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Short communication

Neuropharmacological assessment of potential dopamine D_4 receptor-selective radioligands

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Abstract

Radiolabeled dopamine D_4 receptor-selective agents ([^{3}H]1-benzyl-4-[*N*-(3-isopropoxy-2-pyridinyl)-*N*-methyl]-aminopiperidine maleate; [^{3}H]PNU-101958, and [^{125}I]1-[4-iodobenzyl]-4-[*N*-(3-isopropoxy-2-pyridinyl)-*N*-methyl]-aminopiperidine; [^{125}I]RBI-257) were prepared and characterized. With $D_{4.2}$- and D_{2L} receptor-transfected cell membranes, [^{3}H]PNU-101958 showed high dopamine D_4 receptor affinity and selectivity, and potent inhibition by dopamine D_4 receptor-selective compounds. However, its binding with rat brain homogenates showed little regional selectivity, and pharmacology inconsistent with selective dopamine D_4 receptor labeling. Autoradiography indicated partial displacement of [^{3}H]PNU-101958 by unlabeled dopamine D_4 receptor ligands without regional selectivity, and lack of selective labeling with [^{125}I]RBI-257. The results encourage further efforts to develop better dopamine D_4 receptor-selective radioligands.

Keywords: Autoradiography; Dopamine D_4 receptor; PNU-101958; Radioligand; RBI-257

1. Introduction

Dopamine D_4 receptors share molecular and neuropharmacological similarities with dopamine D_2 and D_3 receptors but differ in regional distribution in brain (Van Tol et al., 1991; Baldessarini and Tarazi, 1996). Dopamine D_4 receptor concentrations in brain tissue can be estimated with D_2-like radioligands (e.g., [^{3}H]nemonapride) with raclopride to occlude D_2/D_3 receptors, and additional agents to mask other nonspecific sites, to yield $>75\%$ displacement by highly dopamine D_4 receptor-selective agents, and greater binding in rat hippocampus and cerebral cortex than caudate–putamen (Tarazi et al., 1997, 1998). However, dopamine D_4 receptor-selective radioligands should provide improvements over such indirect methods. New dopamine D_4 receptor-radioligands [^{125}I]3-[4-(4-iodophenyl)piperazin-1-yl]methyl-1*H*-pyrrolo(2,3-b)-pyridine ([^{125}I]IPMPP) and [^{3}H]2-phenyl-4(5)-[4-(2-pyrimidinyl)-piperazin-1-yl)-methyl]-imidazole dimaleate ([^{3}H]NGD-94-1) have labeled rat brain with limited anatomical selectivity (Kung et al., 1997; Primus et al., 1997). Other compounds with high dopamine D_4 receptor affinity, and selectivity over other dopamine receptors include L-745,870, PNU-101958, RBI-257 and CP-293,019 (Kebabian et al., 1997; Kula et al., 1997). In view of the need for effective dopamine D_4 receptor radioligands, we prepared, and carried out preliminary pharmacological and autoradiographic assessments of radiolabeled derivatives of PNU-101958.

2. Materials and methods

2.1. Radioligands and test agents

Pharmacia-Upjohn compound PNU-101958 (1-benzyl-4-[*N*-(3-isopropoxy-2-pyridinyl)-*N*-methyl]-aminopiperidine maleate), its *N*-desmethyl derivative (1-benzyl-4-

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[*N*-(3-isopropoxy-2-pyridinyl)]-aminopiperidine maleate), and *p*-iodobenzyl analog RBI-257 (1-[4-iodobenzyl]-4-[*N*-(3-isopropoxy-2-pyridinyl)-*N*-methyl]-aminopiperidine) were prepared at Research Biochemicals International (RBI, Natick MA). [*N*-methyl-^{3}H]PNU-101958 (79.5 Ci/mmol) was prepared from the *N*-desmethyl precursor, and [^{125}I]RBI-257 (2200 Ci/mmol) was prepared from 4-trimethylstannyl-RBI-257, at New England Nuclear (NEN, Boston, MA). [*N*-methyl-^{3}H]nemonapride (85.5 Ci/mmol) from NEN and [^{3}H]spiperone (95 Ci/mmol) from Amersham (Arlington Heights, IL).

