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Comparative Pharmacological Study of Ropinirole (SKF-101468) and its Metabolites in Rats

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Abstract

The dopamine receptor agonist ropinirole (SKF-101468) is used to treat Parkinson's disease. Ropinirole is metabolized by two routes to a series of different metabolites although the predominant pathway is species-dependent. It is unknown whether any of the metabolites contribute to its antiparkinsonian activity and whether D_3 or D_2 receptor agonist activity plays a preferential role. Therefore ropinirole and its primary metabolites, SKF-104557, SKF-97930 and SKF-96990, and the rat metabolite, SKF-89124 were tested in the 6-hydroxydopamine lesion model of Parkinson's disease. SKF-89124 and SKF-96990 were also assayed in radioligand binding and microphysiometer functional assays at cloned human dopamine D_2 and D_3.

Ropinirole and SKF-89124 were equipotent in-vivo, and produced dose-related increases in circling at 0·05–0·8 mg kg^{-1}, s.c. (ropinirole) and 0·05–0·75 mg kg^{-1}, s.c. (SKF-89124). Neither SKF-96990 or SKF-97930, at doses up to 15 mg kg^{-1}, increased the circling rate. Some circling was observed with 15 mg kg^{-1} SKF-104557 but the response was less than half that produced by ropinirole (0·8 mg kg^{-1}). SKF-104557 was 150-fold less potent than ropinirole. SKF-89124 possessed-30-fold higher affinity for D_3 over D_2 receptors in radioligand binding studies, but was not selective in the functional microphysiometer assay. SKF-96990 was 10-fold selective for D_3 over D_2 receptors in the radioligand binding assay. Ropinirole and SKF-104557 are 20-fold selective for D_3 over D_2 receptors in radioligand binding assays whereas in microphysiometry, selectivity is 10-fold. SKF-97930 is inactive in radioligand binding and microphysiometer assays.

Primary metabolites of ropinirole did not contribute significantly to its activity in this model of Parkinson's disease. The lack of dopamine D_3/D_2 receptor selectivity for ropinirole rules out the possibility of attributing the degree of either D_2 or D_3 receptor activity to the behavioural efficacy of ropinirole.

Ropinirole (4-[2-(dipropylamino)ethyl]1,3-dihydro 2H-indol-2-one) (SKF-101468) is a new therapeutic agent used in the treatment of Parkinson's disease. Studies of ropinirole in animal models have identified it as a selective, brain penetrant dopamine D_3/D_2 receptor agonist (Eden et al 1991; Coldwell et al 1999b), without dopamine D_1 receptor activity (Eden et al 1991; Reavill et al 1993). In animal models ropinirole has been shown to have antiparkinsonian activity (Eden et al 1991; Fukuzaki et al 2000). Clinical studies have demonstrated antiparkinsonian efficacy in man (Kapoor et al 1989; Vidailhet et al 1990; Kleedorfer et al 1991; Rascol et al 1996, 1998; Korczyn et al 1998; Lieberman et al 1998; Sethi et al 1998). Evidence from non-human primates suggests that ropinirole has a low probability of producing dyskinesia (Jenner & Tulloch 1997), and evidence in man has confirmed that ropinirole monotherapy is associated with a low incidence of dyskinesia and adverse events (Korczyn et al 1998, 1999).

Ropinirole metabolism follows two routes although the predominant route is species dependent. The 7-hydroxylated metabolite, SKF-89124 (4-[-N,N-dipropylamino)ethyl]-7-hydroxy-2-(3H) indolone is the main metabolite in rats and dogs (Mico et al 1986; Swagzdis & Mico 1986; De Marinis & Hieble 1989; Ramji et al 1999), and

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the despropyl metabolite, SKF-104557 (4-[2-(propylamino)ethyl]-2-(3H) indolone) is the main metabolite in man and in non-human primates (Beattie & Blake 1989, Ramji et al 1999). SKF-97930 (4-carboxy-2-(3H) indolone) and SKF-96990 (4-[2-propylamino) ethyl]-7-hydroxy-(3H) indolone) are minor metabolites in cynomolgus monkeys (Beattie & Blake 1989, Ramji et al 1999). However, there is little data available on the pharmacological activity of these metabolites. We therefore tested the effects of these compounds on circling behaviour in hemi-lesioned rats (Ungerstedt 1969). In this model, a unilateral 6-hydroxydopamine (6-OHDA)-induced lesion of the ascending nigrostriatal dopamine pathway results in the rats circling away from the side of the lesion when challenged with dopamine receptor agonists. The circling response provides a readily quantifiable measure of behavioural potency and efficacy of dopamine receptor agonists. We also studied the radioligand binding profile of SKF-89124 and SKF-96990 in cloned human dopamine D_2 and D_3 receptors. We tested whether these compounds have agonist properties at the cloned receptors using a functional assay of dopamine receptor function, namely acidification rates in the Cytosensor microphysiometer (Molecular Devices). Using this and previously published data (Coldwell et al 1999b) we attempted to determine whether D_2 or D_3 receptor mechanisms contribute to the anti-parkinsonian activity of ropinirole.

