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Pharmacology, Biochemistry and Behavior 82 (2005) 140 – 147

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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A-412997 is a selective dopamine D_4 receptor agonist in rats

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Received 23 March 2005; received in revised form 15 July 2005; accepted 8 August 2005
Available online 8 September 2005

Abstract

A-412997 (2-(3′,4′,5′,6′-tetrahydro-2′*H*-[2,4′] bipyridinyl-1′-yl)-N-m-tolyl-acetamide) is a highly selective dopamine D_4 receptor agonist that binds with high affinity to rat dopamine D_4 and human dopamine $D_{4.4}$ receptors (K_i=12.1 and 7.9 nM, respectively). In contrast to the dopamine D_4 receptor agonists PD168077 and CP226269, A-412997 showed a better selectivity profile and no affinity <1000 nM for other dopamine receptors or any other proteins in a panel of seventy different receptors and channels. In functional assays using calcium flux, A-412997 was a potent full agonist at rat dopamine D_4 receptors (28.4 nM, intrinsic activity=0.83) and did not activate rat dopamine D_{2L} receptors, unlike CP226269. Dopamine D_4 receptor selective agonists have been shown to induce penile erection in rats by central mechanisms. A-412997 induces penile erection in a conscious rat model (effective dose=0.1 μmol/kg, s.c.) with comparable efficacy as the nonselective D_2-like agonist, apomorphine. When dosed systemically, A-412997 crossed the blood brain barrier rapidly and achieved significantly higher levels than PD168077. A-412997 is a highly selective dopamine D_4 receptor agonist and a useful tool to understand the role of dopamine D_4 receptors in rat models of central nervous system processes and disease.

Keywords: Dopamine; D_4 receptor; Penile erection; A-412997

1. Introduction

Dopamine is the major catecholamine neurotransmitter in the central nervous system and plays a key role in a variety of processes including sexual behavior, cognition, motor coordination, hormonal and cardiovascular control. These different behaviors and processes are coordinated by the action of dopamine on two different classes of G-protein coupled dopaminergic receptors: D_1-like which increase adenylate cyclase activity through G_s and D_2-like which inhibit adenylate cyclase through $G_{i/o}$ (Kebabian and Calne, 1979; Missale et al., 1998). The D_2-like receptors include D_2, D_3 and D_4, each receptor varying by location and abundance in the CNS (Khan et al., 1998). Due to the complex interactions of dopaminergic receptors, relative abundance and role of different dopaminergic subtypes, it has been difficult to delineate the role of individual receptor subtypes, despite the generation of dopamine receptor gene deleted animals (Gan et al., 2004; Viggiano et al., 2003). To date, most reported D_2-like receptor agonists activate more than one receptor subtype, further complicating the interpretation of in vivo pharmacology (Millan et al., 2002; Moreland et al., 2004a).

The dopamine D_4 receptor is localized predominately to the cerebral cortex as well as hippocampus and hypothalamus (Ariano et al., 1997; Khan et al., 1998). Radioligand binding studies using a selective dopamine D_4 receptor agonist corroborate the immunohistochemical localization of the receptor (Moreland et al., 2004b). The dopamine D_4 receptor has been proposed to play a role in penile erection, cognition and attention (Brioni et al., 2004; Hrib, 2000; Oak et al., 2000). One of the major hurdles in defining dopamine D_4 receptor function in the CNS has been selective agonists which allow the activation of the receptor in animal models. In this report, we describe the

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0091-3057/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2005.08.001

highly selective dopamine D_4 receptor ligand A-412997 that is a full agonist at rat dopamine D_4 receptors and has superior selectivity compared to reported D_4 agonists PD168077 and CP226269.

2. Materials and methods

2.1. Synthesis of 2-(3′,4′,5′,6′-tetrahydro-2′H-[2,4′]bipyridinyl-1′-yl)-N-m-tolyl-acetamide (A-412997.0)

A-412997 was prepared from a five-step synthesis. As none of the intermediates are commercially available, these intermediates are detailed below (Fig. 1).

2.1.1. Synthesis of 4-trifluoromethanesulfonyloxy-3,6-dihydro-2H-pyridine-1-carboxylic acid tert-butyl ester (compound 2, Fig. 1)

To a solution of diisopropylamine (13.4 ml, 96 mmol) in tetrahydrofuran (350 ml) at −78 °C was added 1.6 M *n*-butyllithium in hexane (60 ml, 96 mmol). The reaction mixture was stirred for 5 min at −78 °C. A solution of *t*-butoxycarbonyl-4-piperidone 1 (16 g, 80 mmol) in tetrahydrofuran (100 ml) was added and the reaction mixture was stirred for 10 min. Then a solution of N-phenyltrifluoromethanesulfonimide (31.4 g, 88 mmol) was added. The reaction mixture was stirred at −78 °C for 30 min and the cooling bath was removed to warm it up to room temperature (~1.5 h). The reaction was quenched by saturated $NaHCO_3$ followed by extraction with ethyl ether and 5% citric acid. The organic layer was then washed with 1 NaOH (4 × 200 ml) and water (2 × 200 ml) followed by saturated sodium chloride (1 × 200 ml), dried over $MgSO_4$ and evaporated on rotary evaporator to give yellowish oil. Purification by flash chromatography using hexane : ethyl acetate 8 : 2 as eluent gave 18 g of pure compound 2 as colorless oil and 5 g with ~20% impurity. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.41 (s, 9H), 2.41 (m, 2H), 3.54 (t, 2H), 3.98 (m, 2H), 6.02 (m, 1H).

