Dopaminergic Properties and Experimental Anti-Parkinsonian Effects of IPX750 in Rodent Models of Parkinson Disease

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Abstract: With a view toward improving the neural bioavailability of administered dopaminergic compounds, including dopamine, synthetic efforts have been directed toward enhancing the brain bioavailability of these compounds by accessing cellular sugar transport systems with stereoselective dopaminergic drugs. While synthesis and chemistry of the resultant class of compounds has recently been described in US Patent No. 6,548,484, the associated biologic properties have not previously been reported. One member of this new class, IPX-750, is a pro-drug dopamine-gluconamine designed to retain stereospecificity of binding at: glucose transporters (GLUT 1/ GLUT 3 and intestinal Na^+/glucose co-transporters SGLT1), dopamine transporter (DAT); and, dopaminergic receptors of the D1/D2 families. Designed to be cleavable by tissue amidases, results reported here show that intact IPX-750 pro-drug retains dopaminergic agonist binding and biologic activities both in vitro and in vivo. IPX-750, like dopamine, exhibited predominant D5/D1 binding specificity with lower binding activity at D2. As expected, binding was highly stereospecific, ie, IPX-760, a benzamide differing in just a hydrogen atom and keto oxygen from IPX-750, bound with 6-fold lower activity at D5. In cell culture, activation resulted from binding of IPX-750 at D1 or D5 in transfected cells was measured by increased intracellular cAMP. Interestingly, considering prior reported in vitro toxicity of dopamine oxidized and metabolic product dopamine, no evidence of in vitro toxicity was observed at up to 72 hrs in cell cultures at the EC50 of IPX-750 for increasing intracellular cAMP. IPX-750 was evaluated in the Parkinson's disease animal models, including MPTP mouse model, the 6-hydroxydopamine (6-OHDA) rat model and the *Nurr1(+/−)* knockout mouse model. In MPTP-lesioned and *Nurr1+/−* knockout mice, IPX-750 significantly increased Rota-rod time. In 6-OHDA-lesioned rats, IPX-750 significantly decreased apomorphine (APO)-induced rotation. Worthy of note, after cessation of IPX-750 treatments the anti-parkinsonian activity in MPTP-lesioned and *Nurr1+/−* mice required about 2 weeks to washout, suggesting a possible biologic reservoir of drug. In addition, after eight weeks of twice daily administration of 20 mg/kg IPX-750, mice did not show statistical difference in the total number of TH-positive neurons in substantia nigra (SN). These combined results suggest (i) that stereospecific glycoconjugation may be an effective method to improve penetrability of drugs through the blood brain barrier; (ii) treatment with bioavailable IPX-750 in vitro did not show evidence for neurotoxicity; and, (iii) IPX-750 possesses dopaminergic properties and exerts anti-parkinsonian effects in three different PD rodent models, suggesting therapeutic potential for this new class of drugs in treating dopamine deficiency diseases.

Key Words: IPX-750, dopamine, glycoconjugate, anti-parkinsonian effects, dopamine receptors, Parkinson disease

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Defects of dopamine synthesis in nigrostriatal system are related to the loss of motor control in Parkinson disease (PD); and, dopamine synthetic, regulatory, transporter and receptor defects also likely relate to certain defects in cognition, event prediction, emotion, addiction, attention deficits and schizophrenia.[1,2] Dopamine (DA) does not effectively penetrate blood brain barrier; thus the brain and central nervous system are dependent upon endogenous dopamine synthesis. While levodopa (L-dopa) has been used in the treatment of PD since the 1960s, chronic adaptation to drug and levodopa-induced motor fluctuations and dyskinesias constitute significant limitations.[3] Dopaminergic agonist therapies, while effective, do not sufficiently reverse motor symptoms associated with PD and may be associated with a variety of adverse effects.

Oral delivery of CNS drugs constitutes special chemical structural challenges including general simultaneous requirements for: intestinal penetration; blood borne delivery; blood-brain-barrier penetrability; resistance to degradative enzymes; and, maintenance of functional receptor and transporter binding activities and metabolic utility. For catecholamines like dopamine, norepinephrine and serotonin there also exist extraneuronal inactivating mechanisms including at least (i) enzymes, eg, methylases such as catecholamine-O-methyl transferase (COMT), monoamine oxidases (MAO) and O-glucu-

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ronic acid transferases; (ii) clearance transporters, eg, organic cation transporters (OCT) such as the corticosterone-sensitive monoamine transporter and catecholamine transporters (OCT1 and OCT2) in the liver, kidney and intestine[4]; and, (iii) metabolic conversion to homovanillic acid (HVA) with transport by HVA efflux organic anion transporters.[5] Mechanistically, at the blood brain barrier delivery of cyclic compounds is subject to structural limitations imposed by endothelial cell L-amino acid transporters[6] and restrictions on permeability at endothelial intracellular junctional complexes.[7]

We have described synthesis of a novel class of glycosyl N-linked pro-drug compounds[8] designed to access endothelial blood brain barrier glucose transporter (GLUT) systems and to be cleavable by tissue amidases, while retaining agonist binding specificity of the intact pro-drug at intestinal Na+/glucosyl co-transporters (SGLT1), dopamine transporter (DAT) and dopaminergic receptors of D1 and/or D2 families. Subsequent studies of related compounds and GLUT delivery approaches by others have confirmed, in principle, both the utility of this chemical design concept and structural limitations in the chemistry.[9–11] In this regard, pharmacology studies conducted over the past 20 years have shown relatively stringent structural requirements at D1 and D2 receptors and at DAT.[8] Strict sterospecificity has also been evidenced in glucose transporter studies where (eg, the SGLT vesicle transporter exhibits sugar specificity that is discriminatory between hundreds of different monosaccharides, even with net transport rates estimated to be in the range of 10 thousand to 1 million per second).[8]