Materials and Methods

Drugs and reagents

6-Hydroxydopamine hydrobromide, sodium salt of L-ascorbic acid and apomorphine hydrochloride were obtained from Sigma Chemical Co. Ltd. Quinpirole was obtained from Research Biochemicals Inc. Apomorphine was dissolved in saline containing 0·1% sodium metabisulphite to give a concentration of $0{\cdot}1\,mg\,kg^{-1}$ base. Sublimaze (fentanyl) was obtained from Janssen Pharmaceutica, Domitor (medetomidine hydrochloride) and Antisedan (atipamezole hydrochloride) were from SmithKline Beecham and Nubain (nalbuphine hydrochloride) from Du Pont Merck. Ropinirole, SKF-104557 and SKF-96990 were dissolved in saline. SKF-89124 hydrochloride and SKF-97930 were dissolved in saline by warming. All compounds were injected subcutaneously in a volume of $1\,mL\,kg^{-1}$. Doses refer to base equivalents. For radioligand binding and microphysiometer experiments, agonists were prepared as stock solutions in bicarbonate-free Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine and 44 mM NaCl. Ropinirole, SKF-104557 and SKF-96990 were dissolved in saline. SKF-89124 hydrochloride and SKF-97930 were dissolved in saline by warming.

Cell Culture

Human dopamine receptors were expressed in Chinese hamster ovary (CHO) cells. Human cloned dopamine D_2 (long) receptors were obtained from the Garvan Institute of Medical Research, Sydney, Australia (Selbie et al 1989), and human cloned D_3 receptors were obtained from Unite de Neurobiologie et Pharmacologie (U109) de l'Inserm, Paris, France (Selbie et al 1989; Sokoloff et al 1990). CHO cells expressing human D_2 receptors were grown in 50 : 50 DMEM (with glucose, without sodium pyruvate)–Ham's F-12 containing 10% (v/v) foetal bovine serum. CHO cells expressing human D_3 receptors were grown in DMEM (with glucose, without sodium pyruvate) containing 10% foetal bovine serum, 100 nM methotrexate, 2 mM glutamine, 500 nM (−)-sulpiride and 1% essential amino acids. Cells were harvested by scraping the plates and centrifuging (200 *g*, 5 min, room temp). The cells were resuspended in 10 mL fresh culture medium and a sample was counted. Cells were passaged at 12 500 or 25 000 cells cm^{-2} for studies between passages 5 and 30.

Radioligand binding

Radioligand binding assays were conducted on CHO cell membranes transfected with human D_2 or D_3 receptors. The method was as described by Sokoloff et al (1992). Protein (5–15 μg) was incubated with 0·1 nM [^{125}I]-iodosulpride and test compound in a 50 mM Tris buffer containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ at pH 7·4 for 30 min at 37°C. The solution also contained 0·1% bovine serum albumin in a total volume of 1 mL. Non-specific binding was determined using 100 M iodosulpride. Curves were fitted to data by non-linear regression (Bowen & Jerman 1995). Results are for three to six experiments run on different days for radioligand binding affinity except for SKF-96990 (n = 1).

Cytosensor microphysiometer (Molecular Devices)

Cells were put into 12 mm transwell inserts (Costar) at 300 000 cells/cup in growth medium containing foetal bovine serum. After incubation for 6 h at 37°C in 95% O_2/5% CO_2 the medium was changed to one without foetal bovine serum and (−)-sulpiride. After 16–18 h, the cups were

loaded into the microphysiometer sensor chambers and perfused with bicarbonate-free Dulbecco's modified Eagle's medium (containing 2 mM glutamine and 44 mM NaCl) at a flow rate of 100 μL min^{-1} for 90 s at 37°C. The pump was on for the first 60 s and off for the next 30 s with the acidification rate determined between 68 and 88 s, using the Cytosoft programme (Molecular Devices). Cells were exposed to increasing concentrations of ropinirole or metabolites, at half-log intervals, every 30 min. Each exposure lasted for 4·5 min for cells expressing D_2 receptors and 7·5 min for cells expressing D_3 receptors. At 30 min after the highest agonist concentration, cells were perfused with the dopamine receptor agonist, quinpirole (1000 nM for D_2, and 100 nM for D_3 cells). Quinpirole was included as a standard as it does not produce variable responses and has a maximal response equivalent to dopamine (Coldwell et al 1999a). Acidification rates were calculated as the difference between the average of three measurements taken immediately before agonist exposure and the maximum effect after exposure to the agonist. Concentration–response curves were analysed by Robofit (Tilford et al 1995). SKF-96990 was not screened in the functional test due to lack of compound. Results are for three or four experiments run on different days for functional responses.

