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RESEARCH PAPER

Analysis of the actions of the novel dopamine receptor-directed compounds (*S*)-OSU6162 and ACR16 at the D_2 dopamine receptor

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OSU6162; ACR16; dopamine stabilizers; D_2 dopamine receptor; radioligand binding; stimulation of [^{35}S]GTPγS binding; allosteric effects

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BACKGROUND AND PURPOSE
The two phenylpiperidines, OSU6162 and ACR16, have been proposed as novel drugs for the treatment of brain disorders, including schizophrenia and Huntington's disease, because of their putative dopamine stabilizing effects. Here we evaluated the activities of these compounds in a range of assays for the D_2 dopamine receptor *in vitro*.

EXPERIMENTAL APPROACH
The affinities of these compounds for the D_2 dopamine receptor were evaluated in competition with [^{3}H]spiperone and [^{3}H]NPA. Agonist activity of these compounds was evaluated in terms of their ability to stimulate [^{35}S]GTPγS binding.

KEY RESULTS
Both compounds had low affinities for inhibition of [^{3}H]spiperone binding (pK_i vs. [^{3}H]spiperone, ACR16: <5, OSU6162: 5.36). Neither compound was able to stimulate [^{35}S]GTPγS binding when assayed in the presence of Na^+ ions, but if the Na^+ ions were removed, both compounds were low-affinity, partial agonists (E_{max} relative to dopamine: ACR16: 10.2%, OSU6162:54.3%). Schild analysis of the effects of OSU6162 to inhibit dopamine-stimulated [^{35}S]GTPγS binding indicated Schild slopes of ~0.9, suggesting little deviation from competitive inhibition. OSU6162 was, however, able to accelerate [^{3}H]NPA dissociation from D_2 dopamine receptors, indicating some allosteric effects of this compound.

CONCLUSIONS AND IMPLICATIONS
The two phenylpiperidines were low-affinity, low-efficacy partial agonists at the D_2 dopamine receptor *in vitro*, possibly exhibiting some allosteric effects. Comparing their *in vitro* and *in vivo* effects, the *in vitro* affinities were a reasonable guide to potencies *in vivo*. However, the lack of *in vitro–in vivo* correlation for agonist efficacy needs to be further addressed.

LINKED ARTICLES
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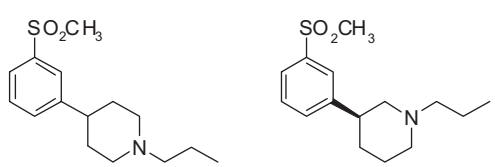
Abbreviations
ACR16, 4-(3-methanesulfonyl-phenyl)-1-propyl-piperidine; OSU6162, (*S*)-(-)-3-(3-methanesulfonyl-phenyl)-1-propyl-piperidine

Introduction

The treatment of schizophrenia has relied upon the antipsychotic drugs which constitute a large class of compounds whose common pharmacological action is to block dopamine receptors of the D_2-like subgroup (D_2, D_3, D_4), with effects at D_2/D_3 receptors appearing to be important (Strange, 2001; receptor nomenclature follows Alexander *et al.*, 2009). Because of the ability of these drugs to act as antagonists or inverse agonists at D_2/D_3 receptors, they can reduce the over-activity in dopamine systems that has been associated with psychosis (Strange, 2008). This may, however, lead to a hypo-dopaminergic state with neurological and cognitive impairments, and will not increase activity in systems where dopamine activity is too low.