*2.1.2. Synthesis of 3′,6′-dihydro-2′H-[2,4′]bipyridinyl-1′-carboxylic acid **tert**-butyl ester (compound 3, Fig. 1)*

4-Trifluoromethanesulfonyloxy-3,6-dihydro-2*H*-pyridine-1-carboxylic acid *tert*-butyl ester (compound 2, Fig. 1) (18 g, 54 mmol) in tetrahydrofuran (~200 ml) was treated with 2-pyridylzinc bromide 0.5 M solution in tetrahydrofuran (124 ml, 62.5 mmol, 1.15 eq.) followed by $Pd(PPh_3)_4$ (625 mg). The reaction mixture was heated at 60 °C for 90 min. All of the triflate was utilized (checked by thin layer chromatography). The tetrahydrofuran was removed by rotary evaporator. Ethyl acetate (300 ml) and 1 N NaOH (200 ml) were added to the residue. Zinc salts were filtered, organic layer was separated and washed with saturated sodium chloride (300 ml), dried (MgSO4) and concentrated on the rotary evaporator to give brown oil. Purification by flash chromatography using Hexane : EtOAc 6 : 4 as eluent gave 9.0 g (64% yield) of compound 3 as colorless oil and 4 g with ~15–20% impurity. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.43 (s, 9H), 2.56 (m, 2H), 3.54 (t, 2H), 4.04 (m, 2H), 6.08 (m, 1H), 7.25 (dd, 1H), 7.56 (d, J = 9 Hz, 1H), 7.77 (m, 1H), 8.54 (m, 1H); MS (DCI-NH_3) m/z 259 $(M+H)^+$, 277 $(M+H+18)^+$.

2.1.3. Synthesis of 3′,4′,5′,6′-tetrahydro-2′H-[2,4′]bipyridinyl-1′-carboxylic acid tert-butyl ester (compound 4, Fig. 1)

3′,6′-Dihydro-2′*H*-[2,4′]bipyridinyl-1′-carboxylic acid *tert*-butyl ester (compound 3) (9 g, 34 mmol) in methanol (150 ml) was hydrogenated using Pd/C at room temperature and 60 psi for 3.5 h to give 8.89 g (96% yield) of compound 4 as pale yellow oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.41 (s, 9H), 1.58 (m, 2H), 1.81 (m, 2H), 2.85 (m, 3H), 4.06 (m, 2H), 7.20 (dd, 1H), 7.28 (d, J = 9 Hz, 1H), 7.70 (m, 1H), 8.48 (m, 1H).

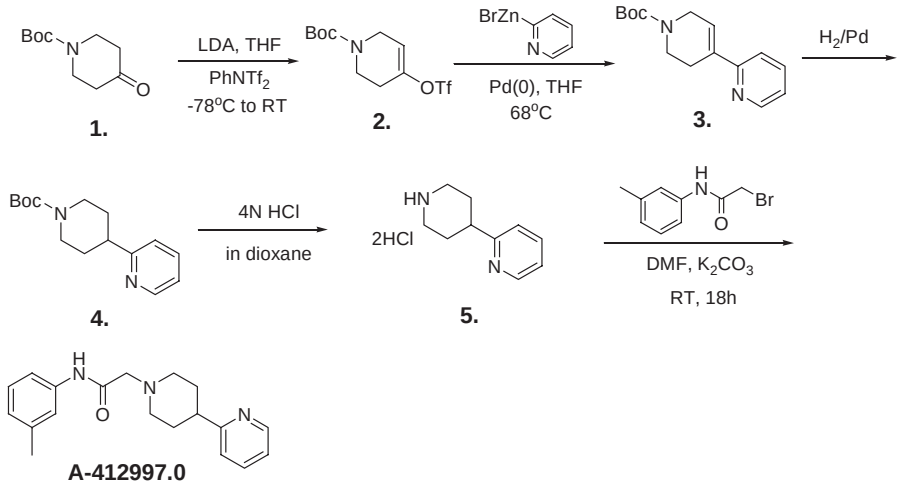

Fig. 1. Synthesis of A-412997.

