Topics in Medicinal Chemistry 18

Corey R. Hopkins *Editor*

Novel Therapeutic Approaches to the Treatment of Parkinson's Disease

An Overview and Update

Springer

18
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Editor

Novel Therapeutic Approaches to the Treatment of Parkinson's Disease

An Overview and Update

With contributions by

L.A. Esposito · P. Galatsis · J.L. Henderson · W.D. Hirst · B.L. Kormos · B.C. Shook

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Preface

Parkinson's disease is the second most common neurodegenerative disease, after Alzheimer's disease, affecting ~1% of the total world's population. Despite the number of individuals afflicted with this disease, the standard of care remains L-DOPA, a drug discovered well over 40 years ago and a drug that only treats the symptoms. As the disease progresses, there is a paucity of treatments available for the patients. Over the past several years, there has been focused research in a number of areas looking to address this lack of disease-modifying treatments.

In this volume of *Topics in Medicinal Chemistry* focused on novel therapeutic approaches to the treatment of Parkinson's disease, the authors cover a range of exciting and potentially novel treatments for this devastating disease. They focus on three areas, adenosine A_{2A} antagonism, synuclein modulation, and LRRK2, and are written by leading members in their area.

The first chapter by Brian Shook (presently Enanta Pharmaceuticals, Watertown, MA) brings the reader update on the state of research in the A_{2A} antagonists for PD as well as combination A_{2A}/A_1 antagonists. The adenosine receptors are present in the indirect pathway of the basal ganglia and are thought to be beneficial in reestablishing the balance within the direct and indirect pathways. The chapter will focus on the xanthine-based and tricyclic- and bicyclic-based A_{2A} antagonists as well as dual A_{2A} and MAO inhibitors.

The second chapter by Luke Esposito (Proteotech, Kirkland, WA) explores the structural biology of α-synuclein as it pertains to PD. The readers will be caught up in the design of α-synuclein-directed therapies – those that have anti-aggregation activity as well as those that inhibit downstream toxicity of the α-synuclein aggregates.

The third chapter by Warren Hirst et al. (Pfizer Worldwide Research & Development, Cambridge, MA) provides an overview of leucine-rich repeat kinase 2 (LRRK), which is the most common known cause of autosomal dominant Parkinson's disease. Mutations in the LRRK are thought to account for ~1% of the total sporadic and ~4% of the familial cases of PD. Significant activity in the

discovery and development of novel small-molecule inhibitors of LRRK by both the academic and industrial communities is discussed.

I would like to express my appreciation to the contributing authors for their work on these excellent chapters for this special volume on emerging therapeutic targets for Parkinson's disease.

Nashville, TN, USA
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Corey R. Hopkins, Ph.D.

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Adenosine A_{2A} Receptor Antagonists

Brian C. Shook

Abstract This chapter summarizes and updates the work on adenosine A_{2A} receptor antagonists for Parkinson's disease. Adenosine A_{2A} receptor antagonists were, and still are, a promising non-dopaminergic approach for the potential treatment of Parkinson's disease. There have been numerous publications, patent applications, and press releases that highlight new medicinal chemistry approaches to this attractive and promising target to treat Parkinson's disease. There have been many research efforts from various pharmaceutical and academic institutions targeting this receptor, and several compounds have advanced into clinical development. The chapter was broken down by scaffold type and will discuss the efforts to optimize particular scaffolds for activity, SAR, pharmacokinetics, and other drug discovery parameters. The majority of approaches focus on preparing selective A_{2A} antagonists, but a few approaches to dual A_{2A}/A_1 antagonists and A_{2A}/MAO-B will also be highlighted. The in vivo profiles of compounds will be highlighted and discussed to compare activities across different chemical series. A clinical report and update will be given on compounds that have entered clinical trials.

