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Aripiprazole's low intrinsic activities at human dopamine D2L and D2S receptors render it a unique antipsychotic

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Abstract

Aripiprazole is the first clinically approved atypical antipsychotic agent having dopamine D2 receptor partial agonist activities. To evaluate aripiprazole's agonist and antagonist properties, we established a Chinese hamster ovary cell line expressing high and low densities of the long and short isoforms of human dopamine D2 receptors, then compared its properties with 7-{3-[4-(2,3-dimethylphenyl)piperazinyl]propoxy}-2(1*H*)-quinolinone (OPC-4392), S(−)-3-(3-hydroxyphenyl)-*N*-*n*-propylpiperidine ((−)-3-PPP), and terguride (other partial agonists) using forskolin-stimulated cAMP accumulation as an index. In cells expressing high receptor densities, all partial agonists predominantly behaved as agonists. However, in cells expressing low receptor densities, the partial agonists showed significantly lower maximal effects than dopamine. Aripiprazole showed the lowest intrinsic activities. In addition, all compounds blocked the action of dopamine with a maximum effect equal to that of each compound alone. Aripiprazole's low intrinsic activities may account for the clinical finding that, unlike the other partial agonists, it is substantially active against both positive and negative symptoms of schizophrenia.

Keywords: Aripiprazole; Antipsychotic; Partial agonist; Dopamine D2 receptor; Receptor density; Schizophrenia

1. Introduction

Aripiprazole, 7-{4-[4-(2,3-dichlorophenyl)-1-piperazinyl]butoxy}-3,4-dihydro-2(1*H*)-quinolinone, is a next-generation, atypical antipsychotic found to be effective against both positive and negative symptoms of schizophrenia. It has a low propensity for extrapyramidal symptoms, causes minimal weight gain and sedation, and produces no elevation in serum prolactin levels or QT_c interval prolongation (Marder et al., 2003; Kane et al., 2002). Aripiprazole's mechanism of action differs from currently marketed typical and atypical antipsychotics, which act as dopamine D2 receptor antagonists. Previous preclinical studies provided evidence that aripiprazole is a dopamine–serotonin system stabilizer with potent partial agonist activities at dopamine D2 and 5-HT_{1A} receptors and antagonist activities at 5-HT_{2A} receptors (Lawler et al., 1999; Burris et al., 2002; Jordan et al., 2002; McQuade et al., 2002).

To date, the dopamine D2 receptor partial agonists S(−)-3-(3-hydroxyphenyl)-*N*-*n*-propylpiperidine ((−)-3-PPP), terguride, and 7-{3-[4-(2,3-dimethylphenyl)piperazinyl]propoxy}-2(1*H*)-quinolinone (OPC-4392) have been developed for schizophrenia treatment (Hjorth et al., 1983; Kehr, 1984; Yasuda et al., 1988), although use of these drugs has not substantially improved positive symptoms (Benkert et al., 1995; Lahti et al., 1998). One possible

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explanation may be that their intrinsic activities at dopamine D2 receptors are not of the appropriate magnitude to make them optimum antipsychotics. However, it is still unclear what intrinsic activities are required for good clinical effects with little or no undesirable side effects such as extrapyramidal symptoms and hyperprolactinemia.

Dopamine D2 receptors have long (D2L) and short (D2S) isoforms, which are generated by alternative splicing. Dopamine D2L receptors differ in that they have an additional 29 amino acids within the third intracellular loop (Dal-Toso et al., 1989; Picetti et al., 1997). Khan et al. (1998) and Usiello et al. (2000) proposed that dopamine D2S receptors are principally dopamine D2 presynaptic autoreceptors, and dopamine D2L receptors are mainly dopamine D2 postsynaptic receptors in vivo, although many dopamine antagonists and agonists representing various different chemical classes could not distinguish between cloned human dopamine D2L and D2S receptors (Leysen et al., 1993). Meller et al. (1987, 1991) and Enz et al. (1990) reported that receptor reserves exist at presynaptic dopamine D2 autoreceptor sites but not at postsynaptic dopamine D2 receptor sites.

In vivo studies of aripiprazole showed it to have potent agonist activities at dopamine autoreceptors (e.g., blockade of γ-butyrolactone- and reserpine-induced 3,4-dihydroxyphenylalanine (DOPA) accumulation) and antagonist activities at dopamine D2 postsynaptic receptors (e.g., blockade of apomorphine-induced stereotypy and locomotor activities) (Kikuchi et al., 1995). More recent in vitro data suggested that aripiprazole has high affinities for rat dopamine D2L and D2S receptors (Lawler et al., 1999) and is a partial agonist with low intrinsic activity at rat or human D2L receptors in stably expressing cells (Lawler et al., 1999; Burris et al., 2002). In addition, receptor density played an important role in determining the maximum efficacy of aripiprazole (Burris et al., 2002).

We proposed to clarify the functional activities of aripiprazole at human dopamine D2L (hD2L) and D2S (hD2S) receptors and to compare aripiprazole's receptor-density-dependent dual dopamine D2 receptor agonist and antagonist properties to those of OPC-4392, (−)-3-PPP, and terguride. To do so, we first constructed four clonal CHO cell lines expressing low densities of dopamine hD2L and hD2S receptors (CHO-hD2L-Low and CHO-hD2S-Low) and high densities of dopamine hD2L and hD2S receptors (CHO-hD2L-High and CHO-hD2S-High).

2. Materials and methods

2.1. Materials

Aripiprazole, OPC-4392, olanzapine, and ziprasidone were synthesized at Otsuka Pharmaceutical. Dopamine, (−)-3-PPP, terguride, haloperidol, risperidone, clozapine, (+)-butaclamol, 3-isobutyl-1-methylxanthine (IBMX), Iscove's modified Dulbecco's medium (IMDM), Ham's F-12 medium, HEPES, and L-glutamine were purchased from Sigma (St. Louis, MO, USA). We obtained [^{3}H]raclopride (71.3 Ci/mmol) from NEN Life Science Products (Boston, MA, USA). Forskolin and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries (Osaka, Japan). G418 was purchased from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum was obtained from JRH BioSciences (Lenexa, KS, USA). Sodium hypoxanthine, thymidine, penicillin, and streptomycin were purchased from Gibco BRL (Gaithersburg, MD, USA). GF/B unifilter plates and Microscint-O were purchased from Packard (Meriden, CT, USA). All other materials used were of the highest purity commercially available.