2.1.4. Synthesis of 1′,2′,3′,4′,5′,6′-hexahydro-[2,4′] bipyridinyl dihydrochloride (compound 5, Fig. 1)

A solution of 3′,4′,5′,6′-tetrahydro-2′*H*-[2,4′]bipyridinyl-1′-carboxylic acid *tert*-butyl ester compound 4 (2.8 g, 10.4 mmol) in dioxane (50 ml) was treated with 4 N HCl in dioxane (30 ml) and the reaction mixture was stirred for 1 h. The solvent was removed on the rotary evaporator to give 2.3 g (96% yield) of compound 5 as pale yellow solid.

2.1.5. Synthesis of 2-(3′,4′,5′,6′-tetrahydro-2′H-[2,4′]bipyridinyl-1′-yl)-N-m-tolyl-acetamide (412997.0)

A mixture of 1′,2′,3′,4′,5′,6′-hexahydro-[2,4′]bipyridinyl dihydrochloride (900 mg, 3.86 mmol), 2-bromo-N-m-tolyl-acetamide (880 mg, 3.86 mmol) and K_2CO_3 (1.6 g, 11.58 mmol) in DMF (20 ml) was stirred at room temperature for 18 h. The reaction mixture was poured into water (30 ml) and extracted with ethyl acetate (20 ml). The organic layer was washed with saturated sodium chloride (2×30 ml) and dried over $MgSO_4$, filtered and concentrated in vacuo and purified by flash chromatography using EtOAc : EtOH, 9.2 : 0.8 to give the desired product 850 mg (yield: 71%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.83 (m, 4H), 2.24 (m, 5H), 2.64 (m, 1H), 2.98 (m, 2H), 3.12 (s, 2H), 3.78 (s, 3H), 6.88 (d, J=6 Hz, 1H), 7.20 (m, 2H), 7.30 (d, J=6 Hz, 1H), 7.45 (d, J= 6 Hz, 2H), 7.71 (m, 1H), 8.51 (m, 1H), 9.59 (bs, 1H); MS (DCI-NH_3) m/z 310 $(M+H)^+$. Elemental analysis calculated for $0.15H_2O.C_{19}H_{23}N_3O$: C, 73.12; H, 7.52; N, 13.46; found: C, 72.72, H, 7.24, N, 13.28.

2.2. Cell lines

The human dopamine D_{2L}, D_3 and $D_{4.4}$ receptors co-expressed with $G\alpha_{qo5}$ in HEK-293 cells were prepared as previously described (Moreland et al., 2004a). The rat dopamine D_4 receptor was cloned as previously described and co-expressed with $G\alpha_{qo5}$ in HEK-293 cells (Moreland et al., 2004b). The rat dopamine D_{2L} receptor was cloned as described for human D_{2L} and stable cell lines constructed with $G\alpha_{qo5}$ in HEK-293 cells.

2.3. FLIPR assay

Assays were carried out as previously reported (Moreland et al., 2004a). Briefly, cells were plated into 96-well, black-wall/clear-bottom microplates (Biocoat, Becton Dickinson) at 20 000 cells per well. After two days of culture, the culture medium was removed by aspiration and replaced by 0.1 ml of Dulbecco's Phosphate Buffered Saline with D-glucose and sodium pyruvate (DPBS) containing 0.04% Pluronic F-127 and 4 μM Fluo-4, fluorescent calcium indicator dye. After incubation for 1 h at room temperature, the cells were washed four times with DPBS in a plate washer (Molecular Devices). After the final wash, 150 μl of DPBS was added to each well. Fluorometric imaging plate reader (FLIPR384, Molecular Devices) transferred 50 μl from the compound plate to the cells and made fluorescence readings for 3 min (every second for the first minute and every 5 s for the next 2 min). All the data were normalized with the response of 10 μM dopamine.

2.4. Radioligand binding assays

Membranes were prepared from stable cell lines expressing either the human dopamine D_{2L} or D_3 receptor in HEK-293 cells (gift of Dr. Liliane Unger, Abbott Laboratories, Ludwigshaven, Germany), or the human dopamine $D_{4.4}$ or rat dopamine D_4 receptor-transfected HEK-293 cells as previously described (Moreland et al., 2004a,b). For membrane preparation, the cells were seeded into a Cell Factory (VWR) and the confluent cells were detached with cell dissociation buffer (Invitrogen). The cell pellet was homogenized using a Polytron for 10 s in 50 mM Tris–HCl, pH 7.4 and centrifuged at 30 min for 100 000 $\times g$. Membrane aliquots were stored at −80 °C until use.

Agonist radioligand assays using [^{125}I]-PIPAT (Amersham) for D_{2L}, [^{3}H]-7-OH-DPAT (Amersham) for D_3 and [^{3}H]-A-369508 for D_4 were carried out as reported (Moreland et al., 2004a,b). Radioligand competitive binding assays for rat serotonin 5-HT_{1A} (5-HT_{1A}) receptor were carried out using membranes derived from rat cortex and the agonist [^{3}H]-8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) (NE Nuclear, Boston, MA) (De Vry et al., 1998). Binding assays for human alpha 1 and 2 adrenoceptor subtypes were carried out as previously described (Esbenshade et al., 2003). Radioactivity was measured by TopCount Microplate Scintillation Counter (Packard). Proteins were determined by BCA Protein Assay Kit (Pierce) using BSA as a standard. Competition curves for non-radioactive compounds were analyzed by nonlinear regression using curve-fitting program (Prism, GraphPad Software). All assays were performed in triplicate and IC_{50} values were converted to K_i values (Cheng and Prusoff, 1973).