Keywords 6-Hydroxydopamine, A_{2A} antagonist, Adenosine, Catalepsy, Dopamine, MPTP, Parkinson's disease

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Abbreviations

6-OHDA	6-Hydroxydopamine
APEC	(2-[(2-aminoethylamino)carbonylethyl-phenyl-ethylamino]-5′-ethylcarboxamido-adenosine)
AR	Adenosine receptors
Bu	Butyl
cAMP	Cyclic adenosine monophosphate
COMT	Catechol *O*-methyltransferase
Et	Ethyl
h	Hour(s)
i-Pr	Isopropyl
L	Liter(s)
L-DOPA	Levodopamine
MAO-B	Monoamine oxidase B
Me	Methyl
min	Minute(s)
mol	Mole(s)
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
p.o.	Oral dose
PD	Parkinson's disease
Ph	Phenyl
PK	Pharmacokinetics
Pr	Propyl
rt	Room temperature
s	Second(s)
s-Bu	*sec*-Butyl
t-Bu	*tert*-Butyl

1 Introduction

Adenosine (**1**, Fig. 1) is a naturally occurring nucleoside that is present in all tissues of mammalian organisms and has numerous biological functions [1]. Early research examined how adenosine and related analogs affected heart function which resulted in the hypothesis that multiple adenosine receptors (ARs) exist. Van Calker et al. found that certain adenosine analogs were able to increase cAMP levels in

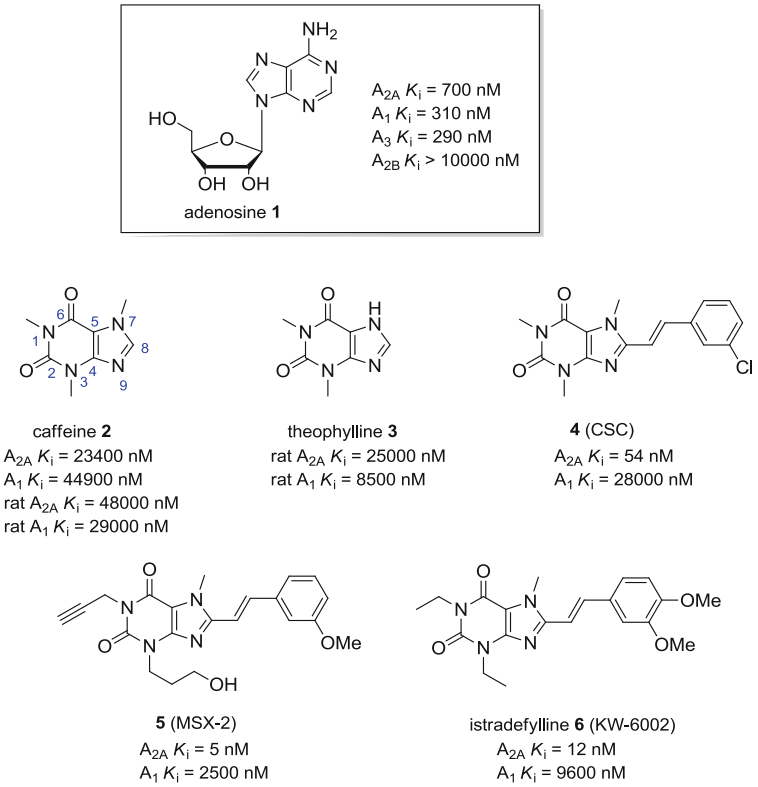


Fig. 1 Xanthine-based A_{2A} antagonists

brain tissue while other analogs inhibited cAMP accumulation, suggesting the existence of distinct adenosine receptors [2]. Adenosine is comprised of four distinct receptor subtypes designated A_1, A_{2A}, A_{2B}, and A_3 belonging to the G protein-coupled receptor superfamily [3]. Adenosine A_1 and A_3 receptors are coupled to inhibitory G proteins, while A_{2A} and A_{2B} receptors are coupled to stimulatory G proteins. The A_2 receptors were divided into two subtypes: A_{2A}, with high affinity for adenosine (0.1–1.0 μM) and A_{2B} with lower adenosine affinity ($\geq$10 μM).