Biochemical, pharmacological and molecular genetics have characterized two G-protein linked dopamine receptor families, D1 and D2. D1 agonist binding specificity is high for SCH23390 (a benzamine) and low for benzamides,[12] and binding stimulates adenylyl cyclase activity; whereas, D2 specificity is high for benzamides, low for SCH23390 and D2 binding inhibits adenylyl cyclase. In behavioral animal model studies and knockout mouse studies, D1 receptors have been associated with cholinergic output from large aspiny neurons[13] and working memory in temporal performance measurements.[14] In contrast, D2 receptors have been associated with inhibition of GABA transmission[13] and behavioral output.[14] Agonist studies have suggested that D1 receptors may influence behavioral output from D2 receptors.[14,15] D1 family receptors are encoded by two distinct genes, D1[16] and D5[17] which differ in binding specificity and relative discriminatory activity. D5/D1B receptors exhibit greater discriminatory binding activity and about 10-fold higher binding affinity for dopamine than do D1/D1A receptors.[17]

IPX-750, dopamine-gluconamine (Fig. 1), is a member of a novel class of dopaminergic-stereoselective glycoconjugate compounds whose synthesis and chemistry has been recently described.[8] The rationale for design of this synthetic class being the following: (i) neural tissue is almost entirely dependent on glucose transport for normal metabolic activity because tissue stores of glucose are lower relative to demand; (ii) glucose transport at the blood brain barrier is mediated by GLUT1, ie, the Na+-independent facilitative transporter in endothelial cells;[18] (iii) glucose transport in striatum is mediated by GLUT3 in neural cells;[19] and, (iv) GLUT1 is also a predominant glucose transporter expressed in human erythrocytes, ie, offering up the potential for a cell-associated biologic drug reservoir, a cell-protected prodrug delivery and a cell-sustained release mechanism.

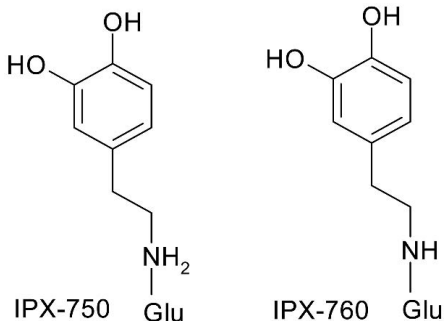

FIGURE 1. Depicts the chemical structures of IPX-750 and IPX-760 wherein Glu is a glucosyl sugar.

In this study, we report the biologic and certain pharmacological properties of dopamine-gluconamine (IPX-750) and dopamine-gluconamide (IPX-760). IPX-750 is shown to exhibit biologic activity at D1 and D5 receptors in vitro with potent anti-parkinsonian effects in three different rodent animal models, warranting further preclinical and possible clinical investigations.

MATERIALS AND METHODS

Cell Culture

COS-7 cells (a fibroblast-like cell line established from the kidney of African green monkey) were cultured in 150 mm plates at 37°C, 5% CO_2 in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). COS-7 cells were grown in either 150 mm plates for radioligand binding assays or 24 well plates for cAMP assays.

D1, D5 and D2 Expression Vectors and Transfected Cells

Human dopamine receptor clones included: D1[16], D5[17] and D2[20], each of which was subcloned into an expression vector pCD-PS and transfected into COS-7 cells by electroporation, as described previously.[21,22]

Radioligand Competition Binding

Transient transfection of cells and radioligand competition binding assays were conducted as described previously.[21,22] Briefly, cells transiently expressing human D1, D5, or D2 receptor cDNA were harvested from 150 mm plates 72 hours after electroporation and cell membranes were prepared

as follows: cells were disrupted in 50 mM Tris-HCl buffer pH 7.4, containing 5 mM EDTA, 1.5 mM CaCl2, 5 mM KCl, 5 mM MgCl2, 120 mM NaCl, using Polytron cavitation (6/30 seconds). Membranes were collected at 18,000 rpm for 15 minutes and the membrane pellet was resuspended to achieve a final protein concentration of 120–150 μg/mL (Bradford protein determination; Bio-Rad Laboratories, Inc.). Radioligand binding was performed in 24-well plates in DMEM. The binding results were evaluated using 0.5 ml aliquots of the membrane preparation with incubation at 37°C for 90 minutes. All assays were terminated by rapid filtration over Skatron filtermats (Lier, Sterling, VA). [^{3}H]-SCH-23390 (NEN: 81.4–86.5 Ci/mmol; 1 Ci = 37 GBq) was used as the D1/D5-selective radioligand and [^{3}H]-Nemonapride (NEN: 68.2 Ci/mmol) as the D2-selective ligand.[12,23] Competition binding experiments were performed using 400 pM [^{3}H]-SCH-23390 and 150 pM [^{3}H]-Nemonapride and the indicated molar concentration of IPX-750 (or IPX-760), ie, 10^{-4}–10^{-11} M. Membrane associated tritium was determined by scintillation spectrometry. Nonspecific binding was evaluated in the presence of 10 mM (+)-butaclamol. Data were analyzed using the nonlinear least-squares fitting program KALEIDAGRAPH (Abelbeck Software, Reading, PA) and Ki values were estimated.

Receptor Activation: cAMP Accumulation

COS-7 cells were transiently transfected with plasmids encoding D1 or D5 receptor and cAMP accumulation assays were conducted as described previously.[24,25] Briefly, after 48 to 72 hours post transfection, cAMP accumulation was measured in DMEM containing 0.5 mM 3-sobutylmethylxanthine and 1 μl propanol. Incubation with dopamine (positive control) or test compounds were conducted over 15 minutes at 37°C in 5% CO_2. cAMP levels were measured by radioimmunoassay according to the manufacturer instructions (Amersham).

Animals

Male C57BL/6 mice, SD rats and Nurr1 +/− mice[26] were used in these studies. They were housed in groups of four in conditions of constant temperature and controlled lighting (light period 12h on/off) and fed an ad libitum diet of RMI expanded pellets and tap water.

Drugs and *Reagents*

IPX-750 and IPX-760 were synthesized as described.[8] 6-OHDA and MPTP were obtained from RBI (Natick, MA). IPX-750 was freshly prepared in saline and administered by intraperitoneal injection twice each day. 6-OHDA was freshly prepared in saline with 0.2% ascorbic acid and was injected unilaterally into the substantia nigra (8 μl total volume; AP: −4.4 mm, ML: 1.1 mm, DV: 7.5 mm; AP: −4.0 mm, ML: 0.8 mm, DV: 7.8 mm). Hemiparkinson rats exhibiting APO-induced rotation of greater than 6 rotations per minute were selected for use in the experiments. 1,2,3,6-methyl-phenyl-tetrahydropyridine (MPTP) was freshly prepared in saline and administered by intraperitoneal injection at a total mouse body dose of 60 mg/kg in four injections. Test compounds were coded and subject to a blind prior to administration.