It has been proposed that an alternative approach to the treatment of schizophrenia is to develop 'dopamine stabilising drugs' (Carlsson *et al.*, 2001). These compounds would normalize/stabilize activity in both overactive and underactive dopamine systems. Two compounds that have been proposed as candidate dopamine stabilizers are the phenylpiperidines OSU6162 and ACR16 (Figure 1) (Sonesson *et al.*, 1994; Nilsson *et al.*, 2004). In animal models, these compounds reverse amphetamine-induced or apomorphine-induced locomotor activation in rats and cause behavioural stimulation in habituated rats (Sonesson *et al.*, 1994; Natesan *et al.*, 2006; Rung *et al.*, 2008). This profile is consistent with the ability of the compounds to inhibit overactive dopamine systems and to stimulate dopamine activity when it is low. In several behavioural tests for agonism, the compounds have been shown to be devoid of intrinsic activity (Sonesson *et al.*, 1994; Natesan *et al.*, 2006). These compounds have also been shown to exhibit high *in vivo* occupancy of brain D_2 dopamine receptors using positron emission tomography studies with [^{11}C]raclopride in the non-human primate (Ekesbo *et al.*, 1999) and *ex vivo* radioligand binding in the rat (Natesan *et al.*, 2006). In contrast, when these compounds have been examined *in vitro*, they consistently show weak actions at D_2 receptors. The affinities of OSU6162 and ACR16 for D_2 dopamine receptors were reported to be 900 nM and 23 μM respectively (Natesan *et al.*, 2006). Recent preliminary reports have suggested that OSU6162 possesses weak partial agonism for the stimulation of [^{35}S]GTPγS binding (Seeman and Guan, 2007). Moreover, it was reported that OSU6162 and dopamine interacted in a complex manner with respect to effects on [^{35}S]GTPγS binding (Lahti *et al.*, 2007), and these authors speculated that the compound might act at orthosteric and allosteric sites on the D_2 dopamine receptor.

In order to clarify some of these issues, we have characterized ACR16 and OSU6162 in a series of *in vitro* assays. The data show that the compounds have low affinity for the D_2 dopamine receptor but some partial agonist activity with some indication of allosteric effects.



ACR16 OSU6162

Figure 1
Structures of the phenylpiperidines ACR16 and OSU6162.

Methods

Cell culture

Two CHO cell lines stably expressing human D_{2short} dopamine receptors at different levels (~2 pmol mg^{-1} (Wilson *et al.*, 2001) and ~ 4 pmol mg^{-1} (Hayes *et al.*, 1992) were used for this work, and membranes derived from either cell line gave comparable results. The cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 200 μg mL^{-1} active geneticin (to maintain selection pressure). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

Membrane preparation

Membranes were prepared from CHO cells expressing D_{2short} dopamine receptors as described previously (Castro and Strange, 1993). Briefly, confluent 175 cm^2 flasks of cells were washed once with 5 mL HEPES buffer (20 mM HEPES, 1 mM EGTA, 1 mM EDTA; pH 7.4). Cells were then removed from the surface of the flasks using 5 mL HEPES buffer and glass beads (2 mm diameter) and were then homogenized using an Ultra-Turrax homogenizer (four 5 s treatments; IKA, Staufen, Germany). The homogenate was centrifuged at 250× *g* (10 min; 4°C), after which the supernatant was centrifuged at 48 000× *g* (60 min; 4°C). The resulting pellet was resuspended in HEPES buffer at a concentration of 3–5 mg protein mL^{-1} (determined by the method of Lowry *et al.*, 1951) and stored in aliquots at –70°C until use.

Radioligand binding assays

Cell membranes (25 μg) were incubated in triplicate with [^{3}H]spiperone (~0.25 nM) or [^{3}H]

N-propylnorapomorphine (NPA; ~0.1 nM) and competing drugs in HEPES buffer (20 mM HEPES, 1 mM EGTA, 1 mM EDTA, 10 mM $MgCl_2$, 100 mM NaCl or 100 mM N-methyl D-glucamine (NMDG) [to maintain ionic strength in the absence of sodium ions (Nunnari *et al.*, 1987)]; pH 7.4 (using HCl or KOH) containing 0.1 mM dithiothreitol) in a final volume of 1 mL for 3 h at 25°C. The assay was terminated by rapid filtration (through Whatman GF/C filters, Maidstone, UK) using a Brandel cell harvester (Gaithersburg, MD, USA) followed by four washes with 4 mL ice-cold phosphate-buffered saline (0.14 M NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 5 mM Na_2HPO_4; pH 7.4) to remove unbound radioactivity. Filters were soaked in 2 mL of scintillation fluid for at least 6 h and bound radioactivity was determined by liquid scintillation counting. Non-specific binding of radioligands was determined in the presence of 3 μM (+)-butaclamol.