2.5. Pharmacokinetics and brain levels

Male Sprague–Dawley rats (approximate body weight 250 g, n=3/group) received subcutaneous doses of PD168077 (0.3 μmol/kg) or A-412997 (0.01, 0.03, 0.1 and 0.3 μmol/kg). The compounds were prepared as solutions in isotonic dextrose (D5W) containing 10% ethanol at concentrations appropriate for a 1 ml/kg dose volume with each treatment. At selected time points after dosing, groups of three rats were euthanized with CO_2 and exsanguinated by cardiac puncture. The brain tissues were removed from each rat. Plasma and brains were stored frozen (−20 °C) prior to analysis. Brain tissue was homogenized with three volumes of saline. Parent drug was removed from measured aliquots of brain homogenate and plasma using a single liquid–liquid extraction with

ethyl acetate : hexane (1 : 1, by volume) at alkaline pH. The organic phase was evaporated to dryness with a gentle stream of dry nitrogen at low heat (~35 °C). Spiked plasma and brain homogenate standards were analyzed simultaneously with the samples. Following reconstitution with mobile phase, the parent drug was separated from co-extracted contaminants using reverse phase HPLC with MS detection and quantitation. Limits of quantitation averaged ~0.3 ng/ml(g) for all three compounds.

2.6. Rat penile erection assay

Assays for penile erection in conscious rats were performed as described (Hsieh et al., 2004). Male adult Wistar rats, weighing 300 g, were used as an animal model to study penile erection in vivo. All experiments were carried out between 9:00 AM and 3:00 PM. On the day of testing, animals were allowed to adapt to a diffusely illuminated testing room with red light for 1 h before the start of the experiment. Rats were placed individually into a transparent Plexiglas cage (20 × 30 × 30 cm) immediately after the drug injection. A mirror was placed behind and under the observation cages to facilitate observation of the animals. Each rat was used only once. A penile erection was considered to occur when the following behaviors were presented: repeated pelvic thrusts immediately followed by an upright position, and an emerging, engorged penis that the rat proceeded to groom. Apomorphine or other compounds were freshly prepared and administered to rats via subcutaneous injection into the back neck area (1 ml/kg injection volume). Clozapine was dissolved in 0.05% acetic acid. In the pharmacological blockade experiments, rats were injected either i.p. with 1, 3 or 10 μmol/kg clozapine (D_4 preferential) before s.c. A-412997 (0.1 μmol/kg, s.c.) injection.

The penile erection episodes were recorded by direct observation for a period of 60 min after the compound dosing, and erection incidence (percentage) was defined as the percentage of animals exhibiting one or more erections during the observation period. Data were expressed as incidence (percentage) ± S.E. calculated by using Wald equation. Statistical evaluation of the results was performed by χ^2 test. A $p < 0.05$ was considered significant. The number of penile erections was also counted and the data, expressed as mean ± S.E.M. of erection over the observation period, were analyzed by the Mann–Whitney nonparametric test. A $p < 0.05$ was considered significant.

2.7. Materials

Fluo-4 and Pluronic F-127 were purchased from Molecular Probes. DPBS, neomycin (G418), hygromycin B and tissue culture reagents were from Invitrogen/Life Technologies. A-412997, CP226269 (5-fluoro-2-(4-pyridin-2-yl-piperazin-1-ylmethyl)-1*H*-indole) (Cowart et al., 2004), PD168077 (N-[4-(2-cyanophenyl)-piperazin-1-ylmethyl]-3-methyl-benzamide) (Matulenko et al., 2004) and PNU95666E (5-methylamino-5,6-dihydro-1*H*,4*H*-imidazo[4,5,1-ij]quinolin-2-one) (Heier et al., 1997) were synthesized by Abbott Laboratories. All other chemicals were purchased from Sigma unless otherwise noted.