Adenosine is a neuromodulator that coordinates responses to dopamine and other neurotransmitters in areas of the brain that are responsible for motor function, mood, and learning and memory [4, 5]. Adenosine A_{2A} receptors are highly expressed in the spleen, thymus, leukocytes, and striatum but to a lesser extent in the heart, lung, blood vessels, and other areas of the brain. Although A_{2A} receptors are not brain specific, in rodent brain, autoradiography studies showed that the greatest densities of A_{2A} receptors are found in the striatum [6, 7] which closely matches the distribution in humans based on PET imaging [8, 9]. A_{2A} receptors

have been cloned from several species including mouse [10], rat [11], guinea pig [12], and human [13].

The loss of dopamine input into the neostriatum is a hallmark of Parkinson's disease (PD) and causes many of the cardinal motor symptoms of this disorder. In the striatum adenosine A_{2A} receptors co-localize and physically associate with dopamine D_2 receptors [14, 15]. A_{2A} and D_2 receptors have opposing effects on adenylate cyclase and cAMP production in cells, such that activation of A_{2A} receptors inhibits dopamine D_2 receptor signaling. Conversely, A_{2A} receptor antagonists enhance D_2-dependent signaling as shown by induction of immediate early gene c-fos expression in the striatopallidal pathway [16] and facilitate other D_2-mediated responses. Of importance to PD, pharmacological blockade of A_{2A} receptors has shown dramatic beneficial effects in preclinical animal models of PD, showing potentiation of dopamine-mediated responses in dopamine depleted (6-hydroxydopamine, (6-OHDA)-treated) [17–19] animals and dramatic relief of parkinsonian symptoms in MPTP-treated [20–22] nonhuman primates [23–26]. A_{2A} antagonists facilitate dopamine receptor signaling and thereby normalize motor function in animal models of dopamine dysregulation. As a result of these findings, the adenosine A_{2A} receptor has become a sought-after target for treating PD. Blockade of A_{2A} signaling by selective A_{2A} receptor antagonists [27, 28] was shown to be beneficial for not only enhancing the therapeutic effects of L-DOPA but also reducing dyskinesia from long-term L-DOPA treatment [29–31].

The adenosine A_1 receptor is also expressed in the striatum; based on anatomical and in vivo microdialysis studies, A_1 receptors appear to be localized presynaptically of dopamine axon terminals where they inhibit dopamine release [32]. A_1 receptor antagonists facilitate dopamine release in the striatum and like A_{2A} receptors potentiate dopamine-mediated responses. Antagonism of both A_{2A} and A_1 would be synergistic – inhibition of the A_1 receptor will facilitate dopamine release, while inhibition of the A_{2A} receptor will enhance postsynaptic responses to dopamine. Interestingly, the A_1 receptor is also concentrated in neocortical and limbic system structures that are important for cognitive function. Pharmacological inhibition of A_1 receptors enhances neurotransmitter release in the hippocampus [33, 34] and enhances performance in animal models of learning and memory [35]. Antagonism of A_1 receptors could present a potential liability as it is known that some A_1 antagonists have cardiovascular [36] and diuretic [37] liabilities.

Most recently, efforts to design dual A_{2A} antagonists/MAO-B inhibitors have been reported [38, 39]. The activity and expression levels of MAO-B in the human brain increase with age [40], and the activity is associated with increased production of reactive oxygen species (ROS) which may contribute the death of neuronal cells [41]. Selegiline and rasagiline are two marketed irreversible inhibitors of MAO-B given to PD patients as monotherapy or in combination with L-DOPA [42, 43]. In addition, safinamide is a reversible inhibitor of MAO-B currently in phase III clinical trials as an adjuvant therapy for PD [44]. The proven effects of MAO-B inhibitors and the promising effects of A_{2A} antagonists in PD patients suggest that a compound having dual activities would be synergistic and could provide enhanced therapeutic and neuroprotective potential.

