British Journal of Pharmacology (1996) 117, 1625–1632

In vitro pharmacological profile of YM-43611, a novel D_2-like receptor antagonist with high affinity and selectivity for dopamine D_3 and D_4 receptors

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1 We investigated some neurochemical properties of a novel benzamide, YM-43611, [(*S*)-*N*-(1-benzyl-3-pyrrolidinyl)-5-chloro-4-cyclopropylcarbonylamino-2-methoxybenzamide] in comparison with putative D_2-like receptor antagonists using both rat and human cloned dopamine D_2-like receptors *in vitro*.

2 Receptor binding studies revealed that YM-43611 had appropriately potent affinities for both rat and human D_2-like receptors, with moderate selectivity for D_3 receptors and high selectivity for D_4 receptors over D_2 receptors (K_i values (nM) for rat receptors: D_2, 165; D_3, 35.5; D_4, 1.85, and for human receptors: D_2, 42.9; D_3, 11.2; D_4, 2.10).

3 YM-43611 displayed weak or negligible affinity for other neurotransmitter receptors, namely D_1, D_5, α_1, α_2, β, 5-HT_{1A}, 5-HT_{2A}, 5-HT_3, H_1, M_1 and M_2 receptors.

4 Dopamine stimulated low-K_m GTPase activity on membranes from Chinese hamster ovary (CHO) cells expressing the human D_2-like receptor subtype. This response to dopamine of low-K_m GTPase activity was inhibited by use of putative D_2-like receptor antagonists. YM-43611 showed a moderate selectivity for D_3 receptors ($Ki = 45.5$ nM) and a high selectivity for D_4 receptors ($K_i = 3.28$ nM) over D_2 receptors ($K_i = 70.6$ nM).

5 Dopamine inhibited forskolin-stimulated adenylate cyclase in intact CHO cells expressing the human D_2-like receptor subtype. YM-43611 shifted the inhibition curve of dopamine on respective D_2-like receptor subtype-mediated cyclic AMP formation to the right in a parallel fashion, showing a pA_2 value of 7.42 (38.1 nM) for D_2 receptors, a pK_B value of 8.06 (8.68 nM) for D_3 receptors, and a pA_2 value of 8.42 (3.77 nM) for D_4 receptors.

6 YM-43611 but not the other D_2-like receptor antagonists exhibited good selectivity with respect to dual antagonism for D_3 and D_4 receptors in both receptor binding and functional assays.

7 These results indicate that YM-43611 is a novel D_2-like receptor antagonist with high potency and selectivity for both D_3 and D_4 receptors. YM-43611 is therefore expected to be valuable in exploration of the physiological role of D_3 and D_4 receptors.

Keywords: YM-43611; D_2-like receptors; D_3 dopamine receptor; D_4 dopamine receptor; antagonist; selectivity; low-K_m GTPase; adenylate cyclase

Introduction

Dopamine had been considered to act on its target cells in the brain and endocrine tissues *via* interaction with only two receptor subgroups, named D_1 and D_2 (Kebabian & Calne, 1979). Recent molecular cloning studies have been shown the existence of five dopamine receptors (Sibley & Monsma, 1992). On the basis of amino acid sequence and pharmacological characteristics, these have been classified into two subfamilies, the D_1-like receptors and the D_2-like receptors. The D_2-like receptors, classically the 'D_2 receptor subgroup', consists of D_2, D_3 and D_4 receptors. These differ with respect to their distribution in the brain (Seeman, 1992), with D_2 receptors mainly distributed in the striatum, D_3 receptors in the limbic brain, and D_4 receptor in the frontal cortex and limbic brain. Moreover, these three subtypes have been reported to mediate different cellular events (Tang *et al.*, 1994a,b; Seabrook *et al.*, 1994). These differences in distribution pattern and signal transduction events suggest that these D_2-like receptor subtypes may play different physiological roles in the brain.

Despite the upsurge of interest in the physiological function of the D_2-like receptor subtypes, pharmacological research is hampered by the limited number of compounds, in particular antagonists, with high potency and selectivity for the individual subtypes. Recently, the selective D_3 antagonists, (+)-S 14297 (Millan *et al.*, 1994) and GR103691 (Murray *et al.*, 1995) and the selective D_4 ligand JL18 (Liegeois *et al.*, 1995) were reported. The antagonistic activities and potencies of these compounds, however, have not been demonstrated in *in vitro* functional studies. We previously developed a benzamide, nemonapride (YM-09151-2; Figure 1), which showed the pharmacological profile of a neuroleptic in *in vivo* studies (Iwanami *et al.*, 1981; Usuda *et al.*, 1981). Nemonapride has selectivity and potent affinity for D_2-like receptors (Terai *et al.*, 1989); in common with other neuroleptics, it is not selective for either D_3 or D_4 receptors over D_2 receptors. In the search for a novel class of D_2-like receptor ligands, we found YM-43611 (Figure 1) in our data base on the basis of structure activity relationship of a series of benzamide derivatives on all three D_2-like receptor subtypes using radioligand receptor binding assays. Low-K_m GTPase assay and adenylate cyclase assay were used to investigate D_2-like receptor antagonistic properties. In the present study, we have elucidated *in vitro* pharmacological profile of YM-43611 as a novel, potent and selective D_3 and D_4 receptor antagonist.

