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Dopamine D₂/D₃ Agonists with Potent Iron Chelation, Antioxidant and Neuroprotective Properties: Potential Implication in Symptomatic and Neuroprotective Treatment of Parkinson's Disease

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Keywords

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Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by degeneration of the nigrostriatal dopaminergic pathway.^[1] While dopaminergic cell loss in the substantia nigra (SN) is by no means the only cell loss associated with PD, it is primarily responsible for the cardinal motor symptoms associated with the disorder. Some of the symptoms associated with PD involve rigidity, bradykinesia, resting tremors, and postural instability along with cognitive and psychiatric complications.^[1–3] Levo-DOPA (L-DOPA) has been one of the mainstay therapies for PD. However, long term use of L-DOPA gives rise to motor fluctuations with dyskinesias and a decrease in duration of response to a given L-DOPA dose.^[4]

Even though the etiology of PD is not fully understood, recent studies on various genetic mutations have shed new insights into the disease process. Oxidative stress has been strongly implicated in midbrain dopaminergic cell death.^[23–27] Alpha-synuclein, a presynaptic protein involved in fibrilization, has been implicated in the pathogenesis of PD.^[5,6] In another recent report, it was demonstrated in cultured human dopaminergic neurons that accumulation of alpha-synuclein induces apoptosis in the presence of dopamine (DA) and reactive oxygen species (ROS).^[7] Furthermore, an interaction between calcium, cytosolic DA and alpha-synuclein has been implicated in the loss of DA neurons in the SN.^[8] This DA-dependent neurotoxicity is mediated by a soluble protein complex containing alpha-synuclein.^[9] Therefore, alpha-synuclein combined with oxidative effects on DA could have synergistic effects in terms of disease susceptibility and progression.

In addition to the above pathogenic factors, various studies have consistently implicated iron in the pathogenesis of PD.^[10–13] This hypothesis comes from the following observations: 1)

Levels of iron are generally higher than normal in the brains of PD patients;^[14] 2) Iron accumulation is observed in the SN;^[13–16] 3) Free iron can cause oxidative stress leading to DA-mediated cell death (see above);^[17,18] 4) Iron has been shown to initiate aggregation of alpha-synuclein forming toxic oligomers;^[19] 5) Iron chelators have been shown to be neuroprotective.^[20] More recently, iron and oxidative stress have been found in association with the ubiquitin proteasome (UPS) dysregulation and protein aggregation.^[21] Iron released from neuromelanin has been reported to reduce proteasomal function, a hallmark of PD.^[22] Oxidative stress can facilitate mutant-protein aggregation, mimicking proteasomal malfunction.^[23] Application of iron chelators may sequester the redundant iron preventing it from inducing oxidative stress as a result of reactive hydroxyl radical generation via its interaction with hydrogen peroxide.^[24] This may act to halt amplified damage triggered by UPS malfunction. In a recent human functional magnetic resonance imaging (MRI) study, iron accumulation was detected in the brains of people who developed PD during the course of Alzheimer disease, which was concurrent with further neurologic decline.^[25] Iron chelators VK-28 and M-30 (Figure 1) have been shown to be neuroprotective in a PD neuroprotection animal model.^[21]

It is increasingly evident that, for a complex disease such as PD, a drug targeting only one target site will only partially address the therapeutic need of the disease. Thus, it is hypothesized that multifunctional drugs with multiple pharmacological activities will be effective disease modifying agents for the treatment of PD.^[26] Our approach to develop such agents involves addressing both symptomatic and neuroprotective aspects of the disease. In our recent study on neuroprotective drug development for PD, we demonstrated the development of brain penetrating, multivalent compounds with agonist activity at D₂/D₃ receptors along with a capacity to chelate iron.^[27] Lead compound D-369 (Figure 1) was efficacious in PD animal models.^[27] Such compounds, besides alleviating motor dysfunction, are expected to reduce underlying oxidative stress by complexing free iron. Herein, we describe the development of one such lead, which not only exhibits potent agonist activity at dopamine D₂/D₃ receptors along with iron complexation properties, but also displays potent antioxidant activity. Moreover, the lead molecule was found to be efficacious in an animal model of PD and exhibited neuroprotection in an MPTP mouse model.