Reports have shown that combining positive allosteric modulators (PAMs) of metabotropic glutamate receptor 4 ($mGlu_4$) with A_{2A} antagonists produces a synergistic effect seen in the reversal of haloperidol-induced catalepsy in rats [45]. Loss of striatal dopamine signaling results in overactivation of the indirect pathway which leads to the motor impairments seen in PD [46]. Both $mGlu_4$ and A_{2A} receptors are involved in the indirect pathway and have been described as targets for pharmacological modulation thereof. As mentioned above, antagonism of A_{2A} receptors effectively reverses motor deficits in rodent models of PD, whereas the activation of $mGlu_4$, expressed presynaptically of GABAergic neurons, also reduces motor impairment in rodent models of PD [45, 47]. Although not completely understood, the observed pharmacologic interaction of these two receptors suggests that synergy may be occurring on the same and/or different neuronal pathways.

The major unmet medical needs of PD are improved symptomatic treatment without inducing adverse effects (primarily dyskinesia) associated with long-term L-DOPA or dopamine agonist therapy; opportunity to slow disease progression by protecting midbrain dopamine and other neurons from degeneration; and treatment of disease comorbidities, including cognitive dysfunction, anxiety, and depression. Based on published preclinical and human clinical data, the majority of approaches focus on selective antagonism of A_{2A} receptors, while a few approaches suggest that dual A_{2A}/A_1 receptor antagonists, dual A_{2A} antagonists/MAO-B inhibitors, and combination of A_{2A} antagonists with $mGlu_4$ PAMs may provide increased benefit to PD patients based on their synergistic and complementary pharmacology. There have been a number of excellent reviews on A_{2A} receptor antagonists reported in the literature [48–52], and this chapter will highlight key compounds, old and new, for this target including their PK and in vitro and in vivo activities. The K_i values represented in the figures and tables refer to binding affinities (human) unless noted otherwise. Clinical compounds will be noted and highlighted.

2 Xanthine-Based A_{2A} Antagonists

Adenosine (**1**) is the endogenous natural ligand for adenosine receptors and acts as an agonist with good affinity for A_{2A}, A_1, and A_3 (hA_3 $K_i = 290$ nM) receptors but is much weaker for A_{2B} (hA_{2B} $K_i > 10$ μM) (Fig. 1) [53]. Modification of the adenine and/or ribose portion of **1** resulted in numerous adenosine agonists having improved potency and metabolic stability. Unlike A_{2A} agonists, A_{2A} antagonists are devoid of the ribose moiety and generally possess a mono-, bi-, or tricyclic core structure that mimic the adenine portion of adenosine. Caffeine (**2**) and theophylline (**3**) are two naturally occurring xanthine bases, nonselective adenosine antagonists. In 1980, Fredholm et al. reported that the naturally occurring adenosine antagonists caffeine and theophylline (**2** and **3**, respectively) produced enhanced locomotor activity in mice [54].

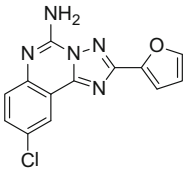
Numerous efforts to identify xanthine-based A_{2A} antagonists have been explored to improve potency, selectivity, and aqueous solubility, a general limitation of xanthines. Chemistry efforts led to the discovery of potent A_{2A} antagonists including 3-chlorostyrylcaffeine (CSC, **4**) [55], MSX-2 (**5**) [56], and istradefylline (KW-6002, **6**) [57] having good selectivity against A_1. The selectivity against A_{2B} and A_3 is also very good for CSC (A_{2B} K_i = ND, rat A_3 $K_i > 10$ μM), MSX2 (A_{2B} K_i = ND, rat A_3 $K_i > 10$ μM), and KW-6002 (A_{2B} K_i = 1,800 nM, rat A_3 $K_i > 3{,}000$ nM). Additional xanthine-based derivatives include DMPX and CS-DMPX, structures not shown [58]. It is well established that the *trans*-styryl substituent at the 8-position is critical for A_{2A} selectivity, but also introduces a chemical liability where the olefin isomerizes from *trans* to *cis* in solution when exposed to light. It has also been shown that the olefin in one analog undergoes a [2 + 2] cycloaddition forming the corresponding dimer which was isolated and as totally inactive against the A_{2A} receptor [59]. Regardless, these compounds have served as valuable research tools, like in the case of KW-6002, a clinical compound that was recently approved in Japan for the treatment of PD [60].