Locomotor Skill: Rota-Rod Test

The Rota-Rod system (Columbus Instruments, Columbus, U.S.A.) for assessing locomotor skills measures the time an animal maintains balance on a moving Lucite rod. The following general conditioning and testing procedures were employed to select animals for use in the different treatment and control groups: namely, animals were first conditioned on a stationary rod for 30 seconds and during this time any animal that fell was placed back on the rod. Animals were next conditioned at a constant speed of 5 rpm for a period of 90 seconds. Animals that failed the first conditioning were given two additional conditioning periods and those that failed the third conditioning period were not selected for use in the experiment. The same basic conditioning methodology was employed in testing treatment and control groups. Thirty minutes after the last conditioning, animals were placed on the rod and timed to determine their locomotor skill, ie, using a starting speed of 5 rpm with an increase of 0.1 revolution per second during the data collection interval.

Apomorphine-Induced Rotation: Rota-Count Performance

The Rota-Count 8 unit (Columbus Instruments, Columbus, U.S.A.) is an eight channel rotational meter utilizing a digital rotary encoder mounted in a small bowl with a cable attachment that allows automatic recording of the number of rotations made be a test animal during a fixed period of time. Five minutes after APO administration, rats were tested of a period of 30 minutes. The average number of rotations per minute over the course of the test period was used for statistical analysis of a group.

Statistical Analysis

Prior to all analyses, data were normalized and homogeneity of variances was confirmed. Statistical analysis was conducted using the Sigma Stat 3.0 and Sigma Plot software, ie, using paired and un-paired *t tests* and Two-way ANOVA (for different degrees of stringency) to evaluate the significance of differences between means. Statistical test values in which $P < 0.05$ were considered to be significant.

RESULTS

Competition Binding Assays: D1 and D2 Ligands

Binding at receptors of the D1 and D2 families has been shown in prior pharmacological studies to be stereospecific in regard to at least the spacing of dopamine benzyl hydroxyl sub-

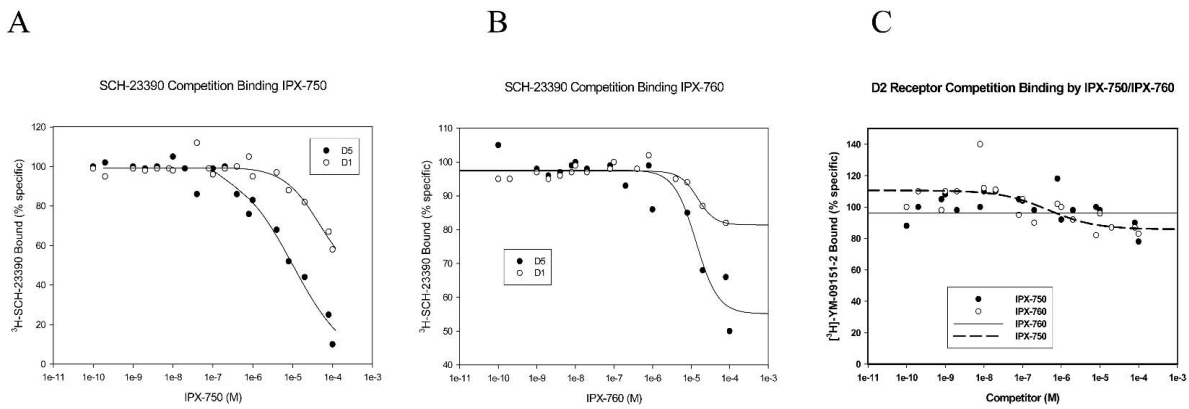

FIGURE 2. (A) and (B) depicts IPX-750 and IPX-760 competition of [^{3}H]SCH-23390 binding, respectively, at dopaminergic receptors in membrane preparations of COS-7 cells transiently expressing human dopamine D1 or D5 receptors; (C) depicts IPX-750 and IPX-760 competition of [^{3}H]-Nemonapride binding at D2 receptors in membrane preparations from COS-7 cells transiently expressing human dopamine D2 receptor.

stituents and to the alkyl side chain length and substitutions at the dopamine nitrogen, ie, with D1 showing apparent preference for benzamines and D2 for benzamides.[8] To determine whether the dopamine-N-alkyl could be used as a bridge for stereospecific glycoconjugation, ie, designed to retain receptor binding at dopaminergic receptors, IPX-750 and -760 were evaluated in competition binding assays against dopaminergic radioligands having nanomolar to picomolar binding affinities at D1, D5 and D2 receptors, ie, D1-selective ^{3}H-SCH-23390 and D2-selective ^{3}H-Nemonapride (YM-09151-2). To avoid confusion associated with possible cellular cleavage of prodrug or presence of multiple homo- or heterodimeric receptors, competition binding measurements were performed with well-washed membranes from COS-7 cells stably transfected with human D1/D1A, D5/D1B or D2 receptor cDNAs. Despite –N-alkyl-glycoconjugation, IPX-750 competed the binding of [^{3}H]-SCH-23390 at D1 and D5 receptors in a concentration-dependent and uniphasic type fashion (Fig. 2A). At D2 neither IPX-750 nor IPX-760 competitors was able to compete the binding of [^{3}H]-Nemonapride, (having about a 20 pM binding affinity at D2),[12,23] even at concentrations of up to 1 mM (Fig. 2C). Ki values for inhibition of binding were calculated to be about 68 μM at D1 and 6.8 μM at D5 (Table 1). For comparison, under these same conditions dopamine exhibited a Ki value of about 300 nM at D5 and 2 μM at D1. Thus, the IPX-750 glycoconjugate prodrug, like dopamine, retained greater binding affinity at D5 than at D1 and glycoconjugation apparently decreased binding at D2. Stereospecificity of binding in the –N-alkyl-glyco bridge was investigated using IPX-760, a compound differing from IPX-750 by a hydrogen atom and a hydrogen-to-keto-oxygen substitution in the glycoconjugated linker. The data presented in Figure 2B showed that these modifications in IPX-760 -N-alkyl bridge produced a more than 6-fold decrease in binding affinity at D5. From this data it would appear that quite minor differences around the linker nitrogen atom have a significant effect on receptor-ligand binding at D5/D1 (Table 1). As a result, it is quite unexpected that a sugar molecule can be attached at this nitrogen atom with retention of receptor binding and, as shown below, retention of biologic activity in vitro and in vivo.