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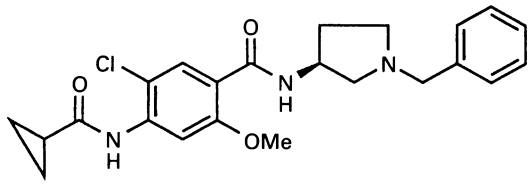
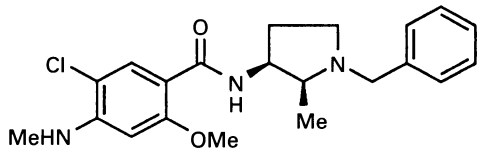
Methods

D_2-like receptors expression constructs

The rat D2L cDNA (the exon 6-containing form) and the rat D3 cDNA were cloned by reverse transcription-polymerase chain reaction (RT-PCR) amplification according to the methods of Chio *et al.* (1990) and Sokoloff *et al.* (1990), respectively. The cloned cDNAs were then ligated into the expression vector pVY1 containing dihydrofolate reductase (dhfr) gene as a selective marker. For cloning of the rat D4 cDNA, we used formamide and Pfu DNA polymerase in RT-PCR amplification as previously described (Matsumoto *et al.*, 1995). The oligonucleotide primers 5'-ATGGGGAACAGCAGCGCTACT-3' (sense, position 131–151; reference RATD4RA in the GenBank database) and 5'-TCAGCAGCGGAGACGAAGAGT-3' (antisense, position 1288–1268) were used to amplify the rat D4 cDNA using poly A^+ RNA from the heart as a template. The PCR cycling conditions were 1 min at 98°C, 1 min at 50°C and 4 min at 74°C for 35 cycles. The amplified cDNA was then ligated into the expression vector pEF-BOS (Mizushima & Nagata, 1990). The human D2L cDNA was cloned by RT-PCR amplification using poly A^+ RNA from the pituitary as a template. The oligonucleotide primers 5'-TCCACCGCCCTGATGGAT-3' (sense, position 22–39; reference HUMDRD2A in the GenBank database) and 5'-GGCTAAGAAGAGGAGGCCGAT-3' (antisense, position 1482–1462) were used to amplify the human D2L cDNA under the PCR cycling conditions of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C for 35 cycles. The human D3 cDNA was cloned by PCR amplification using purified phage DNA of the human nucleus accumbens cDNA library (Clontech) as a template. Oligonucleotide primers 5'-ATGGCATCTCTGAGTCAGCTG-3' (sense, position 1–21; reference Giros *et al.*, 1990) and 5'-AGCTAGAAATGGGTACAAAGA-3' (antisense, position 1244–1224) were used to amplify the human D3 cDNA under the PCR cycling conditions of 1 min at 94°C, 2 min at 50°C and 3 min at 72°C for 35 cycles. The human D4 cDNA containing seven polymorphic tandem repeats in the putative third cytoplasmic region (termed D4.7) was cloned as previously described (Hidaka *et al.*, 1995). These human D2L, D3, and D4 cDNAs were separately ligated into the expression vector pEF-BOS (dhfr) containing dhfr gene as a selective marker.



YM-43611



Nemonapride

Figure 1 Chemical structures of YM-43611 and nemonapride (YM-09151-2). Note that nemonapride is a racemic compound.

Cell cultures and transfections

Chinese hamster ovary (CHO) cells lacking the dihydrofolate reductase gene, CHO (dhfr-) cells, were maintained in minimal essential medium (MEM) alpha medium with ribonucleosides and deoxyribonucleosides (Gibco BRL) supplemented with 10% foetal bovine serum and incubated at 37°C and an atmosphere of 5% CO_2. The rat D2L and D3 cDNA in pVY1 and the human D2L, D3 and D4.7 cDNA in pEF-BOS (dhfr) were separately transfected into CHO (dhfr-) cells using LipofectAMINE (Gibco BRL) according to the manufacturer's protocol. At 72 h after transfection, the medium was changed to MEM alpha medium without ribonucleosides and deoxyribonucleosides and the receptor cDNA was further amplified using up to 700 nM methotrexate. Under 700 nM methotrexate, the transfected cells were divided into single cells and monoclonal cell lines showing specific [^{3}H]-nemonapride binding were used for pharmacological characterization. COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% foetal bovine serum and incubated at 37°C and an atmosphere of 5% CO_2. The rat D4 cDNA in pEF-BOS was transfected into COS-1 cells by the DEAE dextran procedure (Kaufman *et al.*, 1989). The transfected cells were harvested 72 h later for membrane preparations.

Membrane preparation

The D_2-like receptor-transfected cells were rinsed from 150 mm dishes with phosphate-buffered saline (PBS), harvested with 20 mM HEPES-NaOH, 0.5 mM EGTA, 5.4 mM KCl and 140 mM NaCl (pH 7.4), pelleted, resuspended in 5 mM Tris-HCl (pH 7.4), and homogenized with a Polytron (setting 8 for 10 s). The supernatant resulting from centrifugation at 1000 *g* was recentrifuged at 40,000 *g*. The pellet was washed once with 5 mM Tris-HCl (pH 7.4), and the final pellet was resuspended in 50 mM Tris-HCl (pH 7.4). Membrane aliquots were stored at −80°C until use of radioligand binding and low-K_m GTPase assays.