Istradefylline is the most extensively studied xanthine-based A_{2A} antagonist and has shown efficacy in numerous preclinical models of PD [24, 61]. The minimum effective dose (MED) of istradefylline to reverse haloperidol-induced catalepsy in mice was 0.03 mg/kg p.o.). Istradefylline was also able to reverse parkinsonian activity in MPTP-treated primates without invoking dyskinesia. Istradefylline was also investigated in several clinical trials for PD [30, 31]. Istradefylline completed phase III clinical trials in 2009, but was not approved by the FDA due to concerns whether the efficacy finding would support the clinical use of the compound [62]. However, in March 2013, istradefylline was approved in Japan for use as an adjunctive treatment for PD, and it is marketed under the name Nouriast® [60]. The product is used to improve wearing off effects in PD patients on concomitant treatment with L-DOPA-containing medications. There is also one active phase III study using istradefylline (clinicaltrials.gov identifier: NCT01968031) and 17 completed clinical trials according to the clinicaltrials.gov website.

3 Tricyclic A_{2A} Antagonists

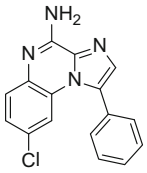
One of the first non-xanthine A_{2A} antagonists discovered was CGS15943 (**7**) which is very potent for A_{2A} (0.4 nM) and has high affinity for other ARs (hA_{2B} K_i = 44 nM, hA_3 K_i = 95 nM) (Fig. 2) [63]. A structurally related triazolo[4,3-*a*] quinoxaline CP-66713 (**8**) was also prepared with good affinity for A_{2A} but modest selectivity versus A_1 [64].

Schering-Plough identified a series of derivatives, where the chlorophenyl ring in **7** was replaced with a pyrazole that led to SCH-58261 (**9**) as a potent A_{2A} antagonist with modest 35-fold selectivity over the A_1 receptor (Fig. 3) [65, 66]. SCH-58261 showed efficacy in rodent models of PD after intraperitoneal (i.p.) administration, but suffers from poor solubility and is not active when dosed



CGS 15943 (**7**)

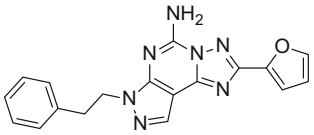
A_{2A} K_i = 0.4 nM
A_1 K_i = 3.5 nM
rat A_{2A} K_i = 3.3 nM
rat A_1 K_i = 21 nM



CP 66713 (**8**)

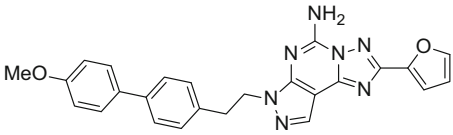
rat A_{2A} K_i = 21 nM
rat A_1 K_i = 270 nM

Fig. 2 First non-xanthine A_{2A} antagonists



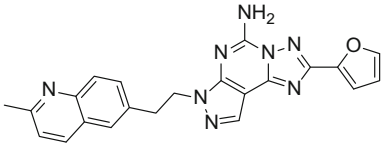
SCH-58261 (**9**)

A_{2A} K_i = 4.3 nM
A_1 K_i = 151 nM
rat A_{2A} K_i = 2.3 nM
rat A_1 K_i = 122 nM