3. Results

3.1. Receptor selectivity

The synthesis and structure of A-412997 are shown in Fig. 1. A-412997 is a selective agent for dopamine D_4 receptors binding with high affinity to both rat and human dopamine D_4 receptors (K_i = 12.1 and 7.9 nM, respectively) in competition binding assays (Table 1). Agonist radioligands were used in the determinations for D_2-like receptors (see Methods) as it has been shown that these reflect the EC_{50} of the receptors (Moreland et al., 2004a,b). For example, comparable studies using the antagonist spiperone on human dopamine $D_{4.4}$ receptor for A-412997, PD168077 and CP226269 are approximately 2× less avid (20.7, 16.2, and 2.5 nM, respectively) compared to [^{3}H]-A-369508 (7.9, 5.7 and 1.1 nM, respectively). Nevertheless, it should be noted that the agonist radioligand measures the high affinity form of the receptor. The selectivity of A-412997 was determined for more than 70 different neurotransmitter receptors and ion channels (Cerep, Paris, France). Those sites showing specific binding <1 μM as well as binding to dopamine receptors are shown in Table 1. A-412997 had no affinity (>10 μM) for adenosine (A_1, A_{2A} or A_3), angiotensin (AT_1, AT_2), β-adrenergic, benzodiazepine, bombesin, CCR1, CXCR2, CGRP, cannabanoid (CB_1 or CB_2), cholecystikinin (CCK_1 and CCK_2), endothelin (ET_A and ET_B), GABA, galanin (GAL-1), histamine (H_2, H_3), ML1, muscarinic acetylcholine (M_1, M_2, M_3, M_4 and M_5), neurokinin (NK_1, NK_2, and

Table 1
Receptor selectivity of A-412997, PD168077 and CP226269

Receptor	A-412997	PD168077	CP226269
	K_i (nM)	K_i (nM)	K_i (nM)
Human D_1	>10000	>10000	>10000
Human D_{2L}	2848±887	1049±72.1	121±9.0
Human D_3	2095±328	2480±557	507±27.5
Human $D_{4.4}$	7.9±0.9	5.7±0.3	1.1±0.1
Rat D_4	12.1±1.5	6.1±0.4	1.0±0.1
Human D_5	>10000	>10000	>10000
Bovine α_{1A}	1035.3±16.7	133.0±16.7	511.6±46.2
Human α_{2A}	2972±310	1165±65	66.9±5.1
Human α_{2C}	6386±917	438.4±71.6	146.2±19.4
Rat 5-HT_{1A}	1006±61.8	252.0±38.8	311.0±15.8
Human 5-HT_{1B}	>10000	>300	>10000
Human κ	>10000	>10000	>300

Data were determined either by CEREP (average of triplicate samples) or as described in Methods for human dopamine D_{2L}, D_3, $D_{4.4}$ receptors, rat dopamine D_4 receptor or rat serotonin 5-HT_{1A} receptor (rat cortex).

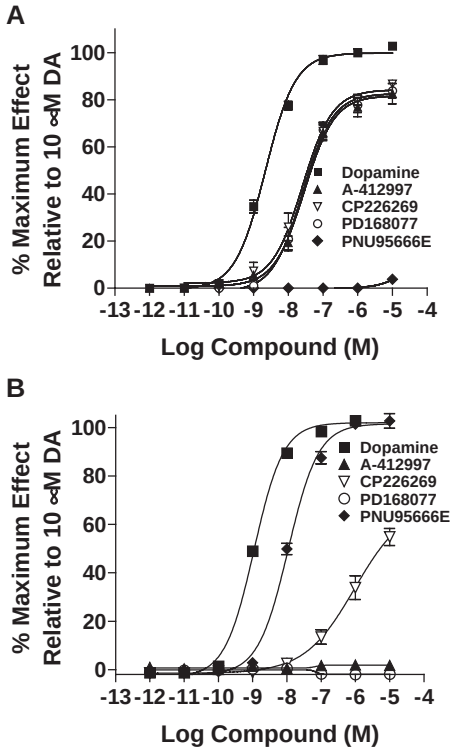

Fig. 2. Effects of agonists rat dopamine D_4 and D_{2L} $G\alpha_{qo5}$ HEK cell lines. Panel A. Rat dopamine D_4 receptor cell line. Effects of D_2-like agonists dopamine (closed squares), D_4 selective agonists A-412997 (closed triangles), CP226269 (inverted closed triangles), PD168077 (closed circles) and D_2-selective agonist PNU95666E (closed diamonds). Panel B. Rat dopamine D_{2L} receptor cell line. Effects of D_2-like agonists dopamine (closed squares), D_4 selective agonists A-412997 (closed triangles), CP226269 (inverted closed triangles), PD168077 (closed circles) and D_2-selective agonist PNU95666E (closed diamonds). Data points represent mean ± SEM. $n = 4–22$.