[^{35}S]GTPγS binding assays

Cell membranes (25 μg) were incubated in triplicate with ligands for 30 min at 30°C in 0.9 mL of HEPES buffer containing 10 mM $MgCl_2$, 10 μM GDP and 100 mM NaCl or NMDG where indicated. The assay was initiated by an addition of 100 μL of diluted [^{35}S]GTPγS to give a final concentration of 50–100 pM. The assay was incubated for a further 30 min and terminated by rapid filtration as above.

[^{3}H]NPA dissociation assays

Cell membranes (30 μg) were incubated in triplicate in HEPES buffer containing 6 mM $MgCl_2$, 100 mM NMDG, with [^{3}H]NPA (0.1 nM) for 3 h at 25°C. The tested drug was added at a concentration able to fully inhibit radioligand binding (3 μM (+)-butaclamol, 1 mM dopamine, 100 μM OSU6162 final concentration, as determined in competition experiments), and incubated for different times. The final reaction volume was 1 mL. The reaction was stopped by rapid filtration through Whatman GF/C glass fibre filters as described previously and bound radioactivity determined by liquid scintillation counting.

Data analysis

Radioligand binding data were analysed using Prism (GraphPad, La Jolla, CA, USA) and all data fitted to a one-binding site model better than a two-binding site model ($P < 0.05$, *F*-test). The inhibition constants (K_i) were calculated from IC_{50} values, derived from competition binding analyses, using the Cheng–Prusoff equation (Cheng and Prusoff, 1973) as described (Roberts *et al.*, 2004; Lin *et al.*, 2006). This corrects for the concentration of the radioligand ([^{3}H]spiperone) and its dissociation constant at the relevant binding site. Data from [^{35}S]GTPγS binding experiments were fitted to a sigmoidal concentration/response curve with a Hill coefficient of one which provided the best fit to the data in all cases ($P < 0.05$). [^{3}H]NPA dissociation data were fitted to models of one or two exponential decay phases, and the better fit was determined using an *F*-test ($P < 0.05$).

Statistical significance of differences between two data sets (e.g. two sets of p*Ki* values) was determined using paired *t*-tests, with significance determined as $P < 0.05$.

Materials

The radioligands [^{35}S]GTPγS (~37 TBq mmol^{-1}) and [^{3}H]spiperone (~600 GBq mmol^{-1}) were purchased from GE Healthcare (Buckinghamshire, UK). [^{3}H]NPA (30–60 Ci mmol^{-1}) was purchased from American Radiolabeled Chemicals (St Louis, MO, USA). Optiphase HiSafe-3 scintillation fluid was purchased from Perkin-Elmer Life Sciences (Cambridge, UK). Dopamine, NMDG and (+)-butaclamol were purchased from Sigma (Dorset, UK). OSU6162 [(*S*)-(-)-3-(3-methanesulfonyl-phenyl)-1-propyl-piperidine] and ACR16 [4-(3-methanesulfonyl-phenyl)-1-propyl-piperidine] were synthesized at Eli Lilly and Company (Indianapolis, IN, USA).

Results

Ligand binding studies

Both OSU6162 and ACR16 were tested for their ability to compete with the antagonist/inverse agonist radioligand [^{3}H]spiperone and the agonist radioligand [^{3}H]NPA for binding to D_2 dopamine receptors expressed in CHO cell membranes. Experiments were performed in buffers containing Na^+ or in buffers lacking Na^+ but containing the cation substitute NMDG to maintain ionic strength (Nunnari *et al.*, 1987). Representative data are given in Figure 2 and values for inhibition constants are given in Table 1. In some cases, especially with ACR16, competition curves were incomplete but, generally, competition curves were described by one-binding site models. Affinities for the two compounds were similar when determined versus [^{3}H]NPA binding as compared with [^{3}H]spiperone. The affinity of ACR16 was significantly higher ($P < 0.05$) in the presence of Na^+ in competition versus [^{3}H]NPA although the effect was small.