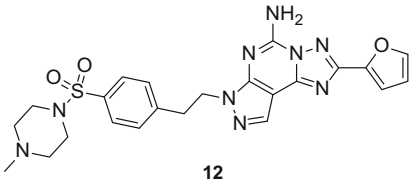
10

A_{2A} K_i = 0.5 nM
A_1 K_i = 1062 nM



11

A_{2A} K_i = 2.4 nM
A_1 K_i = 406 nM

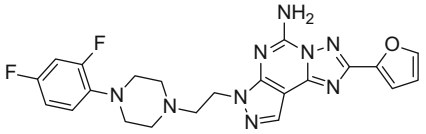


12

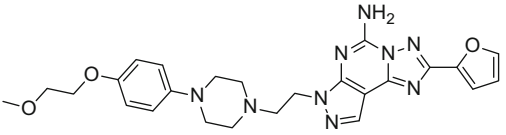
A_{2A} K_i = 3.8 nM
A_1 K_i = 369 nM

Fig. 3 Pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines

orally. It shows similar stimulatory affects to that of caffeine and exhibited positive effects in the rat 6-OHDA lesion model at 5 mg/kg i.p. SCH-58261 was widely accepted as an A_{2A} antagonist reference compound largely due to its ability to cross the blood–brain barrier (BBB). There have been numerous efforts to increase the selectivity and PK properties of this particular scaffold. A series of biaryl substituted pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines were also prepared [67]. The methoxy biaryl compound **10** was very potent for A_{2A} and had much better selectivity (>2,100-fold) against the A_1 receptor compared to **9**. This compound showed 30 and 5% inhibition of rat catalepsy at 1 and 4 h, respectively, at 1 mg/kg. The mediocre efficacy and short duration of action were attributed to the very low plasma exposure exhibited by this compound. Efforts to replace the methoxy aryl



SCH-412348 (**13**)
A_{2A} K_i = 0.6 nM
A_1 K_i > 960 nM



preladenant **14** (SCH-420814)
A_{2A} K_i = 1.1 nM
A_1 K_i = 1474 nM

Fig. 4 Aryl piperazine substituted pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines

substituent with heterocycles like pyridines and pyrazines resulted in compounds that retained potency and selectivity, but generally suffered from poor solubility and poor exposures in rat after oral dosing. Contracting the biaryl substituent into a quinoline, represented in **11**, gave potent compounds with good selectivity versus A_1. More importantly, **11** exhibited a superior PK profile in rats, compared to **9** and **10**, and reversed catalepsy in rats by 86% (1 h) and 38% (4 h) after 3 mg/kg p.o. Additionally, the sulfonamide **12** had a good balance between affinity and solubility while maintaining fairly good selectivity [68]. Unfortunately, no animal studies or PK profiles were reported for **12**.

Additional efforts to optimize potency, selectivity, and PK were achieved by replacing the phenethyl side chain (compounds **9**–**12**) with an aryl piperazine side chain represented in **7** (Fig. 4) [69]. A very promising compound, SCH-412348 (**13**), showed very potent A_{2A} affinity and impressive selectivity (>1,600-fold) versus A_1. Although SCH-412348 showed excellent ability to reverse haloperidol-induced catalepsy (75 and 80% inhibition at 1 and 4 h, respectively) in rats after a dose of 1 mg/kg p.o., the compound has not progressed further due to poor aqueous solubility. Further optimization led to the methoxyethoxy analog **14** (SCH-420814, preladenant), which also had excellent binding affinity for A_{2A} with good selectivity (>1,300-fold) over A_1 and improved solubility. Preladenant also displayed >1,000-fold selectivity versus A_{2B} and A_3 and was also clean in a selectivity panel of more than 60 additional targets. In rats, preladenant showed good oral exposure (AUC = 1,560 ng h/mL at 3 mg/kg p.o.) and bioavailability ($F = 57\%$) and a brain-to-plasma ratio of one. Despite the relatively short half-life ($t_{1/2} = 2.1$ h at 1 mg/kg, i.v.), preladenant (1 mg/kg) reversed haloperidol-induced catalepsy (77 and 70% inhibition at 1 and 4 h, respectively) in rats displaying good duration of action and was further characterized in multiple preclinical animal