2.2. Construction of recombinant expression vectors

Human dopamine D2L and D2S receptor cDNA sequences were amplified using the polymerase chain reaction (PCR) using a human pituitary cDNA library (Clontech, Palo Alto, CA, USA) and two primers. Primers included the 5′ primer, 5′-ACCGGAATTCGCCACCATGGATCCACTGAATCTGTC-3′, and 3′ primer, 5′-ACGGTCTAGAGTCAGCAGTGAAGGAT-3′ (Amersham Pharmacia Biotech, Tokyo, Japan). The 5′ primer contains an *Eco*RI restriction enzyme site, a predicted Kozak sequence (Kozak, 1987), and start codon (Dal-Toso et al., 1989), and the 3′ primer contains an XbaI restriction site and stop codon (Dal-Toso et al., 1989). Reactions were started at 94 °C and performed for 40 cycles (94 °C, 30 s; 54 °C, 1 min; 72 °C, 2 min) in a thermocycler. An approximately 1300-base-pair fragment was digested with *Eco*RI and XbaI and ligated into a mammalian expression vector with the human elongation factor 1α subunit promoter (pEF1) (Invitrogen, Carlsbad, CA, USA). This expression construct was transformed into *E. coli* strain DH5α (Toyobo, Osaka, Japan), and two clones were sequenced and confirmed as encoding human dopamine D2L or D2S receptors.

2.3. Cell culture and stable expression

Chinese hamster ovary cells deficient in the enzyme dihydrofolate reductase (CHO dhfr$^-$; Dainippon Pharmaceutical, Osaka, Japan) were maintained in IMDM supplemented with 10% (v/v) fetal bovine serum, L-glutamine (4 mM), sodium hypoxanthine (0.1 mM), thymidine (16 μM), penicillin (50 units/ml), and streptomycin (50 μg/ml). Chinese hamster ovary (CHO-K1) cells (American Type Culture Collection, Rockville, MD, USA) were maintained in Ham's F-12 medium supplemented with 10% (v/v) fetal bovine serum, L-glutamine (1 mM), penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were grown in a humidified atmosphere of 5% CO_2 and air at 37 °C.

The recombinant expression construct for dopamine hD2L receptors was transfected into CHO dhfr$^-$ cells with TransFast (Promega, Madison, WI, USA). Similarly, the expression construct for dopamine hD2S receptors was transfected into CHO-K1 cells. Single colonies resistant to the antibiotic G418 (400 μg/ml) were isolated and maintained in a culture medium with G418 (200 μg/ml). Clones expressing receptors at high or low levels were selected (CHO-hD2L-High, CHO-hD2L-Low, CHO-hD2S-High, and CHO-hD2S-Low).

2.4. Membrane preparation and ligand binding assays

Cells were grown to confluence in 150-cm^2 tissue culture flasks, then collected by scraping in 50 mM Tris–HCl buffer,

pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ (buffer A). The suspension was homogenized and centrifuged (30 min at 48,000 × *g* at 4 °C), with the resulting pellets resuspended in buffer A and stored at −80 °C until use. The Bradford (1976) protein assay (Bio-Rad, Hercules, CA, USA) was used to measure the protein concentration in membrane preparations (bovine serum albumin was the calibration standard). For each binding assay experiment, cell membranes were thawed and suspended. For saturation analyses, 3 to 47 μg membrane protein was added to 96-well assay plates containing [^{3}H]raclopride in buffer A at a final volume of 200 μl. The plates were incubated at room temperature for 60 min, rapidly filtered through GF/B Unifilter plates, and washed three times with cold 50 mM Tris–HCl buffer, pH 7.4, using a Packard Filtermate Harvester. Filter-bound radioactivity was counted using a Packard TopCount scintillation counter with 40 μl/well of Microscint-O. Non-specific binding was defined in the presence of 1 μM (+)-butaclamol. Saturation experiments were performed using six concentrations of the radioligand, ranging from approximately 0.1 to 10 nM. Competition experiments were performed using 1 nM radioligand and eight concentrations of cold competitor drugs. Assays were performed in duplicate, and replicated at least three times for determination.

2.5. Measurement of cAMP accumulation

To measure cAMP accumulation, 10,000 to 40,000 cells per well were seeded in 24-well plates and grown for 2 to 3 days, washed twice with 350 μl of a serum-free culture medium containing 25 mM HEPES (HEPES-Medium), and preincubated for 40 min at 37 °C in 350 μl of HEPES-Medium containing 1 mM IBMX. They were further incubated for 20 min at 37 °C after adding 150 μl HEPES-Medium supplemented with appropriate concentrations of dopamine and/or partial agonists, 1 mM IBMX, and 33 μM forskolin. The assay mix also contained a 1% DMSO final concentration. The medium was then removed to terminate the reactions, and the intracellular cAMP in each well was determined using the Biotrak cAMP enzyme immunoassay system (Amersham Pharmacia Biotech, Buckinghamshire, UK) following the manufacturer's procedure. Assays were performed in triplicate, and replicated two or three times for determination.