NK_3), neuropeptide Y (Y_1 and Y_2), neurotensin (NT1), opioid (δ, κ, and μ), ORL1, PACAP, PCP, prostanoid (thromboxane and prostacyclin), purinergic (P2X, P2Y), serotonin (5-HT_3, 5-HT_{5A}), somatostatin, TNF-α, VIP1, and vasopressin (V1A) receptors. A-412997 also did not bind to the norepinephine transporter or L-type calcium, K_v^+, SK_{Ca}^+, sodium or chloride channels. Two D_4 partial agonist ligands have been reported, PD168077 and CP226269 (Hrib, 2000). PD168077 is selective across dopamine receptors, binding only to the dopamine D_4 receptor and it is equipotent at rat and human receptors. However, PD168077 has affinity for α_{1A}-adrenoceptors ($K_i = 133$ nM), α_{2C}-adrenoceptors ($K_i = 438$ nM) as well as serotonin 5-HT_{1A} and 5-HT_{1B} receptors ($K_i = 252$ and 300 nM, respectively). CP226269 binds potently to rat and human dopamine D_4 receptors but also has affinity for the human dopamine D_{2L} receptor when assayed in the presence of agonist radioligand [^{125}I]-PIPAT ((*R*,*S*)-2′-*trans*-7-hydroxy-2-[N-*n*-propyl-N-(3′-iodo-2′-propenyl)-amino]tetralin). Further, CP226269 binds to human dopamine D_3 receptors, despite 500 fold selectivity for dopamine D_4 receptors (Moreland et al., 2004a). CP226269 also has affinity for α_2-adrenoceptors ($K_i = 67$ and 146 nM at human α_{2A} and $_C$, respectively) as well as serotonin 5-HT_{1A} ($K_i = 311$ nM) and human kappa opiate receptors ($K_i = 300$ nM, respectively). In contrast to PD168077 and CP226269, A-412997 showed a superior selectivity profile and did not bind to other dopamine receptors or any other in a panel of seventy different receptors and channels ($K_i > 1000$ nM).

3.2. Agonist activity

In order to assess functional activity at rat dopamine D_{2L} and D_4 receptors, stable cell lines were prepared co-expressing the receptors with the chimeric G-protein $G\alpha_{qo5}$ $_{qo5}$ and assessing calcium flux using a fluorescent imaging plate reader (FLIPR) as previously reported for human dopamine D_2-like receptors (Gopalakrishnan et al., 2003; Moreland et al., 2004a). Dopamine induced calcium flux in the rat dopamine D_4 receptor cell line as well as D_2-like nonselective agonists apomorphine and quinpirole with EC_{50}s ranging from 2.4 to 15.9 nM. All of these ligands were full agonists (intrinsic activity relative to dopamine ≥0.8). The dopamine D_2 receptor selective agonist PNU956666E had no detectable activity in this assay. A-412997, PD168077 and CP226269 were all full agonists and activated the rat dopamine D_4 receptor with $EC_{50} = 26–29$ nM (Fig. 2A, Table 2). The response of 1 μM dopamine in this cell line could be blocked by the dopaminergic antagonists haloperidol, domperidone, clozapine ($K_b = 10.7$, 5.5 and 4.2 nM, respectively).

A similar cell line was prepared using the rat dopamine D_{2L} receptor. Dopamine induced calcium flux in the rat dopamine D_{2L} receptor cell line as well as D_2-like non-selective agonists apomorphine and quinpirole with EC_{50}s ranging from 0.4 to 3.0 nM (Fig. 2B, Table 3). All of these ligands were full agonists. The dopamine D_2 receptor selective agonist PNU956666E potently induced calcium flux ($EC_{50} = 10.8$ nM, intrinsic activity = 0.79). A-412997 and PD168077 had no activity in this assay ($EC_{50} > 10\,000$ nM). However, CP226269 potently activated the rat dopamine D_{2L} receptor as a full agonist ($EC_{50} = 54.9$ nM, instrinsic activity = 0.89). This result may be due to a species

Table 2
Characterization of dopaminergic agonists in rat dopamine D4 receptor cell line

Compound	Rat D_4 FLIPR EC_{50} (nM)	Intrinsic activity
Dopamine	2.4 ± 0.2	1
Apomorphine	5.5 ± 0.3	0.87
Quinpirole	15.9 ± 1.2	0.89
PNU95666E	> 10 000	–
A-412997	28.4 ± 1.9	0.83
PD168077	26.1 ± 3.4	0.83
CP226269	28.9 ± 6.7	0.85

Agonist activity was assayed by concentration response curves of various compounds with and intrinsic activities expressed relative to 10 μM dopamine (1.0). All determinations represent the mean ± SEM, $n = 4$.

Table 3
Characterization of dopaminergic agonists in rat dopamine D_{2L} receptor cell line

Compound	Rat D2L FLIPR EC_{50} (nM)	Intrinsic activity
Dopamine	1.1±0.06	1
Apomorphine	0.4±0.01	1
Quinpirole	3.0±0.2	1
PNU95666E	10.8±1.0	0.87
A-412997	>10000	–
PD168077	>10000	–
CP226269	54.9±5.3	0.89

Agonist activity was assayed by concentration response curves of various compounds with and intrinsic activities expressed relative to 10 μM dopamine (1.0). All determinations represent the mean±SEM, $n=4$.

difference, as CP226269 did not activate human D_{2L} (Moreland et al., 2004a). The response of 1 μM dopamine in this cell line could be blocked by the dopaminergic antagonists haloperidol and domperidone ($K_b=0.2$, and 1.0, respectively). Clozapine did not inhibit dopamine induced calcium flux in the rat dopamine D_{2L} receptor cell line ($IC_{50}>10000$ nM).