Scheme 1 outlines the syntheses of compound (±)-**9** and its enantiomers (experimental details are given in the Supporting Information). The starting material 4-chloro-8-methoxy-quino-line (**1**) was synthesized from Meldrum's acid in four steps as described previously.^[27] The methoxy group of compound **1** was deprotected using hydrobromic acid under refluxing conditions to give phenol **2**, which was reprotected by the acid-labile *p*-methoxybenzyl group to furnish *p*-methoxybenzyl ether **3**. Chloro intermediate **3** was condensed with piperazine under refluxing conditions in isopropanol to furnish amine **4**. Initial attempts to directly alkylate amine **4** with 2-bromoethanol gave very poor yields of alcohol **6**. To circumvent this problem, amine **4** was first treated with (2-bromoethyl)-*tert*-butyldimethylsilane in the presence of a base (K₂CO₃) to afford the silyl protected compound **5**. The silyl group of compound **5** was removed by treatment with *n*Bu₄NF to give compound **6** in much improved yields. Alcohol **6** was then oxidized under Swern oxidation conditions to aldehyde **7**, which was then condensed with (±)-pramipexole, (–)-pramipexole and (+)-pramipexole under reductive amination conditions using NaBH(OAc)₃ as the reducing agent to furnish condensed products (±)-**8**, (–)-**8** and (+)-**8**, respectively. Finally, the treatment of **8** with trifluoroacetic acid (TFA) to remove the *p*-methoxybenzyl groups^[28] gave the TFA salts (±)-**9**, (–)-**9** and (+)-**9**, which were converted to the free bases using aqueous solution of Na₂CO₃ and subsequently converted to the corresponding HCl salts by treatment with ethereal HCl. Finally the HCl salts were purified by recrystallization from ethanol.

Binding potency was determined by inhibition of [^3H]spiperone ($15.0 \text{ Ci mmol}^{-1}$, Perkin-Elmer) binding to rat dopamine D_2 and D_3 receptors expressed in HEK-293 cells, in a buffer containing 0.9% NaCl as described previously.^[28] Functional activity of test compounds for human dopamine D_2 and D_3 receptors expressed in CHO cells was measured by stimulation of [^{35}S]GTP γS ($1250 \text{ Ci mmol}^{-1}$, Perkin-Elmer) binding in comparison to stimulation by the full agonist dopamine as described previously.^[28]

In our approach to develop multivalent molecules with agonist potency at dopamine D_2/D_3 receptors, we have exploited our hybrid molecular template design to incorporate an iron-chelating 8-hydroxy quinoline moiety located at a distal position with respect to an agonist binding moiety. Such flexibility was achieved without compromising agonist potency for the dopamine receptors. Racemic **9** was synthesized first and evaluated in the binding assay. The racemate demonstrated high affinity for D_2/D_3 receptors. Subsequently, the individual enantiomers of **9** were synthesized and evaluated for their binding at dopamine D_2 and D_3 receptors. Enantiomer (–)-**9** exhibited the highest affinity for D_2/D_3 receptors ($K_i=27$ and 4.9 nM for D_2 and D_3 receptors, respectively; see Table 1). The other enantiomer (+)-**9** exhibited a much weaker affinity ($K_i=190$ and 13 nM for D_2 and D_3 , respectively). Thus, a profile of differential affinity was exhibited by the enantiomers. The more active enantiomer (–)-**9** was next evaluated for functional receptor activity in the GTP γS binding assay. The results indicate potent full agonist activity at both D_2 and D_3 receptors ($\text{EC}_{50}=34$ and 6.83 nM at D_2 and D_3 receptors, respectively; see Table 2).

Next we evaluated the complex formation of (–)-**9** with FeCl_3 . The hydrochloride salt of (–)-**9** ($600 \mu\text{M}$) in water was scanned in the UV spectra ($200\text{--}760 \text{ nm}$). The drug solution was then mixed with an equal volume of aq $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ($600 \mu\text{M}$) solution and the pH of the mixture was increased to 3.76, 4.0 and 7.4 by adding *N,N*-diisopropylethylamine. UV spectra were taken at each pH. At pH 3.76, the λ_{max} value was 654 nm ($\epsilon=567 \text{ M}^{-1} \text{ cm}^{-1}$), and at pH 7.4 the λ_{max} value was 592 nm ($\epsilon=1600 \text{ M}^{-1} \text{ cm}^{-1}$) with the highest intensity in absorption (Figure 2). Thus, we observed a distinct shift of λ_{max} to the left. This observation agrees with the reported data on complexation of an 8-hydroxyquinoline-related compound with iron.^[29] To further demonstrate the formation of iron complexes, we carried out mass spectral analysis of the (–)-**9**/ FeCl_3 solutions used in the UV analysis in an attempt to detect any peaks corresponding to complex molecular ions. As shown in Figure 3, we indeed observed molecular ion peaks corresponding to $\text{L}_2\text{-Fe}^{3+}$ ($m/z=987$) and $\text{L}_3\text{-Fe}^{3+}$ ($m/z=1452$) complexes formation (L=ligand molecule). These results give clear evidence for the formation of iron complexes with compound (–)-**9**.