Donated drugs were: cinanserin (Bristol-Myers Squibb, Wallingford, CT), clozapine (Novartis, Basel), *cis*-flupenthixol (Lundbeck, Copenhagen), loxapine (Lederle, Wayne, NJ), olanzapine and quinelorane (Lilly Research, Indianapolis, IN), propranolol (Ayerst, New York, NY), risperidone (Janssen, Beerse, Belgium), CP-293,019 and ziprasidone (Pfizer, Groton, CT). BTCP (1-[1-(2-benzo[b]thienyl)cyclohexyl]piperidine) was from Tocris (St. Louis, MO); other test agents were from RBI, and assay chemicals were from Sigma (St. Louis, MO) or Fisher (Boston, MA).

2.2. Receptor sources

Brain tissue from 275-g male Sprague–Dawley rats (Charles River, Wilmington, MA) was homogenized in ice cold Tris–HCl buffer (50 mM, pH 7.4) containing (mM): NaCl (120) and KCl (5), frozen at −70°C until assayed (100–200 μg protein/tube). Human $D_{4.2}$ (COS cells) or D_{2L} (Sf9 cells) dopamine receptors (from RBI) were prepared in ice-cold assay buffer (5–10 μg protein/tube).

2.3. Assay procedures

Radioreceptor assays (Kula et al., 1997) of brain and D_{2L} receptors used Tris–HCl buffer (50 mM, pH 7.4, with 150 mM NaCl); $D_{4.2}$ buffer included (mM): KCl (5), EDTA (5), $CaCl_2$ (1.5), with protease inhibitors phenylmethylsulfonylfluoride (1 μM) and leupeptin (1 mg/ml). Tissue was recovered in a Cell Harvester (Brandel, Gaithersburg, MD) on glass fiber sheets (Schleicher and Schuell, Keene, NH), rinsed with ice cold 150 mM saline, and counted in 3.5 ml Polyfluor (Packard, Downers Grove, IL) in a liquid scintillation spectrometer (Wallac, Gaithersburg, MD) at 50% efficiency. $IC_{50} \pm$ S.E. for test agents was computed from concentration-inhibition functions with ≥ 6 concentrations of test agents in triplicate (Baldessarini et al., 1992).

2.4. Characterization of radioligands

[^{3}H]PNU-101958 was tested with human dopamine $D_{4.2}$ or D_{2L} receptor cell membranes (at 1.0 nM) or rat brain homogenates (0.7 nM); specific binding was defined with 10 μM RBI-257 or 10 μM haloperidol. Incubations were 45 min at 30°C (brain), 60 min (D_{2L}), or 120 min ($D_{4.2}$ membranes) at 20°C (Kula et al., 1997). Comparisons used [^{3}H]spiperone with transfected membranes (at 0.5 nM [$D_{4.2}$] or 0.1 nM [D_{2L}]) or brain tissue (0.06 nM with 100 μM cinanserin to mask serotonin 5-HT_2 sites) for 20 min, with 10 μM haloperidol as blank. [^{125}I]RBI-257 binding (0.1 nM, for 60 min at 20°C) with $D_{4.2}$ cell membranes and rat cerebral cortex homogenates followed conditions for [^{3}H]PNU-101958; 10 μM haloperidol was the blank.