2.6. Data analysis

Radioligand binding saturation and competition curves were calculated using nonlinear regression analysis to fit with one-site binding or one-site competition models using the GraphPad Prism program (San Diego, CA, USA). From this fit, B_{max}, K_d, pIC_{50}, and K_i values were derived. Cyclic AMP data were expressed as a percentage of forskolin-stimulated cAMP accumulation. Concentration–response curves were calculated using nonlinear regression analysis to fit with a sigmoid using the GraphPad Prism program, then pEC_{50} and pIC_{50} values were derived from this fit. The maximal effect (E_{max}) of each compound was calculated as the percentage inhibition of the forskolin effect. To determine relative efficacy values, each agonist E_{max} value was expressed as a percentage of dopamine's E_{max} value. Apparent dissociation constant (K_A) values were derived from measurements of agonist-induced inhibition of cAMP. This was calculated from a double-reciprocal plot of equieffective concentrations of each compound determined in cells with high receptor density ($1/[A]$) or in cells with a lower density of receptor expression ($1/[A']$), according to the method of Furchgott and Bursztyn (1967). From the linear regression of this relationship, the K_A value was determined using the equation $K_A = (\text{slope} - 1)/y\text{-intercept}$. The fractional receptor occupancy, at varying agonist concentrations, was then calculated using the equation, % receptor occupancy $= ([A]/([A]+K_A)) \times 100$, where $[A]$ = fixed agonist concentration and K_A = apparent dissociation constant for the agonist. The receptor occupancy was then plotted against the fractional response in order to determine the degree of receptor reserve (Burris et al., 2002).

3. Results

3.1. Radioligand binding

The dopamine hD2L and hD2S receptors were characterized using [^{3}H]raclopride. No binding was detected in membranes from wild-type CHO dhfr$^-$ and CHO-K1 cells (data not shown). In contrast, when CHO dhfr$^-$ cells were transfected with the expression vector for dopamine hD2L receptors, clones that expressed specific binding sites for [^{3}H]raclopride were identified. Among the nine clones evaluated, two that expressed high and low densities of [^{3}H]raclopride binding sites (CHO-hD2L-High: B_{max}, 11 ± 2 pmol/mg of protein, K_d, 1.3 ± 0.1 nM, n = 3, and CHO-hD2L-Low: B_{max}, 0.36 ± 0.13 pmol/mg of protein, K_d, 2.4 ± 0.1 nM, n = 3, respectively) were used for further studies. Likewise, CHO-K1 cells were transfected with the expression vector for dopamine hD2S receptors, and clones expressing specific binding sites for [^{3}H]raclopride were identified. From eight clones evaluated, two clones (CHO-hD2S-High: B_{max}, 18 ± 1 pmol/mg of protein, K_d, 1.2 ± 0.3 nM, n=5, CHO-hD2S-Low: B_{max}, 0.96 ± 0.09 pmol/mg of protein, K_d, 1.9 ± 0.8 nM, n = 3, respectively) were used for further studies. All clones had similar affinities for the radioligand.

Competition binding experiments were performed using aripiprazole, OPC-4392, (−)-3-PPP, and terguride (the four dopamine D2 receptor partial agonists) plus the five antipsychotic agents (haloperidol, risperidone, ziprasidone, olanzapine, and clozapine). K_i values, derived from competition binding experiments with [^{3}H]raclopride, are shown in Table 1. For each of the agents, the binding affinity for dopamine hD2L receptors was similar to that for hD2S receptors. The K_i values of aripiprazole were approximately 3-fold higher than those of OPC-4392, approximately 280 to 320 times higher than those of (−)-3-PPP, and approximately 1.4 to 1.6 times lower than those of terguride. Aripiprazole's affinity values were comparable to those of risperidone or ziprasidone. The rank order of potency was haloperidol > ziprasidone, risperidone, aripiprazole > olanzapine > clozapine.

3.2. Dopamine hD2L and hD2S receptor-mediated inhibition of cAMP

In the CHO cell line, transfected dopamine D2L and D2S receptors are associated with the inhibition of cAMP accumulation. To evaluate the agonist or antagonist actions of aripiprazole, terguride, OPC-4392, and (−)-3-PPP, we measured the ability of the agonists to either inhibit forskolin-stimulated cAMP accumulation or block the inhibition produced by dopamine. Accumulation

of cAMP stimulated by 10 μM forskolin ranged between 46 pmol/well and 86 pmol/well in CHO-hD2L-Low cells, 45 pmol/well and 89 pmol/well in CHO-hD2L-High cells, 63 pmol/well and 109 pmol/well in CHO-hD2S-Low cells, and 82 pmol/well and 107 pmol/well in CHO-hD2S-High cells. Basal cAMP levels never exceeded 6% of the forskolin-stimulated levels. Wild-type CHO dhfr$^-$ and CHO-K1 cells showed no response when dopamine, aripiprazole, (−)-3-PPP, OPC-4392, or terguride (10 μM) were added (data not shown).

The concentration–response curves for dopamine, aripiprazole, OPC-4392, (−)-3-PPP, and terguride in both CHO-hD2L-High and CHO-hD2L-Low cells are shown in Fig. 1A and B. The E_{max} and EC_{50} values for agonism at dopamine hD2L receptors are summarized in Table 2. In CHO-hD2L-High cells, all test drugs caused dose-dependent inhibition of forskolin-stimulated cAMP accumulation. Aripiprazole's maximal agonist effect was 76% that of dopamine (E_{max} 97% inhibition), lower than observed for OPC-4392, (−)-3-PPP, and terguride (95%, 93%, and 94% effect relative to dopamine, respectively). The rank order of potency was terguride>dopamine and OPC-4392>aripiprazole>(−)-3-PPP. In CHO-hD2L-Low cells, dopamine also inhibited forskolin-stimulated cAMP accumulation in a dose-dependent manner (E_{max} 66% inhibition, EC_{50} 50 nM). Under the same conditions, aripiprazole, OPC-4392, (−)-3-PPP, or terguride produced no apparent agonist effect. In CHO-hD2L-Low cells, the potency of dopamine was about 24-fold lower than in CHO-hD2L-High cells (Table 2). The reciprocal values of equiactive doses of dopamine in CHO-hD2L-High and CHO-hD2L-Low cells were calculated and yielded a K_A value of 227 nM for dopamine at human dopamine D2L receptors (Fig. 2A). The K_A value was used to compare the percentage inhibition of forskolin-stimulated cAMP accumulation by dopamine as a function of receptor occupancy. In CHO-hD2L-High cells, a steep hyperbolic occupancy–effect relationship was seen for dopamine (Fig. 3A). Inhibition of cAMP accumulation by 90% and 50% was achieved with occupancy by dopamine of 11% and 0.9% of the receptors, respectively (Fig. 3A). In CHO-hD2L-Low cells, inhibition of cAMP accumulation by 50% was achieved with receptor occupancy of 39% by dopamine (Fig. 3A).