Radioligand binding assays

The radioligand binding assays for the D_2-like receptors were carried out in polystyrene tubes containing 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, and 5 mM EDTA (pH 7.4), [^{3}H]-nemonapride, the resuspended membranes, and vehicle, competitor drug or nonspecific ligand. For competition experiments, [^{3}H]-nemonapride concentrations were used as follows: hD2, 25 pM; hD3, 92 pM; hD4, 255 pM; rD2, 70 pM; and rD3, 190 pM; rD4, 280 pM. Nonspecific binding was determined in the presence of 10 μM sulpiride for D_2 receptors, 10 μM quinpirole for D_3 receptors, and 1 mM dopamine, 0.1 mg ml^{-1} L-(+)-ascorbic acid and 50 μM pargyline for D_4 receptors. To avoid an excess amount of [^{3}H]-nemonapride binding, we performed binding studies with larger volumes. The final volume and percentage of [^{3}H]-nemonapride bound were as follows: hD2, 4 ml, 19% hD3, 2 ml, 16% hD4, 2 ml, 8.9%; rD2, 2 ml, 25%; rD3, 1 ml, 12%; rD4, 1 ml, 13%, respectively. The reaction mixture was incubated at 25°C for 60 min, and the assay was terminated by rapid filtration under vacuum through a Whatman GF/B filter. The filter was immediately washed four times with 4 ml each of the washing buffer: 50 mM Tris-HCl, 120 mM NaCl (pH 7.4). The filter was placed in a scintillation cocktail for quantification of radioactivity using a β scintillation counter. The methodologies used for examination of binding to neurotransmitter receptors, except for that of D_2-like receptors, are given in Table 1.

Low-K_m GTPase assays

The assay was performed by a minor modification of the method of Clark & Medziharadsky (1987). The assay medium

Table 1 Assay conditions for the competition studies of radioligand receptor binding assays

Receptor	*Receptor source*	*^{3}H-labelled ligand*	*Nonspecific ligand*	*Incubation (min/°C)*	*Reaction buffer reference*
Dopamine D_1	Human cloned	Sch23390 (0.68 nM)	*cis*-Flupenthixol (30 μM)	60/25	Hidaka *et al.* (1995)
Dopamine D_5	Human cloned	Sch23390 (0.49 nM)	*cis*-Flupenthixol (30 μM)	60/25	Hidaka *et al.* (1995)
Dopamine D_1/D_5	Rat striatum	Sch23390 (0.21 nM)	*cis*-Flupenthixol (30 μM)	60/25	Hidaka *et al.* (1995)
Adrenoceptor α_1	Rat cortex	Prazosin (0.12 nM)	Phentolamine (10 μM)	60/25	Michel *et al.* (1989)
Adrenoceptor α_2	Rat cortex	RX821002 (1.0 nM)	Phentolamine (10 μM)	60/25	Michel *et al.* (1989)
Adrenoceptor β	Rat cortex	Dihydroalprenolol (0.80 nM)	Propranolol (10 μM)	60/25	Michel *et al.* (1989)
5-HT 5-HT_{1A}	Rat hippocampus	8-OH-DPAT (0.84 nM)	Metergoline (10 μM)	30/25	Peroutka (1986)
5-HT 5-HT_{2A}	Rat frontal cortex	Ketanserin (0.98 nM)	Metergoline (10 μM)	45/25	Leyen *et al.* (1982)
5-HT 5-HT_3	N1E-115 neuroblastoma	GR65630 (1.9 nM)	ICS 205-930 (10 μM)	60/25	Ito *et al.* (1992)
Histamine H_1	Rat cortex	Pyrilamine (3.5 nM)	Amitriptyline (10 μM)	60/25	Daum *et al.* (1983)
Muscarinic M_1	Rat cortex	Pirenzepine (3.5 nM)	Atropine (10 μM)	60/25	Potter *et al.* (1988)
Muscarinic M_2	Rat heart	QNB (0.10 nM)	Atropine (10 μM)	30/25	Ehlert (1988)

contained 0.3 μM [γ-^{32}P]-GTP, the resuspended membranes, 50 mM Tris-HCl, 120 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 2 mM DTT, 1 mM App(NH)p, 0.5 mM ATP, 1 mM (−)-ouabain, 10 mM creatinine phosphate, 100 units ml^{-1}creatinine kinase, 0.1 mg ml^{-1} L-(+)-ascorbic acid and 50 μM pargyline, and vehicle or competitor drug in a final volume of 100 μl. In separate tubes, the reaction mixture also contained 100 μM GTP to measure the activity of the dopamine-insensitive, high-K_m GTPase activity. After a 10 min incubation at 37°C, the reaction was terminated by addition of 900 μl of ice-cold 6.7 mM phosphate buffer (pH 7.4) containing 5% activated charcoal, and the mixture was centrifuged at 1500 *g* for 15 min. Released $^{32}P_i$ from [γ-^{32}P]-GTP in 500 μl aliquots of the supernatant fluids was measured using a β scintillation counter. The cpm's obtained in the presence of 100 μM GTP were subtracted from the cpm's for total enzyme activity to yield a cpm value representing the activity of the dopamine-sensitive, low K_m GTPase.

Adenylate cyclase assays

Cyclic AMP accumulation was measured in intact CHO cells plated in a 24-well plate 48–72 h before the experiment. CHO cells were pre-incubated with 1 mM 3-isobuthyl-1-methylxanthine, 50 μM pargyline and 0.1 mg ml^{-1} ascorbic acid in PBS (PBS-I buffer) for 20 min at 37°C. The reaction buffer containing 10 μM forskolin, dopamine, and test drugs in PBS-I buffer was then added, and cyclic AMP was allowed to accumulate for 8 min at 37°C. The reactions were terminated by the removal of the buffer and the addition of 0.5 ml/well of ice-cold 0.1N HCl. Cyclic AMP levels were measured with a radioimmunoassay kit (Yamasa Shoyu, Chiba, Japan).

Data analysis

Dopamine concentration-response curves in low-K_m GTPase assays were fitted to the logistic equation

$$\text{Response} = (E_{max} \times C^{n}{}_{H})/(EC_{50}{}^{n}{}_{H} + C^{n}{}_{H}),$$

where E_{max} is maximal response, C is the dopamine concentration, EC_{50} is the concentration of dopamine producing half maximal stimulation, and n_H is the Hill coefficient.