2.5. Autoradiography

Frozen coronal sections (10 μm) of rat forebrain were preincubated for 60 min at 20°C in 50 mM Tris–HCl (pH 7.4) containing (mM): NaCl (120), KCl (5), $CaCl_2$ (2), and $MgCl_2$ (1), then for 60 min with 10 nM [^{3}H]PNU-101958 or 0.1 nM [^{125}I]RBI-257 added. Incubations of adjacent sections included each radioligand with 1 μM dopamine D_4 receptor ligands (RBI-257 or L-745,870), D_1-selective SCH-23390, or D_2/D_3-selective *S*(−)-raclopride. Slides were washed twice for 5 min in ice-cold buffer, dipped in ice-cold water, and dried in an air stream. Labeled slides and calibrated [^{3}H]standards (Amersham) were exposed to Hyperfilm (Eastman-Kodak; Rochester, NY) at 4°C for 1 ([^{125}I]RBI-257) or 5 weeks ([^{3}H]PNU-101958), developed in Kodak D-19 developer, and quantified by computerized densitometry as detailed elsewhere (Tarazi et al., 1997, 1998).

3. Results

Labeling of dopamine $D_{4.2}$-transfected membranes with [^{3}H]PNU-101958 (1.0 nM) for 45 min or [^{3}H]spiperone (0.5 nM) at 20°C for 20 min was optimal and 65%–70% or 70%–75%, respectively, specific with either haloperidol or RBI-257 (10 μM) as blanks. With these optimized conditions, there was a nonlinear Scatchard relationship of specific binding/ligand concentration [B/F] vs. specific binding [B], at $F = 25$ pM–20 nM. Computer-fitting yielded high-affinity ($K_d = 0.489$ nM; $B_{max} = 131$ fmol/mg protein), and low-affinity components ($K_d = 2.04$ nM; $B_{max} = 418$ fmol/mg). With $D_{4.2}$-membranes and either radioligand, unlabeled D_4-agents PNU-101958, RBI-257, L-745,870, and CP-293,019 all showed high potency ($IC_{50} = 0.06$–2.5 nM; Table 1). With rat cortex, these compounds showed moderately high potency vs. [^{3}H]PNU-101958 ($IC_{50} = 1.5$–233 nM; Table 1). [^{3}H]PNU-101958 (at 0.07–1.5 nM; 20 μM haloperidol as blank) showed no detectable specific binding with D_{2L}-membranes.

Affinity of these D_4-agents was much lower vs. [^{3}H]spiperone with D_{2L}-membranes or D_2-rich rat caudate–putamen (Table 1). $D_{4.2}/D_{2L}$ selectivity (IC_{50} ratio) of unlabeled PNU-101958 was 5519 with [^{3}H]PNU-101958 and 543 with [^{3}H]spiperone, and its affinity was 3356-times

Table 1
Affinity of dopamine D_4 receptor-selective compounds

Receptor source	[^{3}H]Ligand	Concentration (nM)	Affinity (IC_{50}, nM) of D_4-selective agents ± S.E.			
			PNU-101958	RBI-257	L-745,870	CP-293,019
$D_{4.2}$ CHO cells	PNU-101958	1.00	0.88 ± 0.22	0.06 ± 0.02	1.38 ± 0.09	2.48 ± 0.23
$D_{4.2}$ CHO cells	Spiperone	0.50	8.95 ± 0.36	1.10 ± 0.16	1.06 ± 0.21	4.79 ± 0.59
Rat cerebral cortex	PNU-101958	0.70	1.49 ± 0.22	1.95 ± 0.38	233 ± 8.8	41.9 ± 9.2
D_{2L} Sf9 cells	Spiperone	0.10	4857 ± 353	1400 ± 240	ca. 10,000	> 10,000
Rat caudate–putamen	Spiperone	0.06	ca. 5000 ± 400	417 ± 28	ca. 5000	> 10,000

In addition, [^{3}H]PNU-101958 (0.07–1.5 nM; 20 μM haloperidol as blank) yielded < 0.5% specific binding with D_{2L} Sf9 cell membranes.

greater with rat cortex vs. caudate–putamen (Table 1). However, affinity of the dopamine D_4 receptor-agents was consistently higher with $D_{4.2}$-membranes than cortical tissue (IC_{50} ratios ranging from 1.69 with PNU-101958 to 169 with L-745,870; Table 1), suggesting dissimilar characteristics of [^{3}H]PNU-101958 binding with transfected cells and brain tissue.