Table 1
Affinities of aripiprazole and various dopamine D2 receptor partial agonists and antipsychotic agents for human dopamine D2L and D2S receptors using the radiolabeled antagonist [^{3}H]raclopride

Drug	CHO-hD2L-High	CHO-hD2S-High
	K_i (nM)	K_i (nM)
Agonist		
Terguride	0.86±0.30	0.76±0.19
Aripiprazole	1.2±0.2	1.2±0.3
OPC-4392	3.5±1.0	3.5±0.9
(−)-3-PPP	339±13	388±62
Antagonist		
Haloperidol	0.43±0.06	0.51±0.06
Risperidone	1.0±0.1	1.3±0.1
Ziprasidone	1.0±0.3	1.1±0.3
Olanzapine	8.3±2.2	7.1±2.1
Clozapine	75±5	73±6

The competition studies were performed with cell membrane preparations and [^{3}H]raclopride as radioligand. K_i are inhibition constants for a one-site model. All values are means±S.E.M. of three experiments carried out in duplicate.

The concentration–response curves for dopamine, aripiprazole, OPC-4392, (−)-3-PPP, and terguride in both CHO-hD2S-High and CHO-hD2S-Low cells are shown in Fig. 1C and D. The E_{max} and EC_{50} values for agonism at dopamine hD2S receptors are summarized in Table 2. In CHO-hD2S-High cells, all test drugs caused dose-dependent inhibition of forskolin-stimulated cAMP accumulation. The maximal agonist effect relative to dopamine (E_{max} 96% inhibition) was similar for all four partial agonists (97–101%). The rank order of potency was terguride>OPC-4392>dopamine>aripiprazole>(−)-3-PPP. In CHO-hD2S-Low cells, all test drugs also inhibited forskolin-stimulated cAMP accumulation in a dose-dependent manner. OPC-4392, (−)-3-PPP, and terguride produced maximal agonist effects of 43%, 44%, and 42% that of dopamine (E_{max} 87% inhibition), respectively, whereas aripiprazole's effect (20% relative to dopamine) was lower than the other partial agonists. The rank order of potency was terguride>aripiprazole>OPC-4392>dopamine>(−)-3-PPP. In CHO-hD2S-Low cells, the potencies of dopamine, aripiprazole, OPC-4392, (−)-3-PPP, and terguride were about 19-, 0.8-, 12-, 12-, and 3-fold lower than in CHO-hD2S-High cells, respectively (Table 2). The reciprocal values of equiactive doses of agonists in CHO-hD2S-High and CHO-hD2S-Low cells were calculated and yielded K_A values of 417 nM for dopamine, 7.4 nM for aripiprazole, 33 nM for OPC-4392, 228 nM for (−)-3-PPP, and 0.51 nM for terguride at human dopamine D2S receptors, respectively (Fig. 2B, C, D, E, and F). The K_A values for agonists were used to compare the percentage inhibition of forskolin-stimulated cAMP accumulation by the agonists as a function of occupancy of receptors. In CHO-hD2S-High cells, steep hyperbolic occupancy–effect relationships were seen for dopamine and other partial agonists (except aripiprazole; Fig. 3B). Inhibition of cAMP accumulation by 90% and 50% was achieved with occupancy by dopamine of 6% and 0.7%, aripiprazole of 97% and 54%, OPC-4392 of 40% and 4%, (−)-3-PPP of 43% and 6%, and terguride of 84% and 30%, of the receptors, respectively (Fig. 3B). In CHO-hD2S-Low cells, inhibition of cAMP accumulation by 50% was achieved with receptor occupancy of 13% by dopamine (Fig. 3C).

Next, we examined the functional inhibition of the dopamine-induced decrease in cAMP accumulation by aripiprazole, OPC-4392, (−)-3-PPP, and terguride. In both CHO-hD2L-Low and CHO-hD2S-Low cells treated with 1 μM dopamine, the partial agonists reversed the inhibition of forskolin-stimulated cAMP accumulation in a concentration-dependent manner to levels that equaled those of the partial agonists alone (Fig. 4A and B). Since aripiprazole's maximal agonist effect was less than that of the other partial agonists, it in turn exerted the largest antagonist effect on dopamine-induced inhibition. The IC_{50} values for antagonism are shown in Table 3. Aripiprazole and the other partial agonists blocked dopamine-induced inhibition with a similar potency between cell lines. Aripiprazole was 3 to 4 times more potent than OPC-4392, 78 to 130 times more potent than (−)-3-PPP, and 8 to 9 times less potent than terguride. In CHO-hD2L-High cells, aripiprazole blocked the agonist effect of dopamine (100 nM) to the level seen by aripiprazole alone (data not shown). Since aripiprazole's agonist effect was markedly higher than that seen in CHO-hD2L-Low