Receptor Activation: cAMP Accumulation

Dopaminergic receptors belong to a family of 7 transmembrane-domain G-coupled receptors with a near-membrane binding crevice; and, binding at this site either activates (D1) or inhibits (D2) adenylyl cyclase activity. Measuring increased intracellular cAMP provides a relative measure of the receptor activation achieved by binding of a test compound at D1 or D5. However, while D5 binding affinity for dopamine is about 10-fold higher than D1, D5 receptor density in transfected COS-7 cells is often only about 50% to 70% of that achieved with D1, preventing a direct cross-comparison of the relative activation achieved by test compounds at the two different receptors.

TABLE 1. Summary: Competition Binding Results Fig. 2A, 2B, and 2C. Ki of Competitors At Human D1, D5, and D2 Receptors in Transfected Cos-7 Cells

Competitor ($10^{-4} - 10^{-11}$M)	Ki D1	Ki D5	Ki D2
Dopamine	2–3 μM	190–300 nM	11 nM
IPX-750 (dopamine–gluconamine)	68 μM	6.8 μM	n-d
IPX-760 (dopamine–gluconamide)	>250 μM	46 μM	n-d

n-d, not detectable, < 1mM under conditions of assay with ^{3}H-Nemonapride (Ka = 20 pM at D2)

TABLE 2. Intracellular cAMP Accumulation (pM/well) Induced by Binding at Human D1 and D5 Receptors Expressed in COS-7 Cells

	D1		D5	
Addition:	**pM**	**Increase* (%DA)****	**pM**	**Increase* (%DA)****
None. . . . . .Basal	4.2	–	6.5	–
Dopamine (10 μM)	19.5	4.6 (–)	13.4	2.1 (–)
IPX-750 (10 μM)	17.2	4.1 (90)	10.8	1.7 (81)
IPX-750 (100 μM)	22.3	5.3 (115)	13.2	2.0 (95)
DA (10 μM) + IPX-750 (10 μM)	19.5	4.6 (100)	13.3	2.0 (95)
IPX-760 (10 μM)	11.2	2.7 (59)	8.5	1.3 (62)
IPX-760 (100 μM)	19.7	4.7 (102)	12.9	2 (95)
DA (10 μM) + IPX-760 (10 μM)	21.8	5.2 (113)	14	2.2 (105)

*Increase = test/basal; **%DA = test/dopamine × 100%

The ability of IPX-750 to bind and activate D1 receptors was investigated in this study. The results presented in Table 1 show that IPX-750 binding at D1 or D5 activates increased intracellular cAMP and the levels achieved are 80% to 90% of that achieved at an equivalent dopamine concentration (10 μM). As in competition binding experiments, above, the IPX-760 gluconamide exhibited lower binding affinity (Fig. 2C) and a lesser increase in total intracellular cAMP, ie, just 59–62% of that achieved with dopamine. Possible partial antagonist activity, alternative receptor binding or synergistic activity of IPX-750 (or IPX-760) was investigated by adding both test compound (10 μM) and dopamine (10 μM) to cultures. No increase or decrease in intracellular cAMP was observed over that observed with dopamine alone, suggesting that the observed effects were mediated through D1/D5 (and not other) receptors and that IPX-750 and IPX-760 do not exhibit partial antagonist activity in this assay. Thus, like dopamine, IPX-750 apparently binds (Fig. 2A) and activates (Table 2) both D1 and D5 receptors in vitro.

Effects of IPX-750 in MPTP-Lesioned Mice

Dopaminergic neurotoxin 1,2,3,6-methyl-phenyl-tetrahydropyridine (MPTP) induces nigro-striatal neuropathology and locomotor deficit in mice, monkey and man. C57BL/6 mice lesioned with MPTP exhibited a 2-fold decrease in Rota-Rod performance times. MPTP-lesioned animals were divided randomly into groups for treatment twice daily with IPX-750 (20 mg/kg or 80 mg/kg) or Saline. Testing was conducted at weekly intervals and the results presented in Figure 3A show significantly improved Rota-Rod performance times at the second week of IPX-750 (80 mg/kg) or third week at IPX-750 (20 mg/kg) relative to pre-treatment values ($P < 0.05$), ie, with near 2-fold better Rota-Rod times than saline-treated controls (Fig. 3A). Maximal therapeutic efficacy, ie, Rota-Rod times, of IPX-750 treatments at 80 mg/kg were not significantly different than at 20 mg/kg. After cessation of IPX-750 treatments, locomotor disability gradually reappeared over a period of 2 weeks, but Rota-Rod time was still higher than pre-treatment at 2-weeks post-washout (Fig. 3B).