To assess the antioxidant activity of (–)-**9**, scavenging of the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical by (–)-**9** and ascorbic acid (reference compound) was assessed using a published method.^[30] The scavenging effect is expressed as percent of the control with no drug. As shown in Figure 4, (–)-**9** inhibited DPPH radical activity in a dose-dependent fashion more potently than the reference ascorbic acid.

Another antioxidant assay, which measures the hydroxyl radical scavenging capacity of a test compound by competing with deoxyribose,^[31] was carried out with (–)-**9**. Hydroxyl radical formation by the Fenton reaction in the SN region of a PD patient's brain could potentially occur as H_2O_2 is generated in the SN by dopamine metabolism, and the SN area is rich in iron in case of PD patients.^[25] Hydroxyl radicals in this assay are generated by reaction of iron (Fe^{3+})–EDTA complex with H_2O_2 (by the Fenton reaction), which then compete to react with either deoxyribose to form smaller molecular fragments or with test compound with potential radical quenching activity. The formation of pink color dye upon treatment of deoxyribose fragments with 2-thiobarbituric acid under acidic conditions is dictated by the capacity of a test compound to quench hydroxyl radical. This is a

colorimetric assay and the absorbance is measured at 532 nm. As seen in Figure 5, (–)-**9** inhibits decomposition of deoxyribose by hydroxyl radicals in a dose-dependent manner, with the highest dose exhibiting 80–85% scavenging activity with respect to a control containing only deoxyribose.

Compound (–)-**9** was next evaluated *in vivo* in rats carrying a unilateral lesion in the medial forebrain bundle induced by application of the neurotoxin 6-hydroxydopamine (6-OHDA) resulting in production of supersensitized dopamine receptors on the lesioned side. Such surgically modified rats, when challenged with direct acting dopamine agonists, exhibit contralateral rotations away from the lesioned side. This assay is considered a standard model for the preclinical screening of potential drugs for the treatment of PD.^[27,28,32] Compound (–)-**9** (D-390) dose dependently produced contralateral rotations with long duration of action (>10 h). The highest dose (5 $\mu\text{mol kg}^{-1}$) produced a total number of rotations of 3199, whereas the lower dose (2.5 $\mu\text{mol kg}^{-1}$) produced a total number of rotations of 2203, indicating a dose-dependent effect (Figure 6).

In order to evaluate whether (–)-**9** (D-390) can prevent midbrain tyrosine hydroxylase (TH⁺) cell loss in an MPTP mouse model, C57L6 mice were pretreated with D-390 (2 mg kg^{-1}) for seven days followed by cotreatment with MPTP (20 mg kg^{-1}) once a day for two consecutive days. Treatment with D-390 (2 mg kg^{-1}) was continued for seven days following the last administration of MPTP. Stereological TH⁺ SN cell counts were performed to assess midbrain dopaminergic cell numbers.^[33] The results indicate that pretreatment with the multifunctional iron chelator D₂/D₃ agonist (–)-**9** (D-390) significantly protects against MPTP-mediated SN dopaminergic cell loss (Figure 7).

We have shown the development of a dopamine agonist with antioxidant and iron chelation properties. In a PD animal model, (–)-**9** (D-390) was efficacious and produced contralateral rotations dose dependently with a long duration of action. In a mouse model of MPTP neuroprotection, D-390 conferred significant neuroprotection against MPTP-mediated dopaminergic cell loss in the SN. Such a multifunctional drug has the potential to not only alleviate motor dysfunction in PD patients, but also to be neuroprotective by slowing or stopping the neurodegeneration process. This neuroprotective effect is most likely derived from the iron chelation and antioxidant properties of the compound, which should reduce oxidative stress in the brain. In addition, agonist potency at the D₃ receptor might also synergize the neuroprotective effect, as we have recently shown that the D₃ agonist compound D-264 produces significant neuroprotection in two PD animal models.^[34] Our future work will further explore these properties.

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Figure 1.
Structures of D₃-preferring compounds and iron chelators.

Figure 2. UV-visible absorption spectra of complex formation between (–)-**9** (0.6 mM) and FeCl₃ in water at different pH.

Figure 3. Molecular ion peaks of complexes formed from (±)-**9** and FeCl₃ at pH 7.4. Peaks at m/z 987 and 1452 correspond to **9**-Fe complex stoichiometry 2:1 and 3:1, respectively.