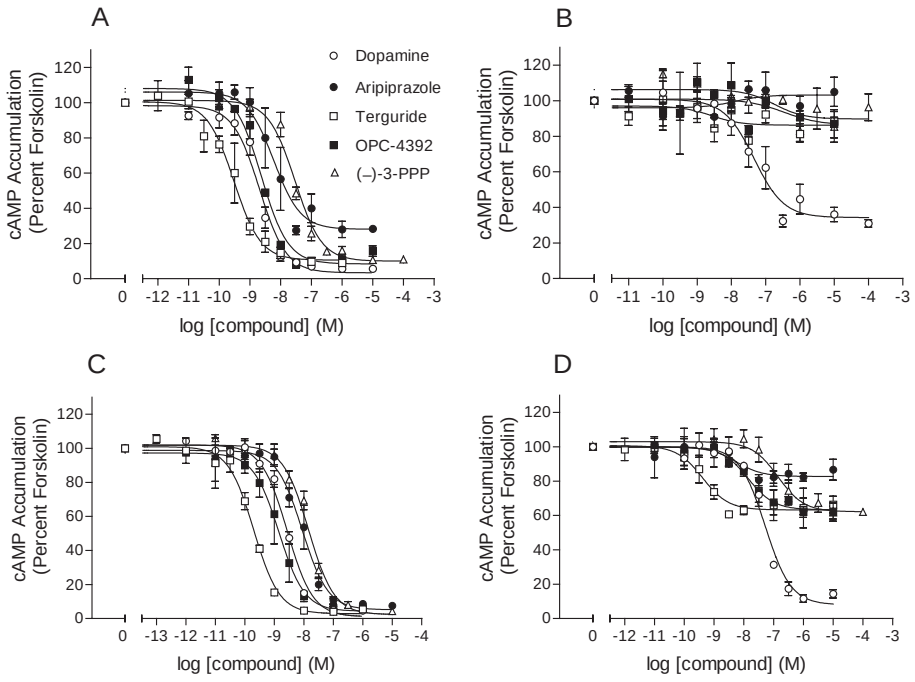

Fig. 1. Concentration–response curves of dopamine, aripiprazole, terguride, OPC-4392, and (−)-3-PPP for inhibiting forskolin-stimulated cAMP accumulation in cells: (A) CHO-hD2L-High, (B) CHO-hD2L-Low, (C) CHO-hD2S-High, and (D) CHO-hD2S-Low. Cyclic AMP accumulation was normalized to the percentage of forskolin-stimulated cAMP accumulation (set at 100%). Values are means of two or three experiments performed in triplicate; vertical bars show S.E.M. ($n=3$) or range ($n=2$).

cells, it exerted a much smaller antagonist effect. In CHO-hD2S-High cells, all partial agonists behaved like full agonists so none blocked the agonist effect of dopamine (100 nM; data not shown). In both CHO-hD2L-High and CHO-hD2S-High cells, the agonist effects of dopamine (100 nM) and aripiprazole (100 nM) were completely blocked by the dopamine D2 receptor antagonist raclopride at 10 μM (data not shown).

4. Discussion

We characterized a series of partial agonists that inhibit adenylyl cyclase activation via dopamine hD2L and hD2S receptors, which were expressed at low or high levels in CHO cells. Overall, our data indicated that aripiprazole

Table 2
Evaluation of the potency and efficacy of dopamine D2 receptor agonist and partial agonists in CHO-hD2L-High, CHO-hD2L-Low, CHO-hD2S-High, and CHO-hD2S-Low cells

Drug		CHO-hD2L-High	CHO-hD2L-Low	CHO-hD2S-High	CHO-hD2S-Low
Dopamine	EC_{50} (nM)	2.1±0.5	50±19	2.7±0.2	50±2
	E_{max} (% inhibition)	97±1	66±4	96±1	87±2
	Relative efficacy	100	100	100	100
Aripiprazole	EC_{50} (nM)	6.3±3.9	NC	9.9±3.2	8.2±4.7
	E_{max} (% inhibition)	73±4	NC	93±1	17±4
	Relative efficacy	76	NC	97	20
OPC-4392	EC_{50} (nM)	2.3±0.1	NC	1.9±0.8	22±10
	E_{max} (% inhibition)	92±0	NC	95±1	38±3
	Relative efficacy	95	NC	99	43
(−)-3-PPP	EC_{50} (nM)	32±6	NC	15±2	178±71
	E_{max} (% inhibition)	90±1	NC	96±2	38±2
	Relative efficacy	93	NC	100	44
Terguride	EC_{50} (nM)	0.34±0.12	NC	0.21±0.01	0.56±0.25
	E_{max} (% inhibition)	90±2	NC	96±1	37±5
	Relative efficacy	94	NC	101	42

The agonist activities of the test compounds were evaluated by inhibiting forskolin-stimulated cAMP accumulation. Concentrations yielding half-maximal effects (EC_{50}) and maximal effects (E_{max}), which were calculated as % inhibition of forskolin activity, were determined from concentration–response curves. The relative efficacy was calculated in relation to the maximal dopamine effect. All values are means±S.E.M. of three experiments performed in triplicate. NC, not calculable.