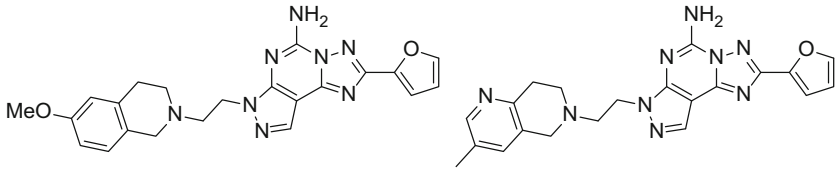


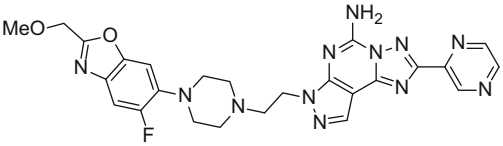
Fig. 5 Fused heterocyclic substituted pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines

models of PD [70]. Preladenant showed significant activity when dosed orally in the rat hypolocomotion model (minimum effective dose 0.1 mg/kg p.o.), 6-OHDA-lesioned rat model (minimum effective dose 0.03 mg/kg p.o.), and MPTP-treated monkey model [50]. Preladenant showed efficacy in PD patients in one phase II clinical trial (P4501) at 2, 5, and 10 mg/kg p.o. Unfortunately on May 13, 2013, Merck (Schering-Plough) announced that the development of preladenant was discontinued due to lack of efficacy in three phase III clinical trials for PD [71].

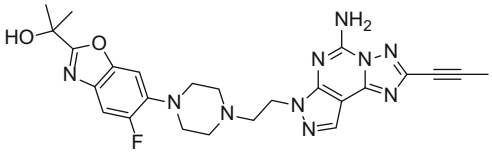
A variety of fused heterocyclic derivatives of the triazolopyrimidine scaffold were prepared that had comparable A_{2A} binding affinities with respect to the aryl piperazines **13** and **14**, but were generally much less selective against the A_1 receptor (Fig. 5) [72]. The tetrahydroisoquinoline derivative **15** was 68-fold selective versus A_1, but had excellent binding affinity for A_{2A}. The compound had moderate plasma exposure in rats and maintained the ability to reverse catalepsy over time by 50 and 58% at 1 and 4 h time points, respectively, after a 3 mg/kg dose p.o. A related azatetrahydroisoquinoline **16** had higher plasma exposure (>fivefold) in rats compared to **15**, good bioavailability ($F = 71\%$), low clearance (4.7 mL/min/kg), and a half-life of 11.3 h. Compound **16** reversed catalepsy in rat (3 mg/kg) by 85 and 30% at 1 and 4 h, respectively, but was less effective at the 4 h time point compared to preladenant despite having a longer half-life. This could be explained by lower brain exposure/free fraction, but brain levels were not reported.

There were significant efforts to identify a suitable replacement for the commonly featured furan, which has the potential to undergo oxidative metabolism. This was a challenging effort and the majority of aryl and heteroaryl replacements retained A_{2A} potency but had significant reduction in the selectivity versus A_1. However, the benzoxazole derivatives **17** and **18**, containing the pyrazine and alkyne replacements, respectively, maintained good potency and excellent selectivity over A_1 (Fig. 6) [73–75]. Compound **17** was claimed in a patent application, but only limited biological data was reported. The alkyne derivative **18** reversed catalepsy in rats by 55 and 48% at 1 and 4 h, respectively, after a 1 mg/kg dose. This represents one of the few compounds from this series that does not have a furan, but is able to maintain good potency in vitro and in vivo, excellent selectivity, and good aqueous solubility [76].

Attempts to further optimize the preladenant structure replaced the pyrazole ring with an imidazole ring to give a series of 3*H*-[1,2,4]-triazolo[5,1-*i*]purin-5-amines

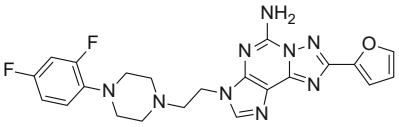


17
A_{2A} K_i = 1.1 nM
A_1 K_i = 1900 nM

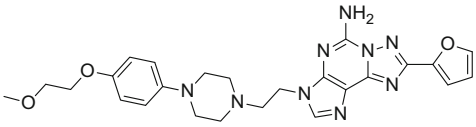


18
A_{2A} K_i = 0.4 nM
A_1 K_i = 1722 nM

Fig. 6 Furan replacements of aryl piperazine substituted 3*H*-[1,2,4]-triazolo[5,1-*i*]purin-5-amines