Circling behaviour

All procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 and SmithKline Beecham ethical guidelines. Male hooded Lister rats (Harlan Olac), 225–250 g, were anaesthetized with Sublimaze (0·6 mL/100 g, i.p.) and Domitor (0·03 mL/100 g, i.m.). The rats were then immobilized in a David Kopf small animal stereotaxic instrument and the heads were raised 5 mm at the incisor bar. A burr hole was made in the exposed calvarium to access the brain surface at the required coordinates. The coordinates, taken from the atlas of De Groot (1967) were anterior (from the interaural line) 4·3 mm; lateral (from the midline) 1·9 mm. A 10 μL Hamilton syringe was lowered vertically to the required depth (8·2 mm from the dura) into the left medial forebrain bundle, and 6-hydroxydopamine hydrobromide solution (8 μg in 3 μL saline containing 2 μg ascorbic acid) was injected slowly over a period of 3 min. The needle was left in-situ for a further 3 min to allow diffusion into the surrounding tissue and then slowly withdrawn. The incision was sutured and the rats treated with Antisedan (0·02 mL/100 g, i.p.) and Nubain hydrochloride (0·02 mL/100 g, i.p.). The rats were placed on shredded paper under artificial heat until they recovered to a normal state of consciousness. The rats were checked by a veterinary surgeon to ensure their well-being.

For quantitative measurements of rotational behaviour, circling was assessed automatically in a RM1057 Rotation Meter (Benwick Electronics) which was controlled by an IBM compatible computer. Rats were harnessed around the thorax and individually placed in high-sided containers. The harnesses were connected to swivels allowing unrestricted 360° rotation in both clockwise and anticlockwise directions. The number of circles per minute and the cumulated circles over 2 h after subcutaneous agonist administration were recorded. Data analysis was performed on the cumulated total contraversive circles recorded for 117 min post-injection.

Testing started at least 5 days after the day of lesion. The success of the lesions was tested by observing the circling response 15 min after an injection of apomorphine hydrochloride (0·5 mg kg^{-1} base, s.c.). Nine rats which developed contraversive rotation (circling away from the lesioned hemisphere), regardless of circling rate, were selected for additional behavioural tests with the drugs under investigation. The rats received different treatments at a frequency of no more than two injections per week separated by three to four days. Due to some limited compound supply the final treatment groups contained 3–8 rats and not all rats received all treatments.

The circling response (Y) taken as the number of rotations during a 117-min observation period provided a measure of behavioural potency. Using the Box-Cox transformation procedure, the response Y was transformed to $Y^{1/4}$ to improve homogeneity of variance and normality of the fitted one-way model (Box & Cox 1964).

Data were analysed using the SAS package, version 6·11 (SAS Institute Inc.). The first stage tested whether there was significant inter-subject variation. The second stage tested for differences between treatment groups by analysis of variance followed by comparisons of each treatment group with the saline control using Dunnett's multiple comparison procedure. Finally, dose–response curves were fitted to agonist response data in cases where agonists produced dose-related increases in circling rate (ropinirole and SKF-89124), to estimate the half-maximum effective dose (ED50). The logistic equation representing the 4-parameter logistic model for this fit was:

$$((A - D)/(1 + (10^{(x-C)B})) + D$$

where x is the $\log_{10}$ (dose), A is the response at zero dose (saline), B controls steepness of climb, C is the $\log_{10}$ (ED50), and D is the response at ∞ dose.

Results

Agonist radioligand binding affinity and functional data for SKF-89124 and the binding affinity for SKF-96990 are reported in Table 1. The rat metabolite, SKF-89124 was 30-fold more selective for D_3 over D_2 receptors as measured by radioligand binding (pK_i 8·4 and 6·9, respectively), but this selectivity was lost in the functional assay (pEC50 = 8·6 for both D_3 and D_2 receptors.