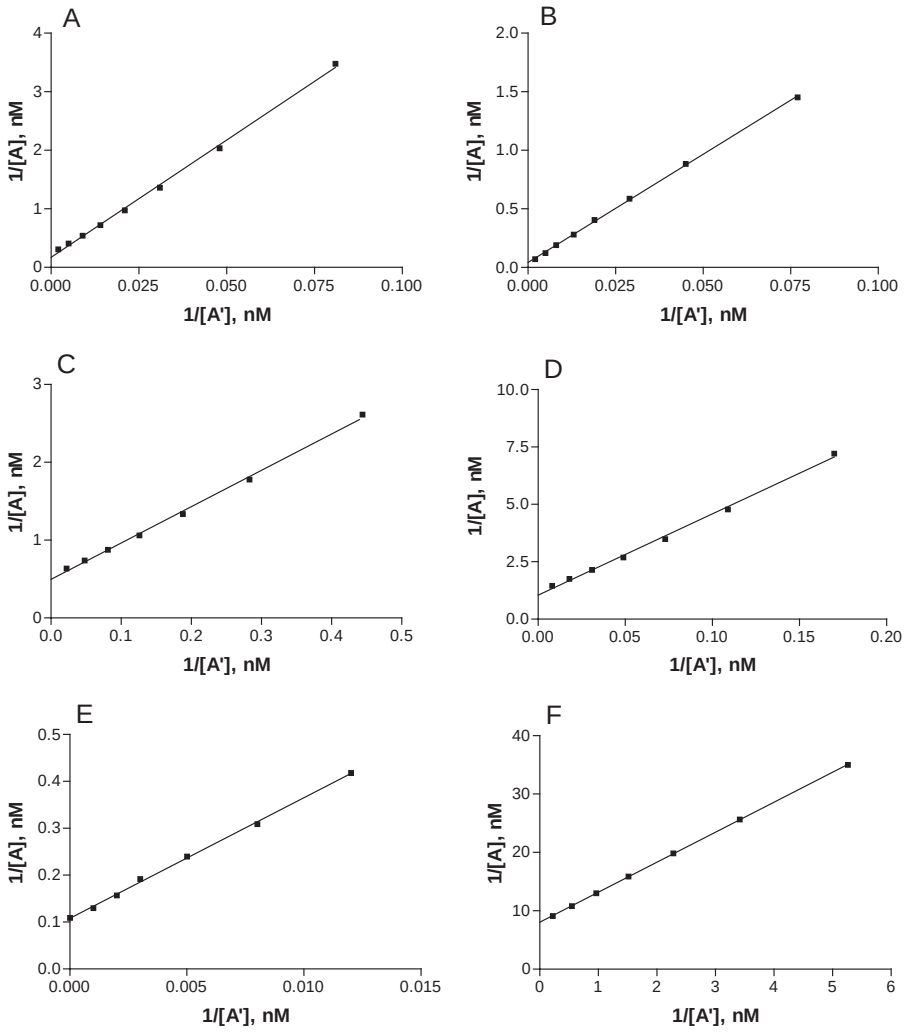

Fig. 2. Double-reciprocal plots of equieffective concentrations of dopamine (A and B), aripiprazole (C), OPC-4392 (D), (−)-3-PPP (E), and terguride (F) for inhibiting forskolin-stimulated cAMP accumulation in cells: (A) CHO-hD2L-High ($1/[A]$) and CHO-hD2L-Low ($1/[A']$), (B, C, D, E, and F) CHO-hD2S-High ($1/[A]$) and CHO-hD2S-Low ($1/[A']$). The apparent K_A values for drugs were determined from a plot of the reciprocals equieffective concentrations of drugs from cells expressing high ($1/[A]$) and low ($1/[A']$) densities of human dopamine D2 receptors using the equation: K_A=(slope − 1)/y-intercept. (A) The K_A value for dopamine at human dopamine D2L receptors was 227 nM. (B) The K_A value for dopamine at human dopamine D2S receptors was 417 nM. (C) The K_A value for aripiprazole at human dopamine D2S receptors was 7.4 nM. (D) The K_A value for OPC-4392 at human dopamine D2S receptors was 33 nM. (E) The K_A value for (−)-3-PPP at human dopamine D2S receptors was 228 nM. (F) The K_A value for terguride at human dopamine D2S receptors was 0.51 nM.

was a partial agonist at dopamine hD2L and hD2S receptors, and its intrinsic activities were lower than those of OPC-4392, (−)-3-PPP, and terguride. Their affinities, along with agonist and antagonist activities, were similar in cells expressing dopamine hD2L or hD2S receptors. By comparing the maximal agonist responses to the partial agonists in the cells expressing high and low levels, we demonstrated that the relative efficacy was dependent on receptor expression levels. In other words, the partial agonists exerted full agonist activity in cells with high receptor density, while showing lower efficacy in cells with a lower density of receptor expression. All partial agonists displayed higher relative efficacies at the cell lines expressing dopamine hD2S receptors compared to corresponding hD2L receptor cell lines (CHO-hD2S-High vs. CHO-hD2L-High and CHO-hD2S-Low vs. CHO-hD2L-Low). This may be because each of the dopamine hD2S receptor-expressing cell lines had higher densities of receptors (B_{max} (pmol/mg of protein), 18 vs. 11, and 0.96 vs. 0.36). This observation is consistent with the dependence of the relative efficacy on levels of receptor expression.

Burris et al. (2002) reported that in CHO cells expressing dopamine hD2L receptors, aripiprazole's relative efficacy to that of dopamine was reduced from nearly 90% to 25% in correlation with concentration-dependent inactivation of dopamine D2 receptors by an alkylating agent, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). The maximum effects of (−)-3-PPP and terguride were progressively reduced in cells exposed to increasing concentrations of

