of the specific [^{3}H]spiperone (Fig. 1A–C, inset) and [^{3}H]nemonapride (Fig. 1B–D, inset) binding resulted in linear plots consistent with a single class of binding sites. The K_d (pM) and B_{max} (pmol/mg of protein) values of [^{3}H]spiperone and [^{3}H]nemonapride to the dopamine hD_{2L} and dopamine rD_3 receptors are summarized in Table 1. These K_d values are in good agreement with those previously reported with cloned dopamine hD_{2L} or dopamine rD_3 receptors in Sf-9 cells (Elmhurst et al., 2000; Javitch et al., 1994; Grünewald et al., 1996). The B_{max} of dopamine hD_{2L} but not of dopamine rD_3 receptors was significantly lower ($P<0.01$) using [^{3}H]spiperone than [^{3}H]nemonapride, indicating that the benzamide radioligand might label a different feature of dopamine hD_{2L} receptors expressed in Sf-9 cells.

Table 2 and Fig. 2(A–B) show that haloperidol displayed high nanomolar affinity for the cloned transfected

Table 2
Inhibition of [^{3}H]spiperone and [^{3}H]nemonapride binding to Sf-9 cell membranes expressing dopamine hD_{2L} or dopamine rD_3 receptors

	[^{3}H]Spiperone		D_2/D_3 ratio	[^{3}H]Nemonapride		D_2/D_3 ratio
	hD_{2L}	rD_3		hD_{2L}	rD_3	
	K_i, nM (means ± S.E.M.)	K_i, nM (means ± S.E.M.)		K_i, nM (means ± S.E.M.)	K_i, nM (means ± S.E.M.)	
(+/−)*RS* Amisulpride	25.4 ± 1.6	9.4 ± 0.6	2.70 ± 0.40	7.7 ± 0.5	7.40 ± 0.2	1.05 ± 0.10
(−)*S* Amisulpride	14.6 ± 0.3	4.9 ± 0.7	2.65 ± 0.10	4.4 ± 0.2	5.05 ± 0.1	0.93 ± 0.10
(+)*R* Amisulpride	540 ± 21.0	93 ± 14.0	6.80 ± 0.20[a]	151 ± 7.9	97 ± 3.4	1.57 ± 0.10[b,c]
Haloperidol	7 ± 0.2	21.9 ± 2.9	0.84 ± 0.05	3.8 ± 0.1	20 ± 1.2	0.20 ± 0.01

One-way ANOVA for the effect of (+/−)*RS* amisulpride and its enantiomers (+)*R* and (−)*S* on [^{3}H]spiperone binding yielded the following result: $F(2,6)=94.342$, $P<0.0001$.
One-way ANOVA for the effect of (+/−)*RS* amisulpride and its enantiomers (+)*R* and (−)*S* on [^{3}H]nemonapride binding yielded the following result: $F(2,6)=11.6$, $P<0.01$.
K_i values are expressed as means ± S.E.M. of at least three determinations in duplicate.
D_2/D_3 K_i ratios are shown for the two different radioligands.
[a] $P<0.0005$ in comparison to amisulpride (−)*S* and (+/−)*RS* (Newman–Keuls test).
[b] $P<0.01$ in comparison to (−)*S* amisulpride.
[c] $P<0.05$ in comparison to (+/−)*RS* amisulpride (Newman–Keuls test).