3.3. Brain levels of A-412997 after dosing

Plasma and brain levels were examined after subcutaneous dosing in rats. At the three doses examined (0.03, 0.1 and 0.3 μmol/kg), A-412997 partitions rapidly into the brain (Fig. 3), with the maximum levels at the first sampling time point (5 min) and brain to plasma ratios >1:1 at all time points. At an effective dose for penile erection (0.1 μmol/kg), brain concentrations averaged 24 ng/g, with peak plasma levels (C_{max}) of 15 ng/ml. A 0.3 μmol/kg subcutaneous dose of PD168077 provided brain concentrations of ~23 ng/g. While the brain levels for A-412997 and PD168077 are similar at maximal efficacy (24 vs. 23 ng/g; Fig. 4), the doses required to provide these brain levels differ by a factor of three (0.1 vs. 0.3 μmol/kg for A-412997 and PD168077, respectively).

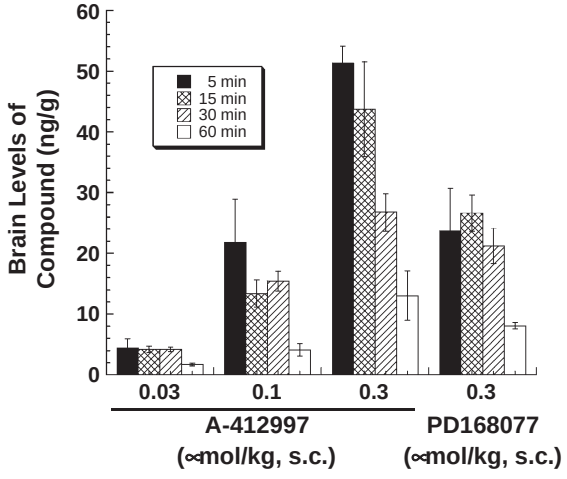

Fig. 3. Brain levels of A-412997 and PD168077 dosed systemically.

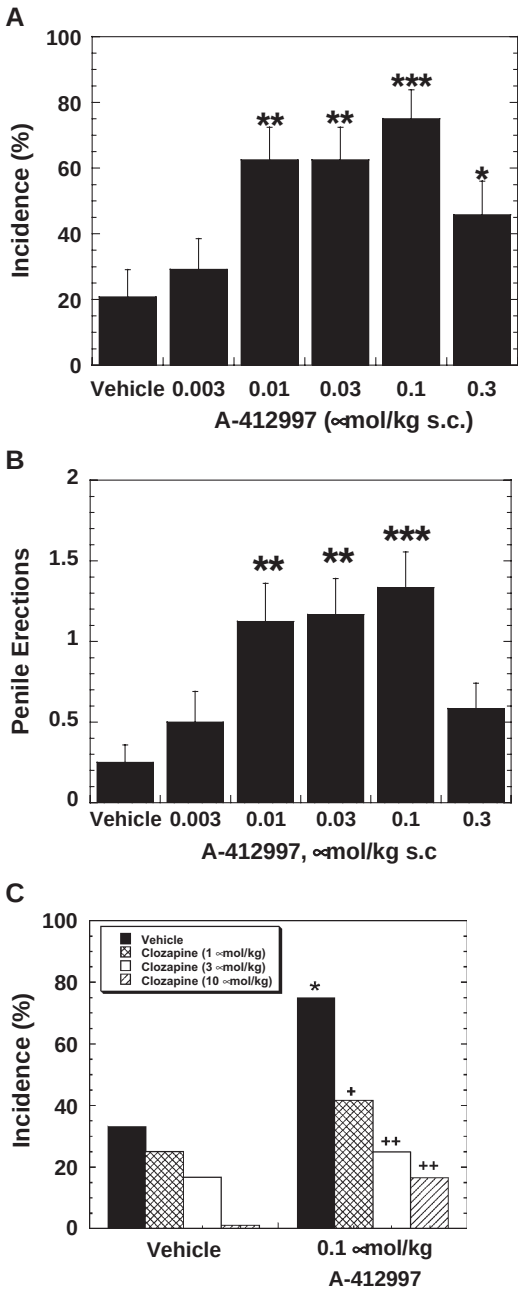

Fig. 4. Effects of A-412997 on penile erection in conscious rats. Panel A Percent incidence of penile erection vs. dose. Panel B. Number of erections vs. dose. In both panels $n=24$. Statistics $^{**}p<0.01$, $^{***}p<0.003$ comparing vehicle to effect. Panel C. Effects of pretreatment with clozapine on optimal dose of A-412997. Incidence of penile erections was determined as described in Methods. Clozapine was dosed 30 min before testing and administered intraperitoneally. Solid bars, no clozapine; hatched bars, 1 μmol/kg clozapine; open bars, 3 μmol/kg clozapine; and diagonal bars, 10 μmol/kg clozapine. For A-412997 and vehicle, $^{*}p<0.05$. $^{++}p<0.01$ vs. A-412997/clozapine plus A-412997.

3.4. Effects of A-412997 on penile erection in rats

It has been demonstrated that the D_2-like agonist apomorphine induces penile erection in conscious rats by centrally mediated mechanisms (Hsieh et al., 2004).