[^{35}S]GTPγS binding studies

Both OSU6162 and ACR16 were tested for the stimulation of [^{35}S]GTPγS binding to membranes of CHO cells expressing D_2 dopamine receptors using

buffers containing Na^+ or NMDG as cited. The substitution of NMDG for Na^+ in [^{35}S]GTPγS binding assay buffers has been shown to enable detection of partial agonist activity for very low efficacy agonists (Lin *et al.*, 2006). Dopamine was used as a control for full stimulation of receptors and representative data are given in Figure 3. Neither compound stimulated [^{35}S]GTPγS binding in buffers containing Na^+ ions, but in the absence of Na^+, a clear agonist signal was seen with OSU6162 (54.3 ± 5.8% of maximal dopamine response, pEC_{50} 5.97 ± 0.21). ACR16 did stimulate [^{35}S]GTPγS binding under these conditions although the signal was often weak, making it difficult to measure accurately (10.2 ± 4.5%). Using the same assay protocol, the low efficacy partial agonist,

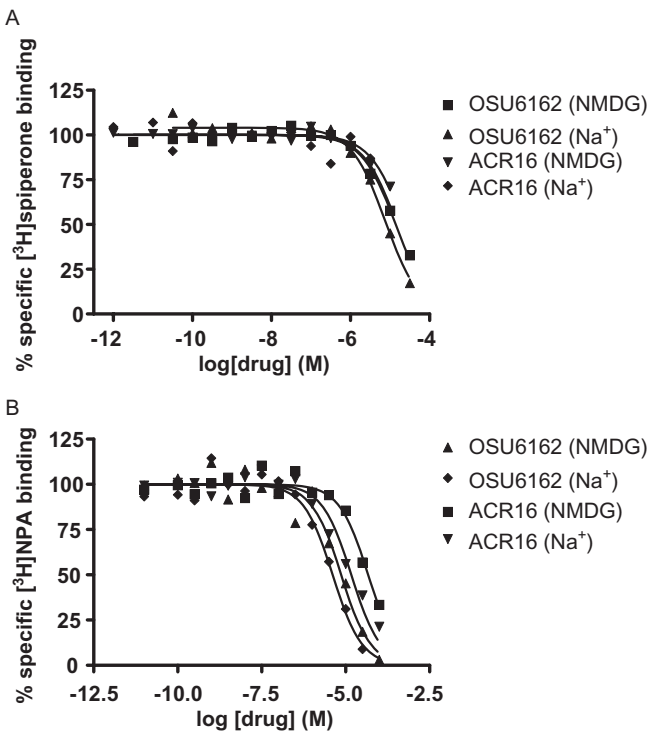


Figure 2

Binding of ACR16 and OSU6162 to membranes of CHO cells expressing D_2 dopamine receptors. Binding of the two compounds was determined in competition with [^{3}H]spiperone (A) or [^{3}H]NPA (B).