Effects of IPX-750 in 6-OHDA-Lesioned Rats

Possible direct agonist effects of IPX-750 at hypersensitized DA receptors was tested in 6-OHDA-lesioned rats

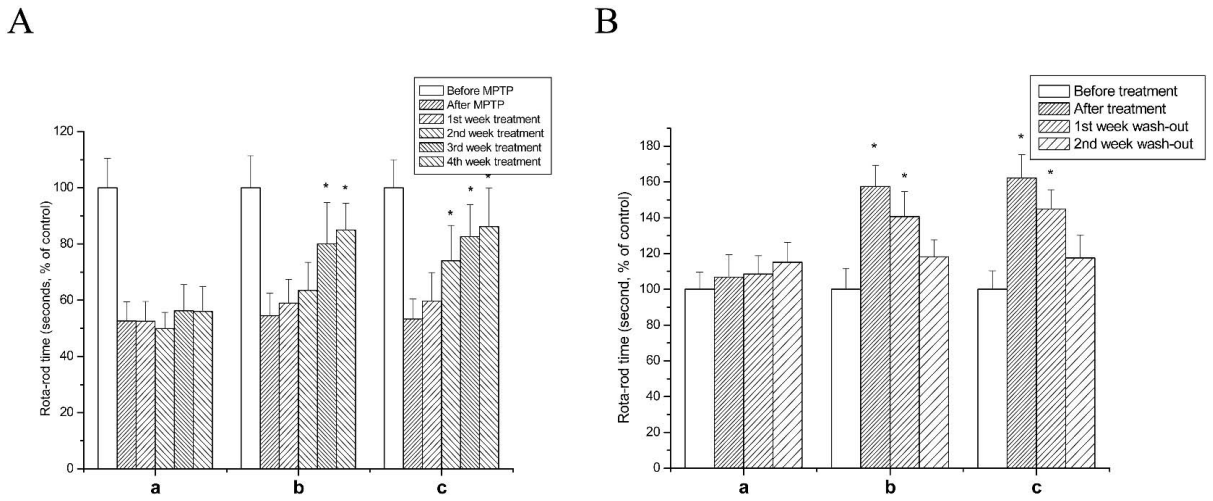

FIGURE 3. Rota-Rod performance in MPTP-lesioned mice in the following treatment groups: namely, (a) saline treatment (n = 7); (b) IPX-750 (20 mg/kg ; n = 8); (c) IPX-750 (80 mg/kg; n = 8). A, Rota-rod performance times in MPTP-lesioned C57BL/6 mice after treatment with IPX-750 or Saline. B, Rota-rod performance times after cessation of treatment and evaluation of persistent drug effects during a 2-week washout period. *$P < 0.05$, *t* test comparison to pre-treatment values.

by administering a single intraperitoneal injection of 80 mg/kg or 200 mg/kg IPX-750. No induced rotational behavior was evidenced, as a result of these single IPX-750 injections, suggesting that IPX-750 had no measurable direct post-synaptic stimulatory effects in this animal model (Fig. 4A). To evaluate possible IPX-750 therapeutic effects, IPX-750 (5 mg/kg or 20 mg/kg) were administered twice daily to 6-OHDA-lesioned rats and compared with the therapeutic effects of levodopa/carbidopa (Sinemet, 20 mg/kg). APO-induced rotation was measurably decreased in IPX-750 treated animals relative to saline-treated controls with statistically significant improvement being observed in the 20 mg/kg treatment group at 2 to 4 weeks (Fig. 3B, treatment group "c"). Sinemet failed to exert any visible therapeutic effect (Fig. 3B). After cessation of therapy, beneficial effects of IPX-750 (20 mg/kg) washed out over a period of about 2 weeks (Fig. 3C). The combined results sug-