Data from competition binding experiments, competition low-K_m GTPase experiments and inhibitory adenylate cyclase experiments were fitted to the equation

$$v = P - \{(I_{max} \times L^{n_H})/IC_{50}^{n_H} + L^{n_H}$$

where I_{max} is the portion of maximum inhibition, IC_{50} is the concentration of test drug producing half maximal inhibition, L is the concentration of the test drug and n_H is the Hill coefficient. For analysis of binding experiments, v is the ^{3}H-labelled ligand bound, P is the estimate of ^{3}H-ligand bound in the absence of competitor drug; when the low-K_m GTPase assay was analysed, v is low K_m GTPase activity, P is the estimate of low-K_m GTPase activity in the absence of competitor drug; when the adenylate cyclase experiments were analysed, v is adenylate cyclase activity, and P is the estimate of adenylate cyclase activity in the absence of dopamine. Data from competition binding experiments with dopamine were analysed with a two-site model by previously described methods (Martin *et al.*, 1984).

In the competition binding experiments, the apparent affinities (K_i values) of competing ligands were calculated from the IC_{50} values by the Cheng-Prusoff equation (Cheng & Prusoff, 1973). In the competition low-K_m GTPase experiments, the apparent affinities (K_i values) of competing ligands were estimated by a modification of the null methods described by Lazareno & Roberts (1987). Briefly, a concentration-response curve to dopamine was generated and a concentration (C) chosen which gave a response greater than 50% of the maximum dopamine response. The concentrations of antagonist (IC_{50}) required to reduce the response of this concentration (C) of dopamine by 50% was then determined. The dopamine concentration-response curve was fitted to the logistic equation as above and a concentration of dopamine (C') identified which yielded a response equivalent to 50% of that produced by concentration C (in the absence of antagonist). The apparent K_i was then determined from the relationship:

$$C/C' = (IC_{50}/K_i) + 1$$

The apparent $pK_B(-\log K_B)$ value was calculated according to the formula

$$K_B = B/\{(A'/A)-1\},$$

where B is the concentration of YM-43611, A' and A are the IC_{50} values of dopamine measured in the presence and in the absence of YM-43611, respectively.

All data were analysed by non-linear least squares regression with no weighting using RS1 software (BBN Research System, Cambridge, Mass, U.S.A.). Results are given as means ± s.e.mean.

Materials

YM-43611 [(*S*-*N*-(1-benzyl-3-pyrrolidinyl)-5-chloro-4-cyclopropylcarbonylamino-2-methoxybenzamide], nemonapride, mosapramine and ICS 205-930 were synthesized in our laboratory. The following drugs were obtained from commercial neuroleptics by extraction, refining, and purity-checking in our

laboratory: bromperidol (Impromen), thioridazine (Melleril), sulpiride (Dogmatyl), zotepine (Lodopin) and risperidone (Risperidal). Raclopride, *cis*-flupenthixol and metergoline were kindly donated by Astra Arcus AB, Lundbeck and Farmitalia Carlo Erba Laboratories, respectively. The following compounds and cloned receptors were purchased from Dupont-New England Nuclear: [^{3}H]-nemonapride (3.18 TBq mmol^{-1}), [^{3}H]-Sch23390 (2.59 TBq mmol^{-1}), [^{3}H]-dihydroalprenolol (4.00 TBq mmol^{-1}), [^{3}H]-ketanserin (2.22 TBq mmol^{-1}), [^{3}H]-GR65630 (2.27 TBq mmol^{-1}), [^{3}H]-pyrilamine (0.914 TBq-mmol^{-1}), [^{3}H]-pirenzepine (3.12 TBq mmol^{-1}), [^{3}H]-QNB (1.94 TBq mmol^{-1}), [γ-^{32}P]-GTP (1.11 TBq mmol^{-1}), human cloned D_1 receptor and human cloned D_5 receptor. The following compounds were purchased from Amersham: [^{3}H]-prazosin (2.70 TBq mmol^{-1}), [^{3}H]-RX821002 (2.29 TBq-mmol^{-1}) and [^{3}H]-8OH-DPAT (8.55 TBq mmol^{-1}). All other chemicals used in this study were obtained from standard commercial sources.

Results

D2-like receptor-expressing cells

CHO cells were separately transfected with the expression vectors encoding rat D2L (rD2), rat D3 (rD3), human D2L (hD2), human D3 (hD3) and human D4.7 (hD4) receptors. Saturable [^{3}H]-nemonapride binding was observed in approximately half of the methotrexate-resistant clones transfected with either the rD2, rD3, hD2 or hD3 receptor cDNA; the line with the highest expression was selected as the cloned cell line expressing the respective receptor for use in the following experiments. Only one cell line among 29 isolated colonies transfected with the hD4 receptor cDNA exhibited stable, permanent [^{3}H]-nemonapride binding sites. COS-1 cells were transiently transfected with the expression vector encoding the rat D4 (rD4) receptor cDNA. The B_{max} values for the respective D_2-like receptor subtypes, estimated by saturation analyses of [^{3}H]-nemonapride specific binding, were as follows (fmol mg^{-1} protein): rD2 receptor, 5990; rD3 receptor, 1760; rD4 receptor, 411; hD2 receptor, 8190; hD3 receptor, 5580; hD4 receptor, 2040.

Binding studies of D_2-like receptors

The affinities of YM-43611 and the putative D_2-like receptor antagonists were analysed in competition experiments with [^{3}H]-nemonapride binding to the different D_2-like receptors. The affinities estimated for a series of compounds are shown in Tables 2 and 3. YM-43611 displayed relatively high affinity for D_3 receptors and high affinity for D_4 receptors compared with that for D_2 receptors. Most of the putative D_2-like antagonists exhibited D_2-selectivity against D_3 and D_4 receptors. (+)-UH232 showed moderate selectivity for D_3 receptors, and clozapine for D_4 receptors.