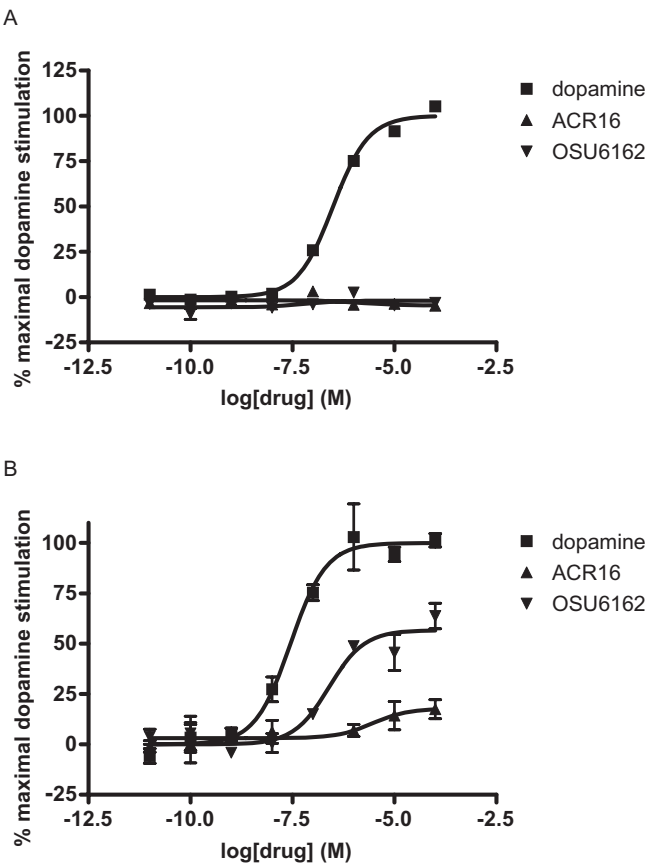


Figure 3

Stimulation of [^{35}S]GTPγS binding by dopamine, ACR16 and OSU6162. Stimulation of [^{35}S]GTPγS binding to membranes of CHO cells expressing D_2 dopamine receptors was determined in the presence of Na^+ ions (A) or NMDG (B).

Table 1

Binding of ACR16 and OSU6162 to D_2 dopamine receptors expressed in CHO-D2 membranes, determined in competition with [^{3}H]spiperone or [^{3}H]NPA

	pKi ± sem			
	[^{3}H]spiperone		[^{3}H]NPA	
	Na+	NMDG	Na+	NMDG
ACR16	<5	<5	5.20 ± 0.13	4.86 ± 0.10*
OSU6162	5.36 ± 0.08	5.08 ± 0.08*	5.79 ± 0.17	5.58 ± 0.19

Binding of ACR16 and OSU6162 was determined in competition with either [^{3}H]spiperone or [^{3}H]NPA. Data are mean ± SEM from three or more experiments.

*$P < 0.05$ for effects of Na^+ ions (paired *t*-test). Others have determined affinities for these drugs: pK_i OSU6162, 5.2 (Sonesson *et al.*, 1994); OSU6162, 6.0, ACR16, 4.6 (Natesan *et al.*, 2006).