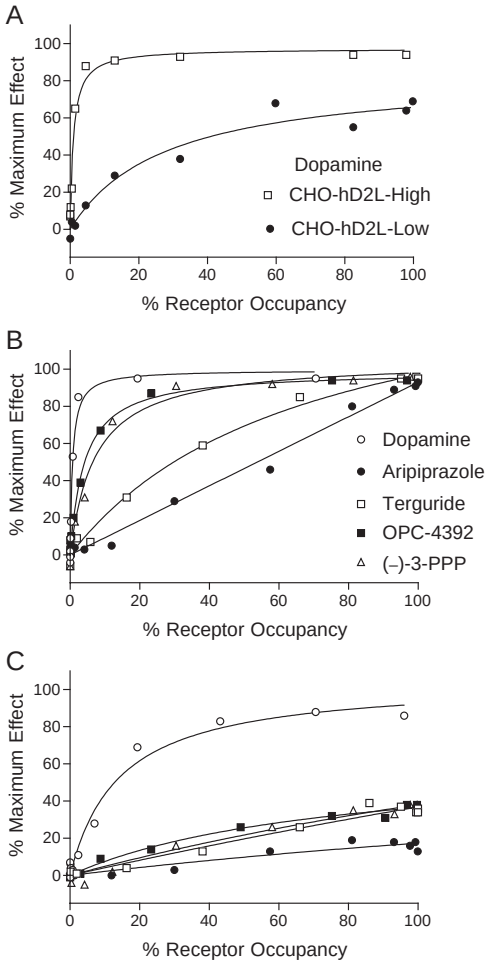

Fig. 3. Comparison of the percent inhibition of forskolin-stimulated cAMP accumulation of dopamine (A, B and C), aripiprazole (B and C), OPC-4392 (B and C), (−)-3-PPP (B and C), and terguride (B and C) as a function of receptor occupancy in cells: (A) CHO-hD2L-High and CHO-hD2L-Low, (B) CHO-hD2S-High, and (C) CHO-hD2S-Low. The percentage of receptor occupancy was determined for each concentration of drug used in Fig. 1 using the equation: % receptor occupancy = $([A]/([A]+K_A)) \times 100$, where $[A]$ = fixed agonist concentration and K_A = apparent dissociation constant for the agonist from Fig. 2. The percent inhibition of forskolin-stimulated cAMP accumulation was determined for each mean value of cAMP accumulation (% forskolin) used in Fig. 1 using the equation: % inhibition = 100 − cAMP accumulation. (A) In CHO-hD2L-High cells, inhibition of cAMP accumulation by 90% and 50% was achieved with occupancy by dopamine of 11% and 0.9% of the receptors, respectively. In CHO-hD2L-Low cells, inhibition of cAMP accumulation by 50% was achieved with receptor occupancy of 39% by dopamine. (B) In CHO-hD2S-High cells, inhibition of cAMP accumulation by 90% and 50% was achieved with occupancy by dopamine of 6% and 0.7%, aripiprazole of 97% and 54%, OPC-4392 of 40% and 4%, (−)-3-PPP of 43% and 6%, and terguride of 84% and 30%, of the receptors, respectively. (C) In CHO-hD2S-Low cells, inhibition of cAMP accumulation by 50% was achieved with receptor occupancy of 13% by dopamine.

EEDQ (Burris et al., 2002). This report is consistent with our results showing the density-dependent efficacy of the partial agonists. Lawler et al. (1999) reported that in C-6 cells expressing rat dopamine D2L receptors, the response to dopamine was 90%, with aripiprazole and OPC-4392

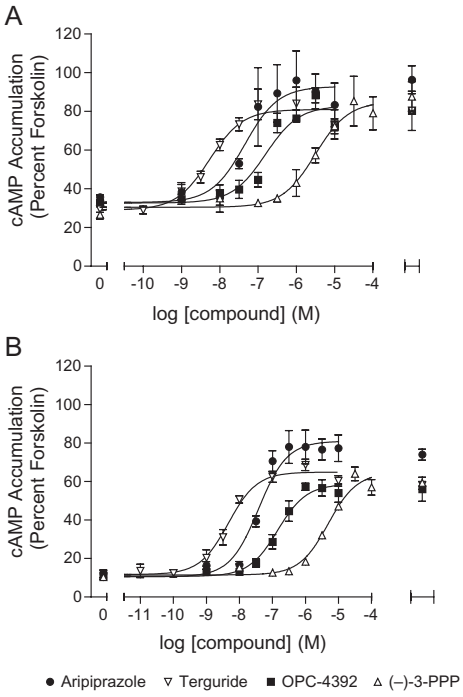

Fig. 4. Reversal of dopamine (1 μM) inhibition of forskolin-stimulated cAMP accumulation in (A) CHO-hD2L-Low and (B) CHO-hD2S-Low cells by aripiprazole, terguride, OPC-4392, and (−)-3-PPP. The isolated points on the right side of the figure represent cAMP accumulation from exposure to 10 μM aripiprazole, 10 μM terguride, 10 μM OPC-4392, or 100 μM (−)-3-PPP in the absence of dopamine. Cyclic AMP accumulation was normalized to the percentage of forskolin-stimulated cAMP accumulation (100%). Values are means of two or three experiments performed in triplicate; vertical bars show S.E.M. ($n = 3$) or range ($n = 2$).

reduced to 30% and 50%, respectively. This report is consistent with the pattern of maximal effects seen in our study.

Khan et al. (1998) and Usiello et al. (2000) proposed that dopamine D2S receptors served presynaptic autoreceptor functions and that dopamine D2L receptors acted mainly at postsynaptic sites in vivo. This is supported by recent studies involving dopamine D2 receptor knockout mice

Table 3
Evaluation of the antagonist potency of dopamine D2 receptor partial agonists' ability to reverse dopamine (1 μM) inhibition of forskolin-stimulated cAMP accumulation in CHO-hD2L-Low and CHO-hD2S-Low cells

Drug	CHO-hD2L-Low	CHO-hD2S-Low
	IC_{50} (nM)	IC_{50} (nM)
Aripiprazole	52 ± 8	39 ± 5
Terguride	6.2 ± 2.5	4.3 ± 1.2
OPC-4392	170 ± 39	155 ± 39
(−)-3-PPP	4040 ± 2090	5000 ± 780

Antagonist potency was evaluated using a concentration-dependent reversal of dopamine (1 μM) inhibition of forskolin-stimulated cAMP accumulation. The concentrations that gave half-maximal reversals (IC_{50}) were determined from concentration–response curves. All values are means ± S.E.M. of three experiments performed in triplicate.

lacking either D2L receptors or both D2L and D2S receptors (Centonze et al., 2002; Lindgren et al., 2003). We concluded that all antipsychotic agents and partial agonists displayed similar affinities for both dopamine hD2L and hD2S receptors, and the partial agonists did not display a profile of agonist selectivity for dopamine hD2S vs. hD2L receptors.

According to a classical receptor theory, responses are directly proportional to the number of receptors occupied by drugs until such responses reach the maximal cell responses (Kenakin, 1997). Therefore, the maximal cell responses that have higher receptor densities, which possess receptor reserves, are greater than for cells having lower receptor densities, which lack receptor reserves. The fractions of receptors occupied by drugs depend on drug concentrations and the equilibrium dissociation constants of drug–receptor complexes, but they have no connection to receptor densities (Kenakin, 1997). Therefore, if drug concentrations are the same, cells that have higher receptor densities must possess more receptors occupied by drugs than cells that have lower receptor densities because receptor occupancy rates in both cells are the same. Furthermore, cells having higher receptor densities require lower drug concentrations for half-maximal responses than cells having lower receptor densities (Kenakin, 1997). We determined that the maximal effect and potency of dopamine in CHO-hD2L-High and CHO-hD2S-High cells were higher than those in CHO-hD2L-Low and CHO-hD2S-Low cells (Table 2). Occupancy–response curves for dopamine indicate the presence of receptor reserves in CHO-hD2L-High and CHO-hD2S-High cells and a lack of receptor reserves in CHO-hD2L-Low and CHO-hD2S-Low cells (Fig. 3A, B and C).