The competition curves of dopamine best fit to a two-site model ($P<0.01$ by F-test). The K_i values for high and low affinity states and the percentage of high affinity state were as follows: hD2 receptor, 39.2 ± 9.5 nM, 1520 ± 160 nM, 30.4 ±

Table 2 Affinities (K_i) of YM-43611 and the putative antagonists for the human D_2-like receptors

Compound	K_i (nM) hD2 receptor	hD3 receptor	hD4 receptor
YM-43611	42.9±1.4	11.2±0.3	2.10±0.10
Haloperidol	0.450±0.022	1.52±0.06	2.08±0.20
Bromperidol	0.239±0.014	1.00±0.03	2.22±0.09
Spiperone	0.053±0.003	0.179±0.009	0.376±0.027
Chlorpromazine	2.09±0.17	2.80±0.13	19.1±2.1
Thioridazine	7.92±1.1	8.67±0.56	15.1±1.3
Zotepine	1.43±0.09	2.88±0.18	13.0±0.68
Clozapine	82.5±6.1	163±4	38.8±1.5
Mosapramine	1.74±0.06	2.35±0.12	14.9±1.1
Nemonapride*	0.017±0.001	0.053±0.06	0.214±0.027
Sulpiride	6.73±0.48	21.8±1.0	787±48
Raclopride	0.595±0.034	1.03±0.035	1610±150
Risperidone	1.36±0.08	6.10±0.32	14.0±0.9
(+)-UH232	13.4±0.5	4.65±0.16	89.4±5.1

Competition curves were analyzed by computerized non-linear regression using a one-site model for [^{3}H]-nemonapride binding to the membranes prepared from CHO cells expressing the human D2L, D3 and D4.7 gene, respectively. Data represent the mean±s.e. mean from duplicate determinations in three to five experiments.
*K_d values derived from the saturation studies of [^{3}H]-nemonapride binding.

Table 3 Affinities (K_i) of YM-43611 and the putative antagonists for the rat D_2-like receptors

Compound	K_i (nM) rD2 receptor	rD3 receptor	rD4 receptor
YM-43611	165±4	35.5±1.3	1.85±0.05
Haloperidol	1.08±0.04	7.04±0.28	15.1±0.6
Clozapine	154±4	401±19	41.4±2.0
Nemonapride*	0.031±0.002	0.196±0.10	0.216±0.023
Raclopride	1.89±0.07	4.67±0.28	2600±80
Risperidone	2.05±0.09	11.8±0.8	15.7±0.6

Competition curves were analyzed by computerized non-linear regression using a one-site model for [^{3}H]-nemonapride binding to the rat D2L, D3 and D4 receptors, respectively. Data represent the mean±s.e. mean from duplicate determinations in three or four experiments.
*K_d values derived from the saturation studies of [^{3}H]-nemonapride binding.

Table 4 Affinities (K_i) of YM-43611, haloperidol and clozapine for neurotransmitter receptors except for D_2-like receptors

Receptor	K_i (nM) YM-43611	Haloperidol	Clozapine
Dopamine D_1	>10,000	63.2±5.1	158±10
Dopamine D_5	>10,000	242±23	644±56
Dopamine D_1/D_5	>10,000	76.0±4.7	169±4
Adrenoceptor α_1	5,930±520	7.91±0.47	4.20±0.34
Adrenoceptor α_2	5,930±1080	9,580±180	243±7
Adrenoceptor β	>10,000	>10,000	>10,000
5-HT 5-HT_{1A}	>10,000	2,270±170	185±11
5-HT 5-HT_{2A}	>10,000	103±5	12.5±0.6
5-HT 5-HT_3	3,890±160	>10,000	35.8±1.4
Histamine H_1	>10,000	2,340±120	3.46±0.27
Muscarinic M_1	5,980±680	203±9	2.40±0.18
Muscarinic M_2	8,230±390	2,630±220	51.1±2.3

The values represent the mean ±s.e.mean from two or three independent experiments.

Table 5 Antagonist potencies of YM-43611 and the putative antagonists on dopamine-stimulated low-K_m GTPase activity for the human D2-like receptors

Compound	K_I (nM) hD2 receptor	hD3 receptor	hD4 receptor
YM-43611	70.6±7.8	45.5±8.9	3.28±0.73
Haloperidol	0.674±0.089	4.26±0.58	17.9±2.5
Clozapine	40.4±5.0	422±72	83.8±10.7
Nemonapride	0.297±0.042	1.20±0.26	1.54±0.25
Raclopride	1.49±0.22	4.79±0.85	5220±740
(+)-UH232	25.4±3.6	15.8±2.5	104±14

The antagonist K_i values (nM) were obtained from the inhibition of dopamine-stimulated low-K_m GTPase activity on membranes prepared from CHO cells separately expressing the human D_2, D_3 and D_4 receptors. Dopamine was used at 3 μM with hD2 receptors, at 0.3 μM with hD3 receptors and at 1 μM with hD4 receptors. Data represent the mean±s.e.mean from triplicate determinations in three to five experiments.

3.3% ($n=5$); hD3 receptor, 1.94±0.43 nM, 50.1±5.8 nM, 34.6±3.9% ($n=5$); hD4 receptor, 9.16±1.31 nM, 942±203 nM, 60.5±2.9% ($n=5$), respectively.

YM-43611 displayed weak or negligible affinities for dopamine D_1, D_5, adrenaline α_1, α_2, β, 5-hydroxytryptamine 5-HT_{1A}, 5-HT_{2A}, 5-HT_3, histamine H_1, muscarinic M_1 and M_2 receptors (Table 4). In contrast haloperidol and clozapine showed some affinities for these receptors.