With [^{3}H]PNU-101958 (0.7 nM) and rat cerebral cortex, potency of only some test agents corresponded to expectations of [^{3}H]PNU-101958 as a dopamine D_4 receptor-selective radioligand (Table 2) Nemonapride and haloperidol had the highest affinity. Also consistent with dopamine D_4-selectivity of [^{3}H]PNU-101958, very low affinity was shown by the dopamine D_2 agonist quinelorane and D_2/D_3-antagonist (−)-raclopride, as well as dopamine membrane transporter ligand β-CIT ([−]-2-β-carbomethoxy-3-β-[4-iodophenyl]-*N*-methyltropane) and serotonin 5-HT_2 receptor antagonist cinanserin (all IC_{50} > 10,000 nM; Table 2).

However, other agents with high affinity for dopamine D_2-like receptors were much weaker against [^{3}H]PNU-101958 binding in rat cortex, in rank-order of apparent potency: *cis*-flupenthixol > chlorpromazine ≥ (−)-eticlopride > spiperone ≥ risperidone > (+)-butaclamol = loxapine (Table 2). Moreover, clozapine, with moderate dopamine D_4-affinity and some D_4/D_2-selectivity (Van Tol et al., 1991; Baldessarini and Tarazi, 1996), and other atypical antipsychotics olanzapine and ziprasidone, had very weak interactions with brain tissue sites labeled with [^{3}H]PNU-101958 (Table 2). Other agents with surprisingly potent interactions with [^{3}H]PNU-101958-labeled sites in brain include amphetamine, with 28-fold [+] > [−] stereoselectivity; the dopamine transporter ligand GBR-12909 showed moderate affinity, and the D_3-selective partial-agonist 7-OH-DPAT was 58% as potent as L-745,870 (Tables 1 and 2). Even the sigma-antagonist ditolylguanidine and β-adrenoceptor antagonist propranolol showed moderately potent interactions vs. [^{3}H]PNU-101958, not expected of a dopamine D_4 receptor-selective radioligand (Table 2). For the preceding compounds with unexpectedly low potency in competing with [^{3}H]PNU-101958 in brain tissue, there was no evidence of multiple sites of interaction. Hill slopes (± S.D.) of concentration-inhibition functions vs. [^{3}H]PNU-101958 with brain tissue for 16 compounds with IC_{50} values of 1.5–4000 nM averaged 1.09 ± 0.17 (Tables 1 and 2), and all other compounds showed no evidence of interacting at concentrations < 5 μM.

The inconsistent dopamine D_4 receptor-selective pharmacologic properties of [^{3}H]PNU-101958 (Table 2) suggest that it may interact with sites in brain tissue other than dopamine D_4 receptors. Autoradiographic binding of [^{3}H]PNU-101958 produced only weak and diffuse signals in rat caudate–putamen, accumbens, and frontal cortex, but with minimal regional selectivity. Autoradiography indicated poor displacement of [^{3}H]PNU-101958 by D_1/D_5-selective SCH-23390, and D_2/D_3-selective raclopride (both at 10 μM) in rat brain sections (< 5%), and displacement by dopamine D_4-selective RBI-257, PNU-