A-412997 induced penile erection in conscious rats when administered subcutaneously with a maximal effect at 0.1 μmol/kg whether the percent incidence (percent of rats having at least one erection) or the number of penile erections was determined (Fig. 4 Panels A and B, respectively). The maximum effective dose was 0.1 μmol/kg with a latency time to erection of 19 min. Apomorphine at the maximum effective dose 0.1 μmol/kg) gave comparable results to A-412997. PD168077 and CP226269 also induce penile erection in rats when dosed systemically with maximum effective doses of 0.3 and 1.0 μmol/kg, respectively (Hsieh et al., 2004). The erectogenic effects of A-412997 could be blocked by systemic pretreatment with either clozapine (1–10 μmol/kg i.p.) (Fig. 4C) which blocks the rat dopamine D_4 receptor in vitro. In all three clozapine treatments, the percent incidence of penile erection was no different than rats without 0.1 μmol/kg A-412997.

4. Discussion

In this report we describe A-412997, a full agonist at the rat dopamine D_4 receptor with superior selectivity compared to PD168077 and CP226269. A-412997 penetrates the brain and activates dopamine D_4 receptors as indicated by penile erection in conscious rats. Further, A-412997 crosses the blood brain barrier and achieves significantly higher brain levels at the efficacious dose required for penile erection than PD168077.

One of the challenges in understanding the function of dopamine D_4 receptors is their expression in the brain relative to other D_2-like receptor subtypes. This has complicated data interpretation in vivo due to a lack of receptor selective agonists. In this study, we characterized A-412997 and compared it with two reported dopamine D_4 receptor selective agonists CP226269 and PD168077. While all three compounds are full agonists at the rat dopamine D_4 receptor as measured in calcium flux assays, the three compounds differ in their profile of cross-reactivity and activation of other receptors. Despite a lack of activity at the human D_{2L} receptor (Moreland et al., 2004a), CP226269 is a full agonist at rat dopamine D_{2L} receptor as well as at the rat dopamine D_4 receptor (Fig. 2, Tables 2 and 3). Although potent at the rat dopamine D_4 receptor (EC_{50}=29 nM), CP226269 also binds to adrenergic, serotonergic and opioid receptors (Table 1). The interaction with other receptors may complicate in vivo pharmacology interpretations. For example, CP226269 has comparable potency in vitro at the rat dopamine D_4 receptor as A-412997 but is ten fold less efficacious in the rat penile erection assay (1.0 vs. 0.1 μmol/kg). PD168077 has also been reported as a dopamine D_4 receptor selective agonist but potential interaction with alpha adrenoceptors and serotonin 5-HT_{1A} receptors may complicate the interpretation of in vivo results. In one study, PD168077 dosed up to 50 μmol/kg induced nonstereotyped shuffling locomotion with uncoordinated movements, jerking, and yawning in rats, which was insensitive to antagonism by either D_4 selective antagonists or haloperidol, suggesting interaction with other receptors other than the dopamine D_4 receptors (Clifford and Waddington, 2000). In a second study, an effect enhancing memory was reported using inhibitory avoidance and PD168077, but doses in excess of 20 μmol/kg potentially confuse the interpretation on the results (Bernaerts and Tirelli, 2003).

The amount of intrinsic activity at the dopamine D_4 receptor may also complicate data interpretation in vivo. The partial D_4 agonist RO-10-5824 (intrinsic activity=0.36) showed trends toward increasing novel exploration in mice, but the effects did not reach significance (Powell et al., 2003). It will be of interest to repeat these studies using A-412997, a full D_4 agonist. Recently, ABT-724, a selective dopamine D_4 receptor agonist has been described that induces penile erection in rats. While the selectivity profiles are comparable, ABT-724 differs from A-412997 in that the latter is a partial agonist in the rat (0.62 vs. 0.82 intrinsic activity at rat D_4 in calcium flux assays) (Brioni et al., 2004). It should be noted that both in the ABT-724 study and an earlier report agonist at dopamine D_4 receptors induces penile erection in conscious rats by a supraspinal central mechanism (Brioni et al., 2004; Hsieh et al., 2004).

In conclusion, in this report we describe A-412997, a selective dopamine D_4 receptor full agonist with an excellent selectivity profile compared to PD168077 and CP226269. A-412997 also achieves brain levels sufficient for in vivo activation of the receptor as seen by rat penile erection. This compound is a useful tool to understand the role of dopamine D_4 receptors in rat models of central nervous system processes and disease.

Acknowledgements

The authors thank Drs. Marlon Cowart, Teodozyi Kolasa and Mark Matulenko for preparation of CP226269, PD168077 and PNU95666E. The authors acknowledge the excellent technical assistance of Renjie Chang, Diana Donnelly-Roberts, Loan N. Miller, Thomas Miller, Masaki Nakane, Marian T. Namovic, Marc A. Terranova, and Marie E. Uchic.

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