Low-K_m GTPase activity meditated by human D_2-like receptors

Dopamine stimulated low-K_m GTPase activity on the membranes from CHO cells expressing human D_2-like receptor subtypes in a saturable, concentration-dependent manner (Figure 2). EC_{50} values and maximal stimulation above basal activity for dopamine were as follows: hD2 receptor, 297±39 nM, 121±3%; hD3 receptor, 45.8±7.8 nM, 48.6±1.4%; and hD4 receptor, 117±15 nM, 56.6±1.2%, respectively.

YM-43611 and the putative D_2-like receptor antagonists concentration-dependently inhibited the response to dopamine at the dose of 3 μM for hD2 receptors, 0.3 μM for hD3 receptors and 1 μM for hD4 receptors (Table 5). YM-43611 exhibited antagonistic activity with moderate selectivity for hD3 receptors and high selectivity for hD4 receptors over hD2 receptors.

Adenylate cyclase activity mediated by human D_2-like receptors

Forskolin (10 μM) produced an increase in cyclic AMP levels in respective human D_2-like receptor subtype-expressing CHO cell lines as follows (pmol per well): hD2 receptor, from

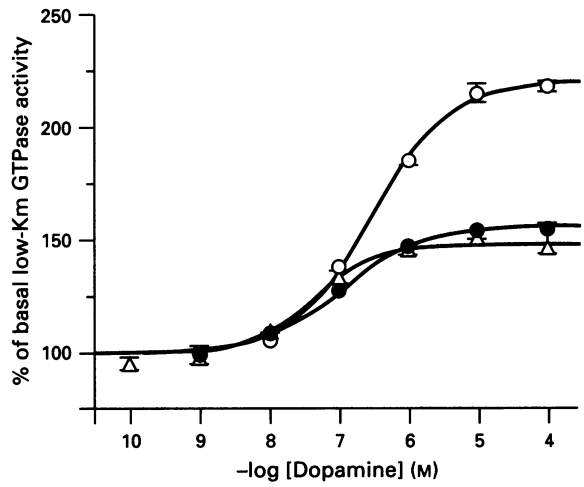


Figure 2 Effects of dopamine on low-K_m GTPase activities on the membrane from CHO cells expressing each human D_2-like receptor subtype. Basal GTPase activities on the membranes from CHO cells transfected with hD2 (○), hD3 (△) and hD4 (●) receptors are 18.4±0.8, 16.4±1.1 and 18.2±2.1 pmol Pi mg^{-1} protein min^{-1}, respectively. Each point represents the mean±s.e.mean from triplicate determinations in four or five separate experiments.

1.03±0.11 to 37.2±1.06; hD3 receptor, from 1.63±0.11 to 48.5±2.7; hD4 receptor, from 2.33±0.23 to 110±6. Stimulation of the human D_2-like receptor subtype by dopamine inhibited the forskolin-induced increase in cyclic AMP levels in a saturable, concentration-dependent manner (Figure 3). The IC_{50} value and maximal inhibition were 4.51±0.63 nM and 89.8±1.6% in the hD2 cell line, 2.31±0.75 nM and

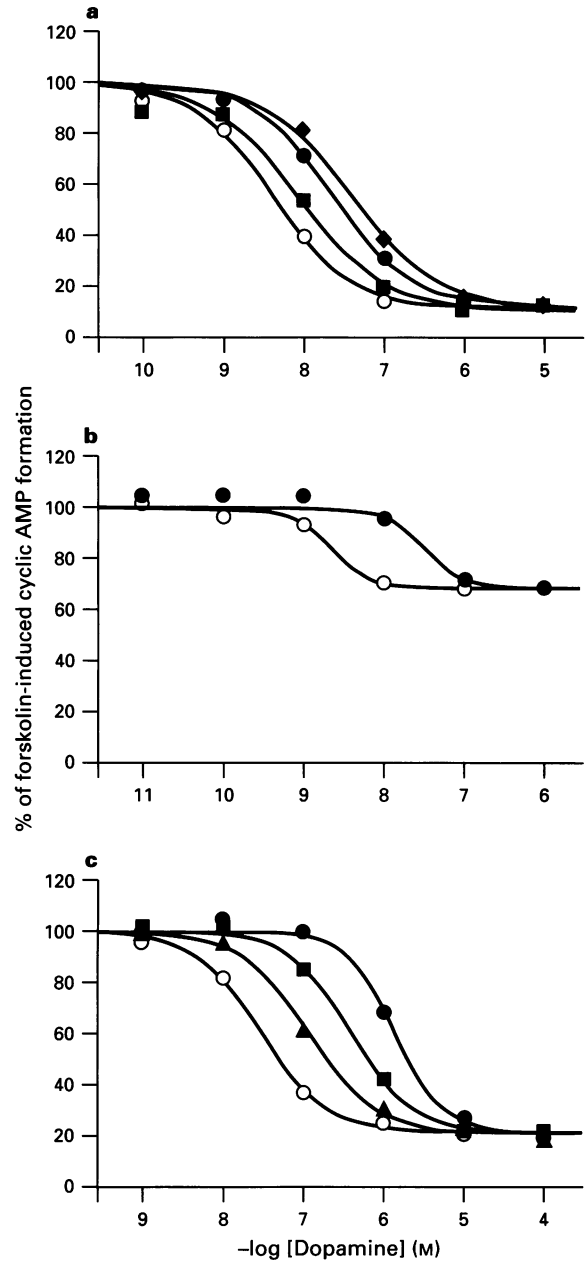


Figure 3 Dose-response curves of dopamine for the determination of antagonistic potency of YM-43611 on hD2 (a), hD3 (b) and hD4 (c) receptors. Cyclic AMP formation was determined by addition of indicated concentration of dopamine; alone (○) or together with 10 nM (▲), 30 nM (■), 100 nM (●) and 300 nM(◆) YM-43611. Each point represents the mean from triplicate determinations in four to five separate experiments. The s.e.mean was between 0.98 to 11%. The curves drawn through the data are derived from the fitting procedure using the logistic equation (see Methods). In all curves the Hill coefficients were close to unity.