It has been reported that partial agonists produced agonist activities at presynaptic dopamine D2 autoreceptors and antagonist activities at postsynaptic dopamine D2 receptors (Hjorth et al., 1983; Kehr, 1984; Yasuda et al., 1988; Kikuchi et al., 1995). Meller et al. (1987, 1991) and Enz et al. (1990) reported that receptor reserves exist at presynaptic dopamine D2 autoreceptor sites but not at postsynaptic dopamine D2 receptor sites. Meller et al. (1987) hypothesized that high sensitivities of agonist responses at presynaptic dopamine D2 autoreceptors exist because large receptor reserves are present and, therefore, partial agonists exert agonist responses. Consequently, agonist responses at postsynaptic dopamine D2 receptors show low sensitivities because receptor reserves are lacking, so partial agonists exerted no or low agonist responses. We demonstrated that, in cells with high receptor densities (possessing putative receptor reserves), partial agonists were efficacious agonists, while in cells with low receptor densities (lacking receptor reserves) the relative efficacy of partial agonists was not apparent or low, and they acted as antagonists. These results and the hypothesis of Meller et al. (1987) may explain why the partial agonists acted as agonists at presynaptic dopamine D2 autoreceptors and acted as antagonists at postsynaptic dopamine D2 receptors. Further research is, however, needed to determine the applicability of our findings in cell lines to the situation in pre- and postsynaptic neurons, as the absolute levels of dopamine D2 receptors in vivo, and their relationship to the levels observed in cell lines, are still unclear. The molecular mechanisms underlying differences in sensitivity at pre- and postsynaptic dopamine D2 receptors are not known. Whether differences in receptor density, distinct signaling pathways, or receptor–effector coupling underlie presynaptic dopaminergic receptor reserve remains to be determined.

The maximal effects and potency of OPC-4392, (−)-3-PPP, and terguride in cells with higher receptor densities were greater than those seen in cells with lower receptor densities (Table 2). Occupancy–response curves for these partial agonists indicate the presence of receptor reserves in CHO-hD2S-High cells (Fig. 3B). These data predict that receptor reserves are present with these drugs under high receptor density conditions. Although a receptor density-dependent change in the maximal effect was seen with aripiprazole, the potency of aripiprazole did not increase (Table 2). The occupancy–response curve for aripiprazole indicates a lack of receptor reserve in CHO-hD2S-High cells (Fig. 3B). These data predict a lack of receptor reserves with aripiprazole in high receptor density conditions. Hence, the difference between aripiprazole and the other partial agonists depends on the difference in their intrinsic activities. For OPC-4392, (−)-3-PPP, and terguride, which had similar intrinsic activities, the order of potency was the same as that of the binding affinity for receptors under high and low receptor density conditions. In high receptor density conditions, however, despite aripiprazole having a higher binding affinity than OPC-4392, its potency was lower than that of OPC-4392. Aripiprazole's lower intrinsic activity is relevant to these results. Receptor occupancy theory explains such phenomena; very weak partial agonists display a decrease in maximum response without a shift in potency as the receptor expression is decreased (Kenakin, 1997). In cells with low receptor densities, all the partial agonists antagonized dopamine's effect to the level produced by each test drug alone, and the order of antagonist activity was similar to that of its affinity. Because aripiprazole's intrinsic activity was lower than the other partial agonists, its antagonist activity was higher than theirs.

In clinical studies, administration of the other partial agonists did not result in a substantial improvement of positive symptoms of schizophrenia (Benkert et al., 1995; Lahti et al., 1998). These results may support the hypothesis that the intrinsic activities of the other partial agonists (except aripiprazole) are higher than the optimal level needed to generate any substantial antagonist effect at postsynaptic dopamine D2 receptors in schizophrenia patients, while aripiprazole's lower intrinsic activity makes it a unique antipsychotic. The blockade of dopamine D2 receptors is an essential minimal requirement for clinical antipsychotic

action in those patients who respond to neuroleptics (Seeman and Tallerico, 1998). However, other factors may also have an important role in the differing antipsychotic activity seen with the various partial agonists, such as differences in their receptor binding profiles, particularly the relative affinities and/or potencies for other receptors. In particular, agonist activity at 5-HT_{1A} receptors and antagonist activity at 5-HT_{2A} receptors has been hypothesized to contribute to the efficacy and lower propensity for side effects of some atypical antipsychotics. Aripiprazole, OPC-4392, and terguride have high affinity and partial agonist activity at 5-HT_{1A} receptors (Jordan et al., 2002; Miwa, unpublished observations; Newman-Tancredi et al., 2002; Millan et al., 2002). Furthermore, these drugs have affinity at 5-HT_{2A} receptors (McQuade et al., 2002; Tadori, unpublished observations; Millan et al., 2002).

In positron emission tomography (PET) studies of patients treated with dopamine D2 receptor antagonists like haloperidol, clinical improvement was seen but hyperprolactinemia and a significant increase in extrapyramidal symptoms were observed at dopamine D2 receptor occupancies exceeding 65%, 72%, and 78%, respectively (Kapur et al., 2000). Aripiprazole, in contrast to other antipsychotics, has shown dopamine D2 receptor occupancy above 90% in PET studies with no significant extrapyramidal symptoms (Yokoi et al., 2002). Consequently, aripiprazole's partial agonist properties, combined with appropriate intrinsic activity at dopamine D2 receptors, might contribute to stabilizing rather than blocking a dopaminergic tone and result in a desired clinical profile.

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