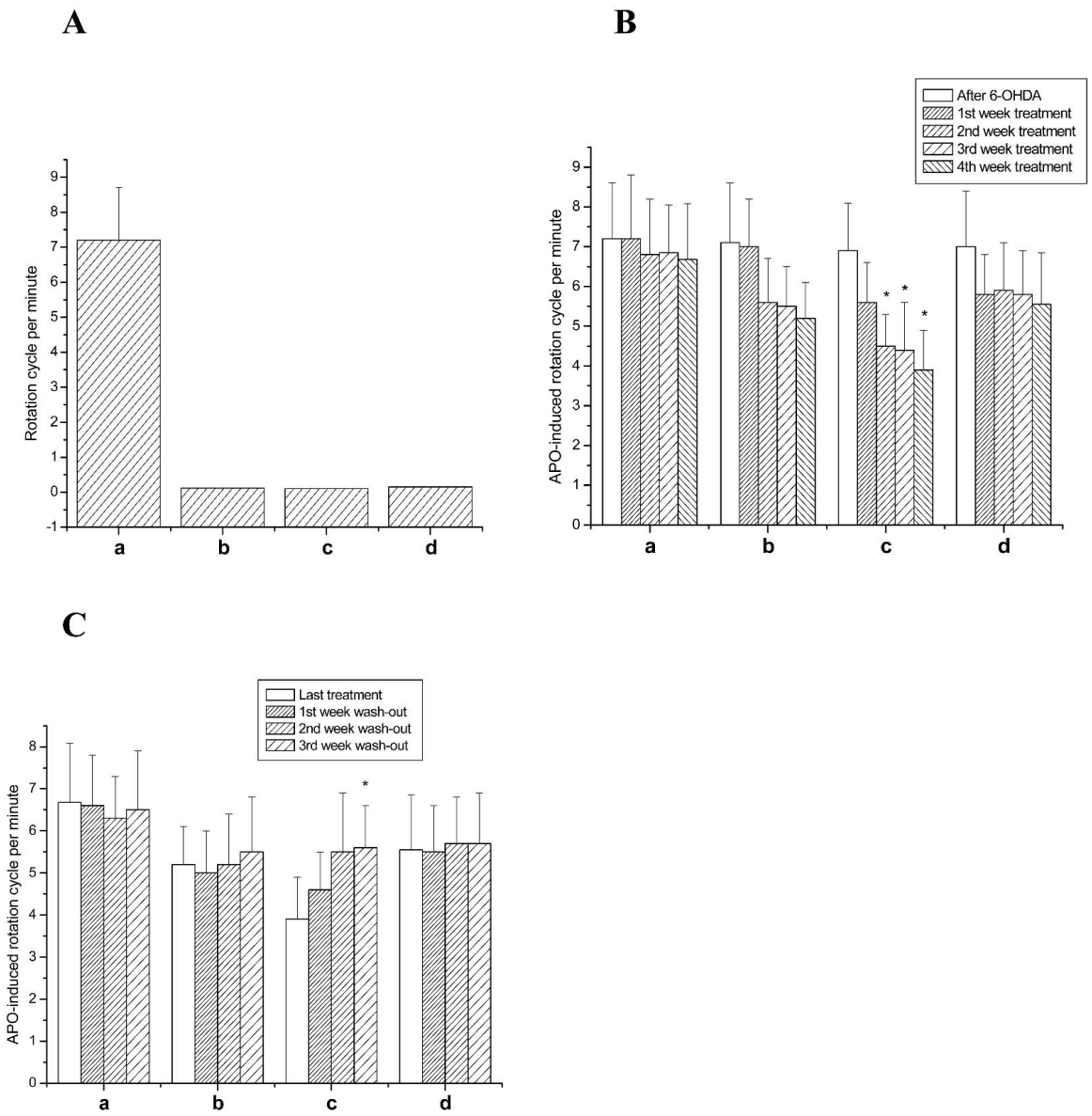

FIGURE 4. Anti-rotational effects of IPX-750 in 6-OHDA-lesioned rats. A, Testing for possible direct induced rotational effects of IPX-750 (ie, in the following treatment groups: namely, (a) apomorphine (APO); (b) IPX-750 (80 mg/kg); (c) IPX-750 (200 mg/kg); and, (d) saline). B, Testing therapeutic inhibitory effects of IPX-750 on APO-induced rotation ie, in the following treatment groups: namely, (a) saline; (b) IPX-750 (5 mg/kg); (c) IPX-750 (20 mg/kg); and (d) Sinemet (20 mg/kg). C, Testing for the washout of drug effects exerted by IPX-750 treatment in 6-OHDA-lesioned rats, ie, in the following treatment groups: namely, (a) saline; (b) IPX-750 (5 mg/kg); (c) IPX-750 (20 mg/kg); and, (d) Sinemet (20 mg/kg). *$P < 0.05$.

gest that IPX-750 treatments compensate for the 6-OHDA-induced locomotor disability; and, apparently IPX-750 does not exhibit post-synaptic apomorphine-like agonist activity in this animal model, perhaps as a result of differences in discriminatory activity of this IPX-750 compound between D1/D5 and D2 receptors (Fig. 2A-2B).

Effects of IPX-750 in *Nurr1*+/− Knockout Mice

Effects of IPX-750 were next evaluated in *Nurr1* deficient mice, a model of age-related, progressive parkinsonism. To provide understanding of the *Nurr1* animal model, Rota-rod times in *Nurr1*+/− mice, were decreased in male *Nurr1*+/− mice relative to male *Nurr1*+/+ mice (Fig. 5A). Comparisons were made of the effect of twice daily treatments of IPX-750, Sinemet. All agents were administed twice daily at 20 mg/kg to *Nurr1*+/− mice. As shown in Figure 5A, *Nurr1*+/− mice treated with IPX-750 treatment (20 mg/kg) for 8 weeks resulted in a 2-fold increase in Rota-rod time in treated animals relative to saline-treated *Nurr1*+/− mice, ie, a result that was comparable to that obtained with saline-treated wild-type mice (Fig. 5A). Despite a relatively small group size (n = 6), the results presented in Figure 5A, show a strong trend toward steady improvement in rota-rod performance in IPX-750 treated animals with statistically significant improvement at 5, 6, and 7 weeks with apparent therapeutic benefit being reached at about 2 weeks and wash-out seemed to require about 2 weeks after removal of drug (Fig. 5B) In this particular study Sinemet did not confer any observable therapeutic benefit over the saline control under these conditions of assay, perhaps as a result of sub-optimal dosing or the relatively small group size (n = 6).

DISCUSSION

The results presented here show that IPX-750, a member of a dopamine glycoconjugate class of compounds, exhibits unique in vitro dopaminergic receptor binding properties, efficacy in three different rodent animal models of Parkinson disease and, implicitly, blood brain barrier penetrability. By design, within the IPX-750 dopamine-gluconamine compound (a) the benzyl hydroxyl groups are both available for binding to acidic residues in the D1/D2 receptors; (b) the length and anomeric structure of the alkyl side-chain[27] is the same as that in dopamine positioning the amine nitrogen for dopaminergic receptor binding[28,29]; and (c) the side chain structure also allows DAT- and GLUT-mediated transport without inhibition[30,31] or cellular toxicity.[32] Aqueous solubility of the resultant glycosyl compound was markedly better than dopamine. In considering prior reported in vitro toxicity of oxidized and metabolic products dopamine, no evidence of in vitro toxicity was observed at up to 72 hrs. in COS-7 cell cultures at the EC_{50} of IPX-750 for increasing intracellular cAMP, ie, 10^{-4} M to 10^{-6} M.

In competition binding assays IPX-750 exhibited Ki values of 68 μM at D1, 6.8 μM at D5 (Fig. 2A) against dopaminergic D1-selective ^{3}H-SCH-23390, suggesting that the dopamine −N-alkyl could be used as a bridge to glycosyl compounds provided the sugar structural integrity was preserved. Evident structural specificity is exquisite since IPX-760, differing from IPX-750 in just a hydrogen atom and keto oxygen, exhibited a 6-fold lower activity at D5 receptors (Fig. 2B). IPX-750, like dopamine, retained greater binding affinity at D5 than at D1. (Dopamine exhibits Ki values of 300 nM at D5