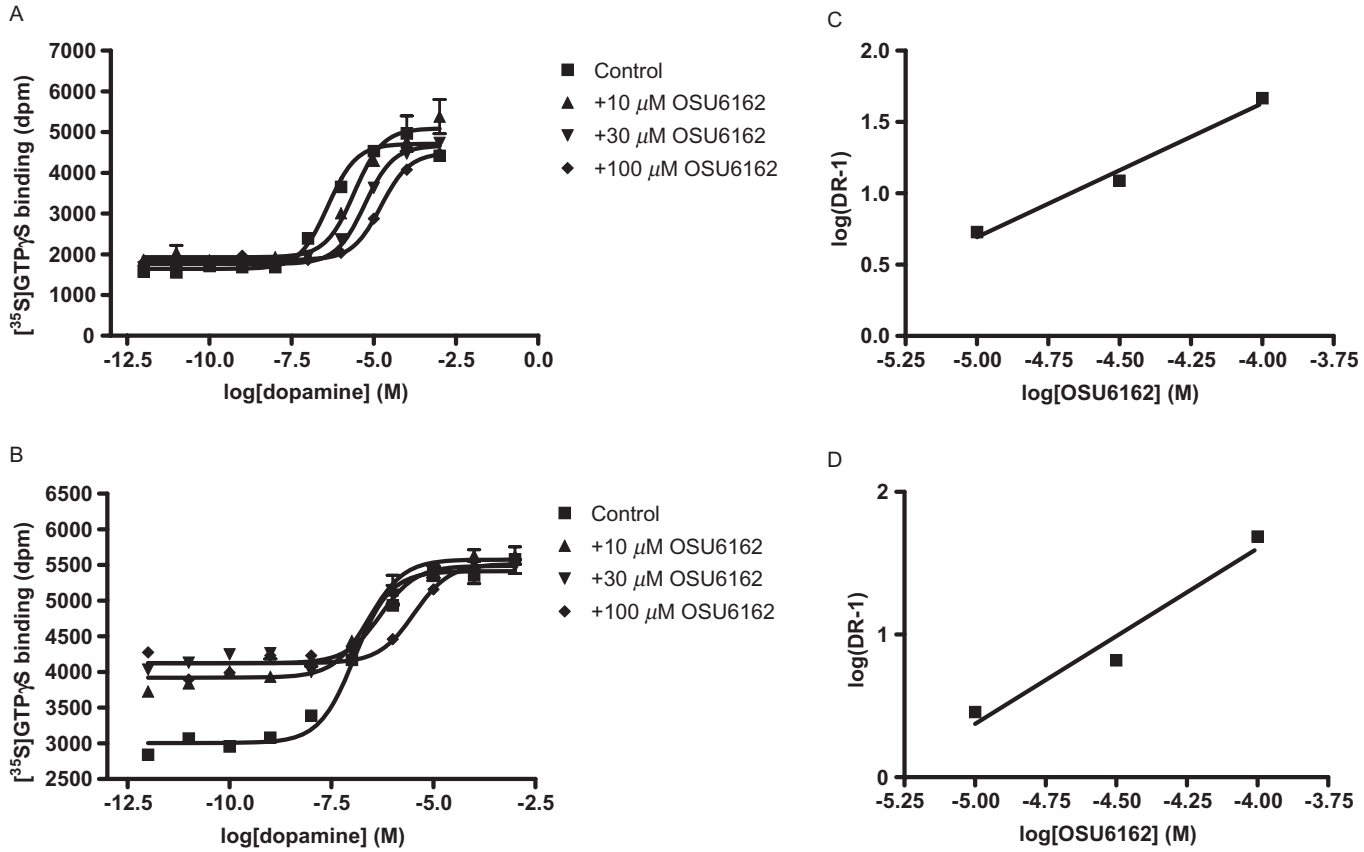


Figure 4

Schild analysis of the effect of OSU6162 to inhibit dopamine stimulation of [^{35}S]GTPγS binding. Dopamine-stimulation of [^{35}S]GTPγS binding to membranes of CHO cells expressing D_2 dopamine receptors was determined in the presence of different concentrations of OSU6162, in the presence of Na^+ ions (A,C) or NMDG (B,D). Panels A and B show the stimulation curves and panels C and D show the derived Schild plots.

aripiprazole, has been shown to achieve a very low stimulation of [^{35}S]GTPγS binding in the presence of Na^+ ions (~5% of the maximal dopamine response) but in the absence of Na^+ ions to achieve a clear stimulation (51.3% of maximal dopamine response) (Lin *et al.*, 2006).

Schild analysis of the effects of OSU6162 on dopamine stimulation of [^{35}S]GTPγS binding

Different concentrations of OSU6162 were tested for the inhibition of dopamine stimulation of [^{35}S]GTPγS binding in the presence and absence of Na^+ ions and Schild analysis of the derived data was performed. Representative data are given in Figure 4. The mean Schild slopes were 0.88 ± 0.02 (Na^+), 0.89 ± 0.18 (-Na^+), and the pK_b values were 6.01 ± 0.07 (Na^+), 5.74 ± 0.28 (-Na^+) (mean ± SEM, four experiments).

Effects of OSU6162 to accelerate [^{3}H]NPA dissociation from D_2 dopamine receptors

OSU6162 (100 μM) was tested for its ability to accelerate [^{3}H]NPA dissociation as compared with dopamine and (+)-butaclamol (Kara *et al.*, 2009). OSU6162 provided an intermediate level of acceleration of [^{3}H]NPA dissociation, compared with dopamine, and representative data are given in Figure 5 and the derived parameters are in Table 2.