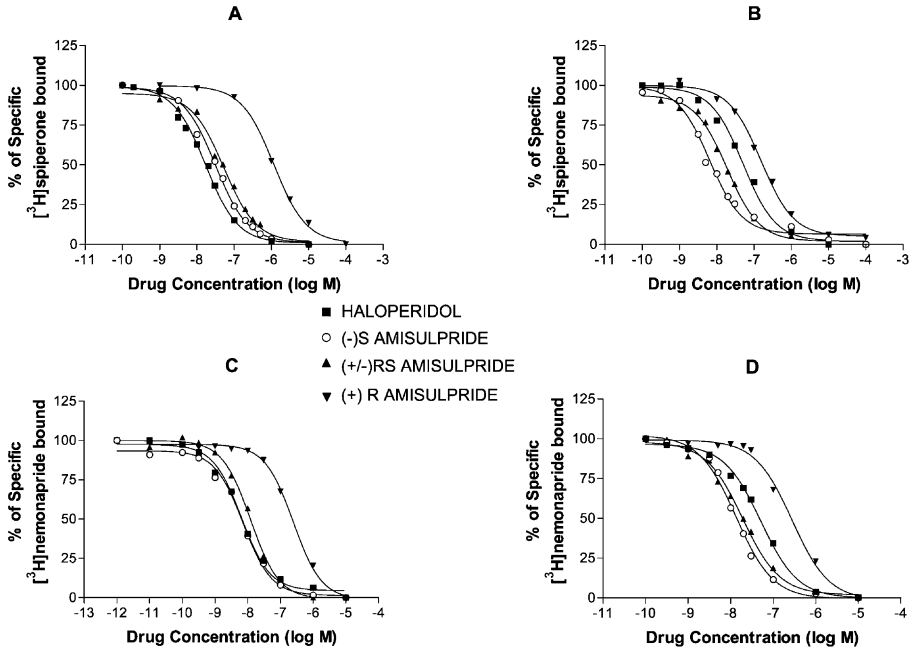

Fig. 2. Inhibition of [^{3}H]spiperone and [^{3}H]nemonapride binding by (+/−)*RS* amisulpride (▲), (+)*R* amisulpride (▼), (−)*S* amisulpride (○), and haloperidol (■) either at dopamine hD_{2L} (panel A–C) or at dopamine rD_3 receptors (panel B–D) expressed in Sf-9 cells. Experiments of binding to Sf-9-dopamine hD_{2L} and Sf-9-dopamine rD_3 receptors membranes were performed as described in the text. Results are representative of data from three independent experiments. Statistical fitting was obtained by nonlinear regression using the Graph Pad Prism program.

dopamine hD_{2L} and dopamine rD_3 receptors labeled with [^{3}H]spiperone (K_i = 7.0 and 21.9, respectively). Similarly, haloperidol displaced [^{3}H]nemonapride binding with a K_i of 3.8 and 20 nM for dopamine hD_{2L} and dopamine rD_3, respectively (Table 2, Fig. 2C–D). Both [^{3}H]spiperone and [^{3}H]nemonapride binding either to the dopamine hD_{2L} or dopamine rD_3 receptors were potently and stereoselectively inhibited by (+/−)*RS* amisulpride and (−)*S* amisulpride as compared to (+)*R* amisulpride (Fig. 2). (+/−)*RS* amisulpride and the (−)*S* enantiomer showed higher affinity than haloperidol for dopamine rD_3 receptors using either the radiolabeled nemonapride or spiperone (Fig. 2B–D). The (+)*R* amisulpride has weaker affinity than the (−)*S* enantiomer for both dopamine hD_{2L} and dopamine rD_3 receptors and also lesser affinity than haloperidol for both receptors.

K_i values at dopamine rD_3 receptors were similar regardless of the radioligand used (Table 2), whereas at dopamine hD_{2L} receptors the K_i values were higher using [^{3}H]spiperone than [^{3}H]nemonapride. However, the rank order of compound potency against either radiolabeled spiperone or nemonapride at either receptor was the same: (−)*S* amisulpride ≥ (+/−)*R*/*S* amisulpride > haloperidol > (+)*R* amisulpride at dopamine D_3 receptors and haloperidol > (−)S amisulpride ≥ (+/−)*R*/*S* amisulpride > (+)*R* amisulpride at dopamine hD_{2L}receptors. The selectivity ratio (K_iD_2/K_iD_3) for the two radioligands is shown in Table 2. (+/−)*RS* amisulpride and its (−)*S* enantiomer displayed similar affinity for dopamine hD_{2L} and dopamine rD_3 receptors, whereas the (+)*R* enantiomer showed a mild selectivity for dopamine rD_3 receptors (ratio K_i dopamine D_2/K_i dopamine D_3 receptors = 6.8 and 1.5 for [^{3}H]spiperone and [^{3}H]nemonapride, respectively).