Table 2
[^{3}H]PNU-101958 pharmacology

Agent	Affinity (IC_{50}, nM ± S.E.M.)
(±)-Nemonapride	3.28 ± 0.33
Haloperidol	3.88 ± 0.23
GBR-12909	167 ± 18
(+)-Amphetamine	356 ± 18
(±)-7-OH-DPAT	402 ± 58
cis-Flupenthixol	651 ± 72
Ditolylguanidine	685 ± 186
S(−)-Eticlopride	948 ± 87
Chlorpromazine	1036 ± 172
Propranolol	1113 ± 97
Spiperone	3113 ± 406
Risperidone	4156 ± 517
Ziprasidone	ca. 5000
(−)-Amphetamine	> 10,000
BTCP	> 10,000
(+)-Butaclamol	> 10,000
Cinanserin	> 10,000
β-CIT	> 10,000
Clozapine	> 10,000
Loxapine	> 10,000
Olanzapine	> 10,000
Phentolamine	> 10,000
S(−)-raclopride	> 10,000
Quinelorane	> 10,000
SCH-23390	> 10,000
R(−)-apomorphine	> 30,000

Tested with membranes prepared from rat cerebral cortex, at radioligand concentration = 0.70 nM.

101958 (both by 60%), or L-745,870 (by 40%) was limited, further indicating that [^{3}H]PNU-101958 binds to sites other than dopamine D_4 receptors in rat brain that probably do not include dopamine D_2 sites, based on its lack of affinity for D_{2L}-transfected membranes (Table 1), but may include some sites on dopamine transporters (Table 2).

In preliminary experiments to characterize interactions of [^{125}I]RBI-257 (0.1 nM) with tissue, coincubation with haloperidol or RBI-257 (1–50 μM) prevented up to 60% of its binding to $D_{4.2}$-membranes. However, binding did not saturate with rising concentrations of [^{125}I]RBI-257 and, once bound, [^{125}I]RBI-257 was not displaced by 10–50 μM haloperidol or RBI-257. Nevertheless, IC_{50} values with $D_{4.2}$-membranes indicated relatively high affinity for dopamine D_4 receptor-selective compounds RBI-257 (0.24 ± 0.05 nM), L-745,870 (1.55 ± 0.28 nM), and PNU-101958 (6.71 ± 1.15 nM), and low affinity for S(−)-raclopride (IC_{50} ca. 2500 nM) and SCH-23390 (IC_{50} $> 10{,}000$ nM). Following even short film-exposure of [^{125}I]RBI-257 in sub-nM concentrations with rat forebrain sections, autoradiographic images produced were highly blurred, with no evidence of displacement of [^{125}I]RBI-257 in a regionally selective manner by 10 μM of haloperidol or L-745,870.

4. Discussion

Neither [^{3}H]PNU-101958 nor its analog [^{125}I]RBI-257 seem to be favorable radioligands for visualizing dopamine D_4 receptors in brain tissue, despite their encouraging characteristics with dopamine D_4-transfected cell membranes (Table 1; Kula et al., 1997). The pharmacology of binding of [^{3}H]PNU-101958 in homogenates of rat cerebral cortex also was inconsistent with high dopamine D_4 receptor-selectivity (Table 2). With autoradiography, neither radioligand showed strong displacement by dopamine D_4 receptor-selective compounds RBI-257 and L-745,870, or expected regional selectivity based on findings with indirect assay methods (Tarazi et al., 1997, 1998). Evidently [^{3}H]PNU-101958 can bind to unidentified sites in brain tissue other than dopamine D_4 or D_2 receptors that may include dopamine transporters. Alternatively, proposed dopamine D_4 receptor agonist properties of PNU-101958 (Gazi et al., 1998) may contribute to apparent discrepancies in binding affinities of several dopamine antagonists (Table 2). [^{125}I]RBI-257 displayed even less specific binding to rat brain tissue sections, perhaps in part reflecting high lipophilicity of this intensely radioactive halogenated derivative (Kula et al., 1997), coupled with low abundance of dopamine D_4 sites in most brain regions (Tarazi et al., 1997, 1998). Despite limited selectivity of both radioligands in brain tissue, interactions of [^{3}H]PNU-101958 with dopamine D_4 receptor-enriched membranes suggest it as a lead to developing dopamine D_4 receptor-selective radioligands.

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