Nine rats produced contraversive circling after apomorphine administration. These rats were selected for further use in the knowledge that robust apomorphine-induced contraversive rotation only occurs after 60–70% striatal dopamine depletion and denervation (Costall et al 1976; Przedborski et al 1995). Although data for each animal was not complete, it was still possible to obtain information on between-rat variability and test it's significance on response. Initial analysis of the data showed that there was no significant variability between rats and this could be excluded as a factor from the analysis model. Analysis of the drug-induced mean rotation rates for transformed data by one factor analysis of variance, revealed an overall significant treatment effect ($F(17, 66) = 25{\cdot}96$; $P < 0{\cdot}0001$). The log dose transformed response curves for the dopamine agonists are shown in Figure 1.

Ropinirole caused a dose-related increase in contraversive rotation which was significantly different from saline at doses of 0·2, 0·8 and 3·2 mg kg^{-1} ($P < 0{\cdot}05$). SKF-89124 produced a very similar behavioural profile with minimal circling at 0·05 mg kg^{-1} and a significant increase in circling at doses of 0·2, 0·75 and 3·2 mg kg^{-1} ($P < 0{\cdot}05$), and reaching asymptote at a dose of 0·75 mg kg^{-1}. The estimated ED50 values for ropinirole and SKF-89124 were 0·10 and 0·16 mg kg^{-1}, respectively. SKF-97930 (0·8–15 mg kg^{-1}) and SKF-96990 (3·2–15 mg kg^{-1}) did not increase circling above the rate observed after saline treatment. SKF-104557 caused a small and significant increase in circling rate only after the maximum dose of 15 mg kg^{-1} ($P < 0{\cdot}05$). At that dose, the SKF-104557-induced circling response was less than half of that produced by the optimum dose of ropinirole (0·8 mg kg^{-1}) and an ED50 could not be obtained. SKF-104557 was approximately 15-fold less potent than ropinirole.

Table 1. Radioligand binding affinity (pK_i) and functional potency (pEC50) at human dopamine receptor subtypes.

Compound	D_2		D_3	
	pK_i	pEC50	pK_i	pEC50
SKF-89124	6·9 ± 0·2	8·6 ± 0·1	8·4 ± 0·2	8·6 ± 0·2
SKF-96990	6·5	NT	7·6	NT

Results are means ± s.e.m., n = 3–6 for radioligand binding affinity, and n = 5–9 for functional potency (microphysiometer) except for SKF-96990 (n = 1). NT, not tested.

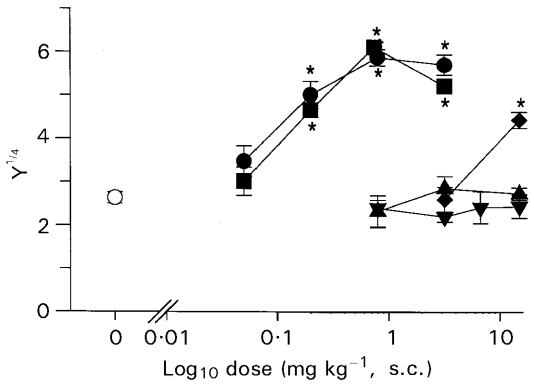

Figure 1. Effect of ropinirole and its metabolites on contraversive circling in rats with 6-hydroxydopamine lesions of the left nigrostriatal pathway. Circling rates are presented as $Y^{1/4}$ transformed data ± s.e.m. from corresponding transformed data, where Y = the number of contraversive circles completed in 117 min post-dose. *$P < 0{\cdot}05$ significantly different from saline. ○, saline (n = 8); ●, ropinirole (SKF-101468; n = 7–8); ■, SKF-89124 (n = 3–4); ▲, SKF-97930 (n = 3–4); ▼, SKF-96990 (n = 3–4); ◆, SKF-104557 (n = 3–4).

Discussion

Previous pharmacokinetic studies of ropinirole have shown that a range of metabolites are produced, the balance of which differ with the species being studied. In rats, the major products consist mainly of the unchanged parent compound and SKF-89124 or its glucuronide (Mico et al 1986; Swagzdis & Mico 1986; De Marinis & Hieble 1989; Ramji et al 1999). In cynomolgus monkey, SKF-104557, SKF-97930 and SKF-96990 glucuronide have been identified, but with little free base of SKF-89124 being formed (Beattie & Blake 1989; Ramji et al 1999). In man, ropinirole metabolism is more similar to non-human primates than rats as the major metabolite is SKF-104557, accounting for 37% of the parent compound, while other metabolites are produced in far lower quantities (Beattie & Blake 1989; Ramji et al 1999). Having discovered the metabolic route of ropinirole, it was possible to investigate the pharmacological profile of its structurally related