4. Discussion

This study shows that (−)*S* amisulpride is the active enantiomer form of (+/−)*RS* amisulpride with regard to the ability to bind to dopamine D_2 and dopamine D_3 receptors. Indeed (−)*S* amisulpride was found to be twice as potent as the racemic form and 20 to 40 times more potent than (+)*R* enantiomer in displacing the radioligands from dopamine D_2 and dopamine D_3 receptors. Several studies have investigated the pharmacological characteristics of benzamide and butyrophenone binding to dopamine D_2-like receptors; both the B_{max} and K_d/K_i values determined using various radioligands vary considerably (Hall et al., 1991; Seeman et al., 1992). However, recently, Malmberg et al. (1996) have reported that [^{3}H]spiperone, [^{125}I](*S*)-3-iodo-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-5,6-dimethylsalicylamide ([^{125}I]NCQ-298), [^{3}H]raclopride and [^{3}H]nemonapride displayed very high dopamine D_2 receptors affinity and labeled an identical dopamine D_2 receptors population both in native and cloned dopamine D_2 receptors in Chinese Hamster Ovary (CHO) cells. The present

data showed K_d values of [^{3}H]spiperone and [^{3}H]nemonapride similar to those previously reported with cloned dopamine hD_2 or dopamine rD_3 receptors in Sf-9 (Elmhurst et al., 2000; Javitch et al., 1994; Grünewald et al., 1996), but higher than in CHO or other cell lines (Levant, 1997). In our experimental conditions, [^{3}H]nemonapride and [^{3}H]spiperone labeled identical number of binding sites at dopamine rD_3 receptors. On the contrary, [^{3}H]spiperone labelled 20% less binding sites than [^{3}H]nemonapride at dopamine hD_{2L} receptor sites. The differences in dopamine D_2 receptors density might be explained with the possibility that benzamides such as nemonapride or raclopride bind to monomers, while the butyrophenone spiperone binds to dimers as suggested by Seeman et al. (1992). The displacement of [^{3}H]spiperone and [^{3}H]nemonapride binding from either dopamine hD_{2L} or dopamine rD_3 receptors by amisulpride was steroselective, (−)*S* amisulpride being twofold more potent than the racemic form and 35–19-fold more potent than the (+)*R* enantiomer in both bindings. Moreover, the racemic amisulpride and its active enantiomer displayed higher affinity for dopamine rD_3 than dopamine hD_{2L} receptors when [^{3}H]spiperone was used as radioligand, but no selectivity was observed when [^{3}H]nemonapride was used. The lower potency of amisulpride to displace [^{3}H]spiperone rather than [^{3}H]nemonapride from dopamine hD_{2L} receptors may be due to differences in binding features of the two ligands at the receptor protein expressed in Sf-9 cells. Apparently, with regard to the radioligands these differences were not observed when haloperidol was used as a displacer. It should be mentioned that (−)-sulpiride is twice as potent as the racemic sulpiride and about 50 times more active than (+)-sulpiride in [^{3}H]spiperone binding using rat striatal membranes (Mizuchi et al., 1982). Yet, (−)-sulpiride is eight times more potent than the racemic form in increasing the prolactin levels in rats (Kakigi et al., 1992). Thus, the role of the two enantiomers of amisulpride should be determined in vivo, where the functional correlates may be a function not only of the relative affinities for a given receptor but also of penetration and regional distribution in brain (Kessler et al., 1989, 1991).

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