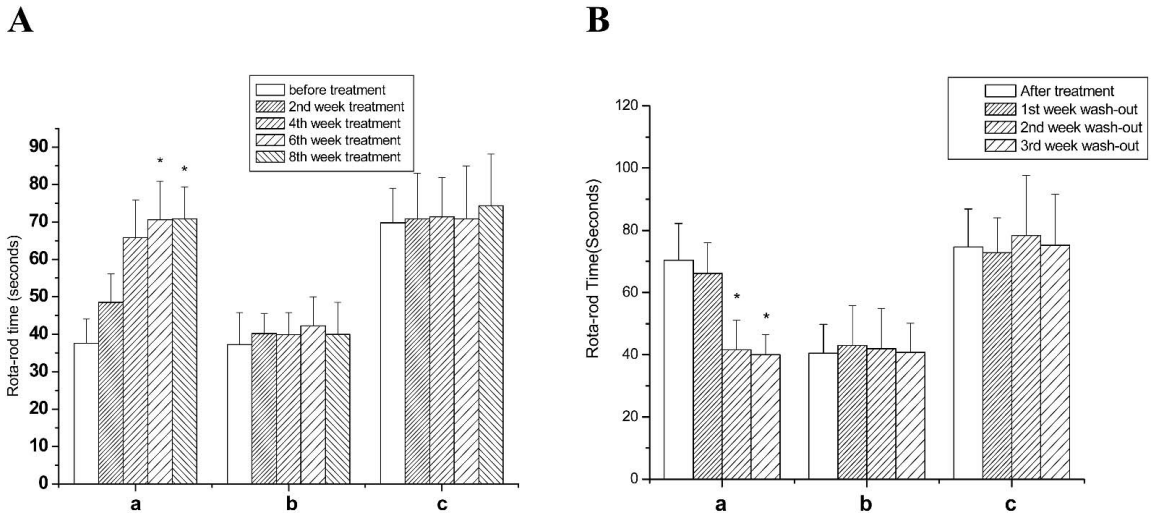

FIGURE 5. Rota-rod performance in Nurr1+/− mice. a: IPX-750 (20 mg/kg) treated Nurr1+/− mice (n = 8); b: saline treated Nurr1+/− mice (n = 8); c: saline treated Nurr1+/+ mice (n = 8). A, Prior to IPX-750 treatment, male Nurr1+/− mice showed a significant decrease in Rota-rod time as compared with male Nurr1+/+ mice. Two weeks after treatment with IPX-750, male Nurr1+/− mice showed a significant increase in Rota-rod time. B, After stopping IPX-750 administration the Rota-rod time was gradually decreased in Nurr1+/− mice to the baseline at the second week. *$P < 0.05$.

and 2 μM at D1 under these conditions of assay in COS-7 cells transiently expressing receptors.[22]) In competition binding measured with D2-selective ^{3}H-Nemonapride (YM-09151-2), having a 20 pM binding affinity at D2,[12,23] IPX-750 and IPX-760 both failed to compete binding, even at concentrations of up to 1 mM (Fig. 2C). IPX-750 binding at D1 and lack of binding at D2 is consistent with binding of benzamines at D1 and benzamides at D2. Alternatively, the near complete lack of binding at D2 may be an assay artifact resulting from the relatively slow off-rate of nemonapride radioligand, which has a picomolar binding affinity.[32,33]

Importantly, D1 receptors activate adenyl cyclase and increased intracellular cAMP while D2 receptors inhibit the enzyme and reduce cAMP. Unlike other more non-specific agonists with dual receptor binding specificities that can thus antagonist their own agonist effects on cAMP, IPX-750 appears to be a relatively specific activator of D5/D1 receptors. Measured intracellular cAMP was increased by binding of IPX-750 (10 μM) to D1 or D5 at levels that were 80% to 90% of that achieved with dopamine (10 μM) (ie, with lesser activation being achieved by IPX-760 at either receptor) (Table 2). Comparisons of relative activation at D1 and D5 are not possible in these studies because, dopamine binding at D5 is about 10-fold higher than at D1,[17] but D5 receptor density in transfected COS-7 cells is often only about 50% to 70% of that achieved with D1.[22] Thus, like dopamine, IPX-750 apparently binds (Fig. 2A) and activates (Table 1) both D1 and D5 receptors in vitro.

In vivo, IPX-750 treatments improved locomotor performance in three different rodent animal models, MPTP-lesioned mice, 6-OHDA lesioned rats and *Nurr1*+/− mice. C57BL/6 mice lesioned with MPTP had 2-fold decreased Rota-rod performance times relative to untreated controls (Fig. 3A). Animals treated twice daily with IPX-750 (20 mg/kg or 80 mg/kg) and tested at weekly intervals for Rota-rod performance, showed steady improvement during therapy with significantly improved Rota-rod performance times at the second week (80 mg/kg dosing) or third week of IPX-750 (20 mg/kg dosing) treatment relative to NaCl-treated controls and relative to pre-treatment values ($P < 0.05$; Figure 3A). The minimal effective dose, minimal maintenance dose and delivery timing have not (to date) been determined. After cessation of IPX-750 treatments, locomotor disability gradually reappeared but after two weeks of washout the Rota-rod time was still higher than pre-treatment (Fig. 3B). The combined results in this animal model suggest that about 2 weeks were required for full loading of therapeutic efficacy and, similarly, therapeutic efficacy was progressively lost over about a 2-week washout period. These results may relate to possible biologic reservoir of drug in vivo (eg, associated with erythrocyte and tissue GLUT1). The alternative that the drug compound is long acting cannot (at present) be ruled out. Irrespective, if confirmed in man, this pharmacophore approach may offer therapeutic benefits, particularly as related to issues of erratic compliance in elderly patients.

In the 6-OHDA rat model, unilateral nigral lesions were induced by I.C.V. injection and inhibitory effects on apomorphine induced rotational behavior were tested. Possible direct apomorphine-like agonist effects of IPX-750 at hypersensitized DA receptors was tested in 6-OHDA-lesioned animals and no induced rotational behavior was observed, suggesting that in spite of observed D1/D5 in vitro agonist activity (Fig. 2A, 2B) IPX-750 had no measurable direct post-synaptic stimulatory effects in this animal model (Fig. 4A). Statistically significant therapeutic effects were observed in the 20 mg/kg IPX-750 treatment group, (but not in the 5 mg/kg group), at 2 weeks (Fig. 4B). After cessation of therapy, therapeutic effects were still evident in the IPX-750 (20 mg/kg) treatment group at two weeks post-washout. As in the MPTP mouse model, above, the combined results of testing in the 6-OHDA-lesioned rat model suggest therapeutic efficacy, as well as, the possibility of long lasting activity.