Figure 4. Results of a DPPH antioxidant assay measuring the DPPH radical scavenging activity by ($\pm$)-9 (D-385) (---) and ascorbic acid (—).

Figure 5. Results of a deoxyribose assay measuring the hydroxyl radical scavenging capacity of ($\pm$)-**9** in deoxyribose-containing solution. Values are reported as percentage versus a blank $\pm$ standard deviation (SD).

Figure 6.

Effect of (–)-**9** (D-390) on turning behavior in 6-OH-DA unilaterally lesioned rats studied over ten hours. Each point is the mean $\pm$ standard error of the mean (SEM) for four rats. All drugs were administered by intraperitoneal (i.p.) injection immediately before counting rotational activity. One way ANOVA analysis demonstrates a significant effect among treatments: $F(3,95)=62.13$ ($p<0.0001$). Dunnett's analysis shows that the effect of (–)-**9** at two doses (2.5 and $5 \mu\text{Mol kg}^{-1}$) are significantly different compared to vehicle ($p<0.01$). All experiments were approved by local Institutional Animal Care and Use Committee (IACUC) review and conducted according to current US National Institutes of Health (NIH) policies on the use of animals in research.

Figure 7.

Stereological quantification of tyrosine hydroxylase (TH)-positive dopaminergic cell counts within the substantia nigra (SN). &: $p < 0.05$ between vehicle and MPTP; #: $p < 0.05$ between MPTP and D-390/MPTP ($n=4$).

Scheme 1.

Synthesis of (±)-**9**, (+)-**9** and (-)-**9**. *Reagents and Conditions*: a) 48% aq HBr, reflux, 26 h, 70%; b) *p*-methoxybenzyl chloride, K₂CO₃, *n*Bu₄NI, DMF, 80°C, 10 h, 89%; c) piperazine, *i*PrOH, 100 °C, 46 h, 97%; d) (2-bromo-ethyl)-*tert*-butyldimethylsilane, K₂CO₃, CH₃CN, reflux, 14 h, 69%; e) *n*Bu₄NF, THF, RT, 1.5 h, 91%; f) oxalyl chloride, DMSO, Et₃N, CH₂Cl₂, -78°C, 2 h, 77%; g) (±)-pramipexole or (-)-pramipexole or (+)-pramipexole, NaBH-(OAc)₃, CH₂Cl₂, RT, 48 h: (±)-**8**, 70%; (-)-**8**, 75%; (+)-**8**, 68%; h) CF₃COOH, CH₂Cl₂, RT, 3.5 h: (±)-**9**, 82%; (-)-**9**, 73%; (+)-**9**, 67%.

Table 1

Inhibition constants determined by competition experiments using [^3H]spiperone binding to cloned $\text{D}_{2\text{L}}$ and D_3 receptors expressed in HEK cells.^[a]

Compd	K_i [nM]		$\text{D}_{2\text{L}}/\text{D}_3$
	$\text{D}_{2\text{L}}$	D_3	
5-OH-DPAT	83.3 (6) $\pm$ 15.6	1.92 (4) $\pm$ 0.41	43.4
D-369	3.75 $\pm$ 0.63	1.28 $\pm$ 0.08	2.92
($\pm$)- 9 (D-385)	45.0 (5) $\pm$ 8.1	4.03 (4) $\pm$ 0.83	11.2
(<i>S</i>)-(-)- 9 (D-390)	27.1 $\pm$ 5.0	4.98 $\pm$ 0.78	5.4
(<i>R</i>)-(+)- 9 (D-412)	190 $\pm$ 29	13.2 $\pm$ 2.3	14.5

^[a] Results are the mean $\pm$ SEM of 3–7 experiments each performed in triplicate. Values in parentheses are the number of experiments carried out if different from three.

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Table 2

Stimulation of [³⁵S]GTPγS binding to hD₂ and hD₃ receptors expressed in CHO cells.

Compd	D ₂		D ₃		D ₂ /D ₃
	EC ₅₀ [nM]	[a] %E _{max} [b]	EC ₅₀ [nM]	[a] %E _{max} [b]	
Dopamine	218±12	100	8.32±0.04	100	26
(S)-(-)-9 (D-390)	34.0±5.8	110±2	6.83±0.58	89.1±7.0	5.0

[a] EC₅₀ is the concentration producing half maximal stimulation. EC₅₀ values are the mean ±SEM for 3–6 experiments each performed in triplicate.

[b] For each compound, maximal stimulation (E_{max}) is expressed as percent of the E_{max} observed with 1 mM (D₂) or 100 μM (D₃) of the full agonist dopamine (%E_{max}). Results are the mean ±SEM for 3–6 experiments each performed in triplicate.