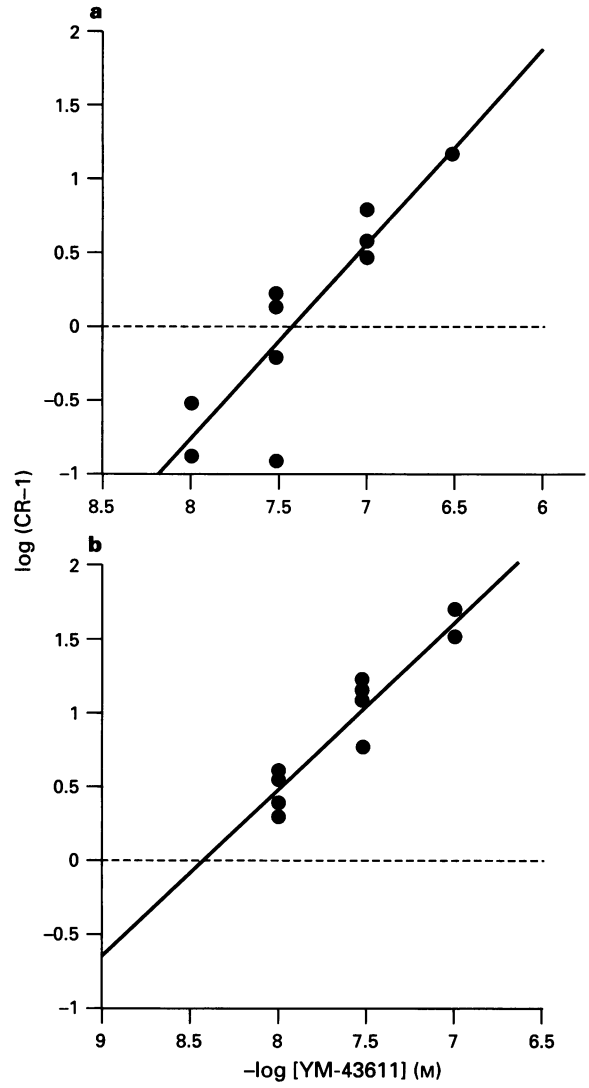


Figure 4 Schild plots for dopamine - YM-43611 antagonism on hD2 (a) and hD4 (b) receptors expressing CHO cells. CR indicates the ratio of ED_{50}s obtained in the presence and absence of YM-43611.

30.7±1.6% in the hD3 cell line and 30.0±2.8 nM and 77.9±1.1% in the hD4 cell line, respectively.

YM-43611 shifted the inhibition curve of dopamine on the human D_2-like receptor subtype-mediated cyclic AMP formation to the right in a parallel fashion (Figure 3). To assess more stringently the antagonistic properties of YM-43611 on hD2 and hD4 receptors, we performed Schild analyses using several YM-43611 concentrations (Figure 4). The pA_2 values and slopes of the Schild plots for YM-43611 were as follows: hD2 receptor, 7.42±0.08 (A_2=38.1 nM), 1.32±0.22; and hD4 receptor, 8.42±0.10 (A_2=3.77 nM), 1.12±0.12, respectively.

We could not performed Schild analysis for hD3 receptors because the maximal response to dopamine in the hD3 cell line was lower than that on either the hD2 or hD4 cell lines. YM-43611 shifted the inhibition curve of dopamine on hD3 receptor-mediated cyclic AMP formation to the right; maximal inhibition was not affected by 100 nM YM-43611 (30.8±5.8%). Thus, the pK_B value was calculated to assess the antagonistic potency of YM-43611 on hD3 receptors; a pK_B value of 8.06±0.14 (K_B=8.68 nM) was obtained.

Discussion

In the present study, we have shown in binding and functional assays that YM-43611 is a potent and selective D_3 and D_4 receptor antagonist. We previously developed a benzamide, nemonapride (YM-09151-2), as a potent and selective D_2-like receptor antagonist (Iwanami *et al.*, 1981; Terai *et al.*, 1989). In our studies on a series of benzamide derivatives, we found that varying 4-substituents on the benzamide could affect affinity and selectivity for the D_2-like receptor subtypes (data not shown). This approach led to the development of a novel benzamide derivative, YM-43611.

Receptor binding studies indicated that YM-43611 has appropriate affinity for both rat and human D_2-like receptors, but has negligible affinity for other neurotransmitter receptors.

Among D_2-like receptors, YM-43611 was more potent at both D_3 and D_4 receptors than at D_2 receptors. This is the first ligand to show both D_3 and D_4-selectivity in binding studies.