The seemingly contradictory nature of in vitro binding of IPX-750 at D5/D1 (Table 1); lack of direct in vivo agonist activity post-synaptically in the 6-OHDA rat model; but observed effects on apomorphine induced rotation (Fig. 4B) are at present somewhat difficult to explain. Clearly, direct injection of an agonist into the brain of 6-OHDA lesioned animals does not mimic rates or processing of a pro-drug by tissues or cells involved in blood–brain-barrier delivery. Theoretically, in a 6-OHDA lesioned animal failure to induce rotational behavior after direct injection of IPX-750 could result from at least: (a) limited access to and binding at post-synaptic dopaminergic neurons in the lesioned side of the brain; (b) the type and nature of binding at post-synaptic D5/D1B, D1/D1A and/or D2 receptors; (c) any possible cell-surface modulatory interactions between dopaminergic, GABA, NMDA and other receptors; (d) the rate of scavenging/removal of injected compound by non-neuronal cells in the lesioned side of the brain; (e) the rate of IPX-750 transport by DAT/VMAT transporters (in the non-lesioned side of the brain); (f) the rate and site of processing of the IPX-750 pro-drug into biologically active dopamine, eg, by tissue amidases pre-synaptically in the non-lesioned side; and, (g) rate of release of dopamine into the lesioned-side synaptic cleft. In a 6-OHDA lesioned animal pre-synaptic neuronal transport and processing of drug on the lesioned side should be minimal, (ie, (d)–(f) above), possibly accounting for the lack of direct agonist activity. However, since the non-lesioned side of the brain is still active the possibility exists for dopamine crossover to occur, ie, blocking apomorphine-induced rotation after a period of therapy. Therapeutic effects of IPX-750 in this model might also result from processing of the IPX-750 pro-drug with release of dopamine into the lesioned-side synaptic cleft, ie, by cells other than neurons (eg, glial, stromal or endothelial cells).

IPX-750 was also evaluated in *Nurr1* deficient mice. Briefly, Nurr1 (also called NOT, TINUR, RNR-1, HZF-3, and NR4A2) is an orphan member of the nuclear steroid receptor transcription factor superfamily. The important roles of Nurr1 in the development of midbrain DAergic neurons; continued expression during adulthood suggest that Nurr1 may play an important role in maintenance of the dopaminergic system; and, lower levels of Nurr1 protein in *Nurr1*+/− mice reportedly increase the vulnerability to neurotoxin MPTP.[34] Homozygous (Nurr1−/−) mice display a selective and near complete loss of dopaminergic neurons in substantia nigra (SN), low DA levels in the striatum and these animals die within 24 hours of birth.[35–37] *Nurr1*+/− heterozygous mice display no apparently histologic and behavioral abnormality.[36–38] In the present study, *Nurr1*+/− mice treated with IPX-750 (20mg/kg) exhibited steadily improvement in Rota-rod performance times (Fig. 5A) with statistically significant improvement at 5, 6 and 7 weeks (Fig. 5A). An apparent therapeutic benefit was reached in about 2 weeks and drug wash-out seemed to require about 2 weeks (Fig. 5B). Sinemet had markedly lesser effects in this animal model than IPX-750. Thus, the combined results of these three different rodent studies are all in agreement: namely, IPX-750 appears to exert anti-parkinsonian effect in vivo and the therapeutic efficacy washes out after about 2 weeks.

Fernandez et al[9,10] and Bonina et al[11] have recently described a related class of dopamine-succinyl-linker-glycosyl compounds, some of which reportedly exhibited D2 and GLIT-1 binding and biologic activity in animals (ie, >50% of the activity of amphetamine in reserpine-treated mice).

In mice, IPX-750 has no apparent acute LD_{50} at single doses of up to 1600 mg/kg ip, whereas the single acute dose dopamine LD_{50} is about 800 mg/kg. However, after 5 days of dosing an LD_{50} was achieved at about 1600 mg/kg ip (unpublished observations). As in the other 3 animal model studies (above) there appears the preliminary dose ranging toxicology studies suggest "loading" of drug, perhaps into a biologic reservoir (e.g. GLUT1 receptors in erythrocytes and tissues). Dopamine has been used without consequence for several decades in patients to maintain hemodynamic stability after coronary artery surgery and to improve tone in vascular grafts. L-dopa has similarly been used without significant overt toxicity in treatments of Parkinson disease,[3] however, recent concerns have been raised by in vitro studies associating toxicity with L-dopa,[39] dopamine,[40] dopamine-quinone,[41] cysteinyl-dopamines,[42] dopaminochromes[43] and potential dopamine metabolites and oxidation products.[44] Some toxicity is reportedly inhibited in vitro by natural cellular antioxidants,[45] superoxide dismutase,[46] diaphorase[47] or glutathinone,[48] pyruvate induction of anti-apoptotic signaling pathways,[49] increased glucose metabolism[50] and release of anti-oxidants from glial cells.[51] Reassuring recent primate studies reported that L-Dopa delivered by intraneural injection, with or without carbidopa or entacopone, failed to provoke marked oxidative damage in nigro-striatal pathways.[52] In the rat 6-OHDA model, it is reported that even the relatively weak sulfhydryl antioxidant cysteine was sufficient to protect against neuronal degeneration in the striatum.[53] In seeming contradiction, recent reports have also suggested neuroprotective effects associated with dopamine receptor agonists such as apomorphine, pergolide, pramipexole, and ropinirole.[54–58] Since the latter agonist compounds act predominantly at D2/D3 receptors, there is perhaps a perception that D2/D3 specificity may be most desirable in treating neurodegenerative diseases like PD. However, the listed neuroprotective exception is apomorphine, which has agonist activity at both D1 and D2 receptors.[59,60] In addition, it has recently been reported that lisuride[61] and SKF-38393,[62] (both dopamine combined D1/D2 receptor agonists), may exhibit neuroprotective effects in vitro that include enhancing the survival of cultured dopaminergic neurons in L-DOPA or 1-methyl-4-phenylpyridinium ion (MPP+).[61] This aberrant D2/D3 agonist perception also ignores possible presynaptic properties of D1 agents including possible anti-oxidant and anti-apoptotic effects, effects on DAT flux and effects on glial cells capable of releasing antioxidants. Such in vitro studies have been difficult to reproduce in vivo because of the poor blood–brain-barrier penetrability of many test agents. With respect to 2-amine and 2-amide drugs, glycoconjugation in a manner similar to IPX-750 may provide a useful tool. Future in vivo and in vitro studies will be needed to investigate any potential neuroprotective effects of dopamine-glycoconjugate compounds.

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