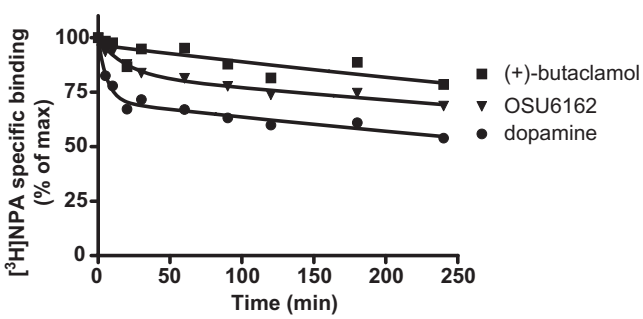


Figure 5

Dissociation of [^{3}H]NPA from D_2 dopamine receptors expressed in membranes of CHO cells. [^{3}H]NPA dissociation was determined as described. Dissociation curves were best described by one-phase (butaclamol) or two-phase models (dopamine and OSU6162).

Table 2

Acceleration of [^{3}H]NPA dissociation by dopamine and OSU6162

	% fast dissociation	$t_{0.5}$ fast (min)	$t_{0.5}$ slow (min)
(+)-butaclamol	–	–	549 ± 101
OSU6162	17.1 ± 2**	11.5 ± 2.5*	668 ± 93
Dopamine	30.8 ± 2	5.6 ± 0.6	400 ± 46

[^{3}H]NPA dissociation was determined as described. Dissociation curves (see Figure 5) were best described by one-phase (butaclamol) or two-phase models (dopamine and OSU6162), and values for the fast and slow half lives and % fast dissociation are given (mean ± SEM, three or more experiments).
*$P < 0.05$; **$P < 0.01$ significantly different from dopamine with a paired *t*-test.

Discussion

The data presented here show that both OSU6162 and ACR16 bind with low affinity to the D_2 dopamine receptor. Both ligands show some sensitivity to sodium ions in their binding with affinities being slightly higher in the presence of Na^+. This is a pattern we have described for some structurally related compounds, for example, AJ76 and (-)-3-PPP at this receptor (Lin *et al.*, 2006), and may reflect a similar interaction with the receptor. Affinities were not significantly different when tested in competition with the agonist radioligand [^{3}H]NPA, compared with the antagonist/inverse agonist radioligand [^{3}H]spiperone. The estimates of affinity we report are broadly in agreement with those reported by others (see Table 1).

In [^{35}S]GTPγS binding assays, neither compound showed any agonist efficacy when tested under standard assay conditions in buffers containing Na^+ ions. When the assays were performed under conditions that maximize actions of low-efficacy agonists (Na^+ substituted by NMDG [Lin *et al.*, 2006)], however, both compounds exhibited agonist efficacy, with OSU6162 exhibiting a greater intrinsic activity than ACR16. It is interesting to note that while both compounds inhibit conditioned avoidance responding in the rat, only ACR16 produced escape failures at high doses, suggesting additional inhibitory effects on locomotor activity (Natesan *et al.*, 2006). These authors also reported that ACR16 was more efficacious than OSU6162 in blocking amphetamine-induced hyperactivity in the rat and showed a weaker behavioural activation of locomotion in habituated rats. Together, this would support a possible lower degree of intrinsic activity for ACR16 compared with OSU6162 (see also Rung *et al.*, 2008). An alternative explanation is that the two compounds differ in their potential cross-reactivity towards other, non-dopamine receptor, targets.

It has been suggested that OSU6162 may exert some allosteric effects and so we tested this compound in two assays that may detect such effects. In Schild analysis of the inhibition of dopamine-stimulated [^{35}S]GTPγS binding, in the presence or absence of Na^+ ions, concentration/response curves for dopamine were progressively shifted to the right by increasing concentrations of OSU6162 without any depression of the maximal response. Schild slopes for these experiments were close to one, suggesting that these compounds are acting competitively and providing no strong evidence for allosteric effects. It should be noted that the low affinity of OSU61612 limited the range of concentrations that we could test. This limited our ability to detect deviation from competitive behaviour. In a second experimental design, we tested OSU6162 for its ability to accelerate the dissociation of the agonist radioligand [^{3}H]NPA from D_2 dopamine receptors. We have shown that this assay detects allosteric effects of agonists at this receptor (Kara *et al.*, 2009). OSU6162 did accelerate [^{3}H]NPA dissociation, providing some support for allosteric effects of this compound. Recent reports have suggested that the D_2 dopamine receptor functions as an asymmetrical dimer with negative cooperativity between the subunits, illustrating how such allosteric effects could arise (Han *et al.*, 2009; Kara *et al.*, 2009). Full activation of the receptor is achieved when one subunit is occupied and this may account for the lack of apparent cooperativity for these compounds in simple signalling assays. The [^{3}H]NPA dissociation assay (Kara *et al.*, 2009) detects negatively cooperative, allosteric effects across the dimer directly, and so may be a more sensitive detector of such effects.