To estimate the antagonistic activity and potency of YM-43611 and other ligands, we utilized two kinds of functional assays: low-K_m GTPase assay and adenylate cyclase assay. The former is a direct assessment of D_2-like receptor-G-protein interaction (Milligan, 1988), and the latter is an assessment of D_2-like receptor-effector coupling. With regard to the low-K_m GTPase assay, this is the first quantitative functional assay comparison of the putative antagonists on the individual D_2-like receptor subtype. Not only dopamine but also apomorphine and quinpirole stimulated low-K_m GTPase activity (data not shown). The rank order of K_i values of the tested antagonists correlated with receptor binding affinities for the individual D_2-like receptor subtype. These results indicate that dopamine-stimulated low-K_m GTPase is mediated by activation of the respective D_2-like receptor subtypes. The low-K_m GTPase assays revealed that only YM-43611 had more potent antagonistic activity at both D_3 and D_4 receptors than at D_2 receptors. In the adenylate cyclase assays, YM-43611 exhibited the profile of a D_2-like receptor antagonist on the receptor-effector coupling, showing a potent antagonism with moderate D_3-selectivity and high D_4-selectivity against D_2 receptors. The potency and selectivity of YM-43611 were in reassuringly close agreement with the data from the binding and two types of functional assays. These results therefore confirm that YM-43611 is a D_2-like receptor antagonist that has moderate D_3-selectivity and high D_4-selectivity over D_2 receptors.

In studies on D_3 receptors, YM-43611 showed approximately 2–4 fold greater selectivity for D_3 over D_2 receptors in the binding and low-K_m GTPase assays. This selectivity is similar to that of (+)-UH232, which is considered to be a putative autoreceptor selective antagonist (Svensson *et al.*, 1986). Recently, several studies have suggested that D_3 receptors play, at least in part, a functional autoreceptor role; activation of D_3 receptor causes behavioural inhibition as a result of the depression of dopaminergic neurone firing (Kreiss *et al.*, 1995). Against this, Svensson *et al.* (1994) suggested that D_3 agonist-induced behavioural inhibition was related to events at the postsynaptic level. YM-43611 should therefore be a useful tool in the study of D_3 receptor-mediated functions at the pre- and/or post-synaptic levels.

In studies on D_4 receptors, YM-43611 exhibited 20–90 fold greater selectivity for D_4 than D_2 receptors in the binding assay. This D_4-selectivity is one order of magnitude greater than that of clozapine, the only currently available neuroleptic tested to exhibit D_4-selectivity. Approximately 10 fold greater D_4-selectivity was reported for clozapine in the original D_4 study (Von Tol *et al.*, 1991); however, as with a similar observation by Lahti *et al.* (1993), we found only small D_4-selectivity. We observed that the antagonists exhibited several fold greater D_2 affinity for the human than that for the rat receptors (Tables 2 and 3). Van Tol *et al.* (1991) determined the D_4-selectivity from the affinities for the rat D_2 and human D_4 receptors. The species-specific differences in the affinity for D_2 receptors, therefore, appears to be responsible for the differential degree of D_4-selectivity with clozapine. Furthermore, clozapine showed relatively little D_2-selectivity in the low-K_m GTPase assay using the human D_2-like receptors. These finding indicate that clozapine may have little D_4-selectivity on the human D_2-like receptors. Although rather lower D_4-selectivity was observed with clozapine, relative D_4-selectivity was probably of sufficient magnitude compared to that of other neuroleptics to be pharmacologically relevant. YM-43611 should be valuable for the pharmacological research on the D_4 receptor because of its greater D_4-selectivity.

In the present study, the apparent differences in the functional activities of dopamine were observed among the binding and two types of functional assays. Firstly, the potency of dopamine, which is generally in agreement with those reported by Chio *et al.* (1994a,b) in the binding and adenylate cyclase assays, differed among the three assays. In particular, dopamine exhibited an apparently higher potency in the adenylate cyclase assay than the low-K_m GTPase assay. Secondly, in the adenylate cyclase assay, the maximal response of the D_4 receptor to dopamine was greater than that of the D_3 receptor, whereas they were similar in the low-K_m GTPase assay. Although the exact reason is not clear, differential interaction of the receptor with multiple subtypes of G-protein may account for the above discrepancy. The CHO cell is known to express a variety of G-protein subtypes (Prather *et al.*, 1994). While all G-proteins posses an intrinsic GTPase activity in their α-subunit, the inhibition of adenylate cyclase activity is predominately mediated by the G_i subtype. Senogles *et al.* (1990) reported that the D_2 receptor couples with G_{i2} subtype with ~10 fold higher affinity than any other G_i subtype; this result indicates that various subtypes of G-proteins are not equivalent with regard to interaction with the dopamine-stimulated receptor. Moreover, recent studies have demonstrated that a number of independent cellular events are differentially regulated by D_2-like receptor subtypes (Tang *et al.*, 1994a,b; Seabrook *et al.*, 1994; Lajiness *et al.*, 1995). Taking into account these lines of evidence, our present results could be explained as follows: Among the G-protein subtypes, the adenylate cyclase-linked G-protein subtype might be activated by the D_2-like receptor stimulated with the lower dose of dopamine than other G-protein subtypes. Among the adenylate cyclase-linked G_i subtypes, the D_3 receptor might have a different preference in the interaction with the G_i subtype from that of the D_4 and/or D_2 receptors; the CHO cell might express a smaller amount of a suitable adenylate cyclase-linked G_i subtype which preferentially interacts with the D_3 receptor. Whether or not this interpretation is true or whether another one is required cannot be known until further study is done.

The present studies provide evidence that a novel benzamide, YM-43611, is a potent and selective D_2-like receptor antagonist. Furthermore, this compound is the only ligand to show dual selectivity of both D_3 and D_4 receptors over D_2 receptors. Further neurochemical and behavioural investigation is necessary to characterize firmly this novel, selective D_3 and D_4 receptor antagonist. This compound will prove useful in the study of D_3 and D_4 receptors and in our understanding of their involvement in psychopharmacological function.

The authors would like to thank Dr S. Nagata, Astra Arcus AB, Lundbeck and Farmitalia Carlo Erba for supplying the vector pEF-BOS, raclopride, *cis*-flupenthixol and metergoline, respectively.

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(Received July 3, 1995
Revised December 12, 1995
Accepted December 20, 1995)