metabolites. Our aim was to determine if SKF-89124 and SKF-96990 had any appreciable affinity for the dopamine D_2 or D_3 receptor, and whether SKF-89124 possessed agonist or antagonist properties as defined in the microphysiometer functional assay. It has been previously shown (Coldwell et al 1999b) that ropinirole was 20-fold selective for the D_3 over the D_2 receptor (pK_i 7·1 and 5·8, respectively). In the microphysiometer assay, ropinirole was 10-fold selective for the D_3 over the D_2 receptor (pEC50 8·4 and 7·4, respectively). In the radioligand binding assay, SKF-104557 had similar affinity and selectivity to ropinirole, exhibiting 20-fold selectivity for the D_3 over the D_2 receptor (pK_i 7·0 and 5·7, respectively). In the functional assay, again similar to ropinirole, SKF-104557 was 10-fold selective for the D_3 over the D_2 receptor (pEC50 7·2 and 6·2, respectively). SKF-97930 was inactive at both D_2 and D_3 receptors (Coldwell et al 1999b). Data reported here show that the most potent compound, as measured by radioligand binding and function at D_2 and D_3 receptors, was SKF-89124. However, this is only a minor human metabolite
of ropinirole accounting for less than 5% of the ropinirole dose (Ramji et al 1999). SKF-89124 showed a maximum response which was equivalent to that shown by quinpirole (data not shown). As quinpirole has been previously shown to have a maximum response equivalent to dopamine (Coldwell et al 1999a) this suggests that SKF-89124 is a full agonist. In that study all measures of radioligand binding and function at the D_2 and D_3 receptors of the human metabolites SKF-104557 and SKF-97930 were less potent than ropinirole. Generally, data from both studies showed the agonists to have greater D_3 selectivity in radioligand binding than in the functional assays and this was particularly true of SKF-89124. This was mainly due to increased functional potency at the D_2 receptor compared with radioligand binding. This phenomenon has been reported previously for dopamine receptor agonists whereas in the case of antagonists, receptor affinity agreed more closely with functional potency (Chio et al 1994; Sautel et al 1995; Coldwell et al 1999a, b). With the recombinant systems used in this and previous (Coldwell et al 1999b) studies, it has proved difficult to identify high affinity, partial agonists at human D_2 and D_3 receptors. Ropinirole, SKF-104557 (Coldwell et al 1999b) and SKF-89124 (data not shown) are full agonists at the D_2 and D_3 receptors in these recombinant systems. This is possibly due to the high receptor density common in such recombinant systems and which leads to a high receptor reserve. This is the likely explanation for the relatively high functional potency of SKF-89124 at both D_2 and D_3 receptors compared with the parent compound, ropinirole, which in functional studies, is somewhat D_3 selective (Coldwell et al 1999b). Thus, the relative efficacy of ropinirole and its metabolites cannot be accurately determined at recombinant dopamine receptors and, at this stage, the receptor reserve of D_2 and D_3 receptors in the human brain is unknown.

In the 6-OHDA circling model, only two of the compounds tested, ropinirole and the major rat metabolite SKF-89124, produced any appreciable increase in the rate of circling. The peak circling rate in both cases was reached at $0{\cdot}8\ mg\ kg^{-1}$, and thereafter reached asymptote. After subcutaneous administration, ropinirole was 180-fold more potent in this study than when administered by gavage (Fukuzaki et al 2000). As SKF-89124 is a minor metabolite of ropinirole in man, it is unlikely to play a major role in the antiparkinsonian efficacy of ropinirole even though it showed equivalence to ropinirole in the circling model. Similarly, the low behavioural potency of the three primary metabolites of ropinirole, namely SKF-104557, SKF-97930 and SKF-96990, suggests that these compounds do not contribute significantly to the in-vivo activity of ropinirole in this model of Parkinson's disease. This lack of in-vivo activity of SKF-104557 is difficult to explain considering that the radioligand binding data for ropinirole and SKF-104557 are very similar (Coldwell et al 1999b). However, in a previous study in monkeys (Ramji et al 1999) where radiolabelled ropinirole was administered intravenously, ropinirole accounted for the majority of the radioactivity in the brain and only a small proportion in the plasma, whereas SKF104557 was the major component detected in the plasma. Therefore, it seems likely that SKF-104557 is not notably brain-penetrant whereas ropinirole enters the brain freely. Given that dopamine D_2 receptors are more abundant than D_3 receptors (Murray et al 1994; Gurevitch & Joyce 1999), and ropinirole and its metabolites possess only marginal or no selectivity for the D_3 over the D_2 receptors as found in this and previous (Coldwell et al 1999b) studies, no conclusions can be drawn about the relative contribution of either receptor to antiparkinsonian mechanisms until more selective agents become available.

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