The data reported here are broadly in agreement with recent suggestions that OSU6162 is a low-efficacy partial agonist in experiments *in vitro*. Additionally, we report for the first time that ACR16 has some very low agonist efficacy. The data reported here on the ability of OSU6162 to stimulate [^{35}S]GTPγS binding, when compared with those in Lin *et al.* (2006), suggest that the intrinsic activity of OSU6162 is similar to that of aripiprazole. It will be of interest to see if the partial agonist efficacy of OSU6162 and ACR16 is seen more clearly in assays downstream of G protein activation.

It has also been suggested that the *in vivo* occupancy of D_2 dopamine receptors is surprisingly high compared with the *in vitro* affinities of these compounds for the D_2 receptor (see for example Ekesbo *et al.*, 1999). Table 3 compares the activities of ACR16, OSU6162 and haloperidol in several *in vivo*

Table 3

Comparison of the *in vivo* activities of ACR16, OSU6162 and haloperidol with their *in vitro* binding affinities

	ACR16	OSU6162	Haloperidol
Striatal D_2 receptor occupancy (ED_{50}, mg/kg)	19.0 (950)	5.27 (264)	0.02
Amphetamine induced locomotion (ED_{50}, mg/kg)	28.2 (564)	44.7 (894)	0.05
Conditioned avoidance (ED_{50}, mg/kg)	39.8 (2096)	–	0.019
D_2 receptor dissociation constant (K_i)	6.30 μM (11900)	1.62 μM (3060)	0.53 nM

In vivo data are taken from (Natesan *et al.*, 2006); dissociation constants at the D_2 receptor are from this study and from Strange (2001). Figures in brackets show ratios of ED_{50} or K_i for ACR16 and OSU6162, relative to haloperidol.

tests, with the affinities measured in ligand binding assays. Based on these data, the two phenylpiperidine drugs are slightly more potent in these *in vivo* tests (approximately fivefold), compared with their *in vitro* affinities, although the *in vivo* data do not take into account potential pharmacokinetic differences between the drugs. The conclusion reached by some authors that these compounds have much higher than expected *in vivo* effects does not therefore seem warranted.

Although the *in vitro* studies reported here show that these compounds possess some intrinsic activity, this has rarely been seen *in vivo*. There is little *in vivo* evidence of partial D_2 dopamine receptor agonist activity in these compounds, even under conditions of sensitized D_2 dopamine receptors. Thus, although both compounds stimulated activity in habituated rats (Natesan *et al.*, 2006), they both failed to reduce plasma prolactin levels in normal rats and failed to inhibit the rate of dopamine synthesis in animals treated with the monoamine-depleting agent reserpine for 18 h (Natesan *et al.*, 2006). In addition, and in contrast to known partial D_2 dopamine receptor agonists, OSU6162 did not induce contralateral rotations in 6-OH-dopamine-lesioned rats (Nichols *et al.*, 2002) or non-human primates (Ekesbo *et al.*, 1999). The most likely explanation for this dichotomy is that the compounds are very low-efficacy partial agonists, close to neutral in terms of intrinsic activity, and that this activity may be detected in sensitive *in vitro* tests such as the [^{35}S]GTPγS binding assay reported here, but for the most part the compounds appear as neutral antagonists *in vivo*.

Acknowledgement

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Conflicts of interest

None to declare

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