19
A_{2A} K_i = 0.7 nM
A_1 K_i = 62 nM



20
A_{2A} K_i = 0.9 nM
A_1 K_i = 602 nM

Fig. 7 Aryl piperazine substituted 3*H*-[1,2,4]-triazolo[5,1-*i*]purin-5-amines

(Fig. 7) [77]. Compounds **19** and **20** contain the optimal aryl piperazine substituents present in the pyrazolopyrimidines **13** and **14**. Both **19** and **20** have good affinities for A_{2A} while maintaining good selectivity versus A_1 (>80- and >660-fold, respectively), albeit considerably lower than the selectivities obtained from **13** and **14**. Compound **20** reversed catalepsy in rats by 55 and 50% at 1 and 4 h, respectively, after a 1 mg/kg oral dose. However, the compounds from this series were inferior to the corresponding compounds from the pyrazolopyrimidine scaffold. Efforts to replace the furan substituent with substituted aryls and heteroaryls resulted in compounds having good A_{2A} potency but were much less selective versus A_1 receptors.

A variety of ethylamino derivatives were prepared in a pyrazolo[4,3-*e*]-1,2,4-trizolo[4,3-*c*]pyrimidon-3-one series (Fig. 8) [78]. The ethylene dimethylamino analog **21** has good binding affinity for A_{2A} and is 269-fold selective versus A_1 receptors. In general, analogs had good binding affinities but were not able to reverse haloperidol-induced catalepsy in rat at 10 mg/kg p.o. Tissue distribution

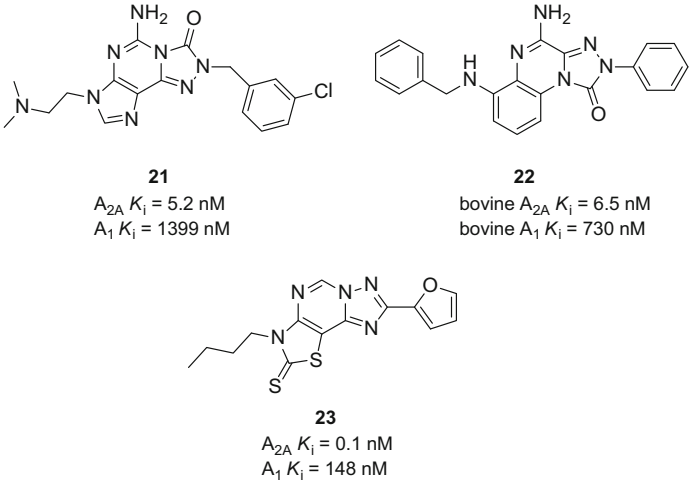


Fig. 8 Pyrazolo[4,3-*e*]-1,2,4-trizolo[4,3-*c*]pyrimidon-3-one, triazoloquinoxalinones, and thiazolotriazolopyrimidines

studies showed very low exposures in rat brain. A series of triazoloquinoxalinones were prepared to be selective A_{2A} antagonists with **22** being the most potent compound reported [79, 80]. Substitution on the benzylic position generally decreased the activity as did replacement of the phenyl with heteroaryl rings. No PK or in vivo data was reported for this series of compounds. A series of thiazolotriazolopyrimidines was synthesized, and multiple compounds showed subnanomolar binding affinities [81]. Compound **23** had potent A_{2A} binding affinity and exhibited good selectivity against A_1 receptors. Compound **23** also showed very potent functional (cAMP) activity ($K_i = 0.1$ nM) and was able to reverse both haloperidol-induced catalepsy and akinesia in mice after a dose of 10 mg/kg p.o.

A series of methylene amine substituted arylindenopyrimidines were reported as dual A_{2A}/A_1 receptor antagonists (Fig. 9) [82]. Compound **24** was the original lead compound that was potent in both A_{2A} and A_1 functional assays (no binding affinity has been reported) and had an ED_{50} of 5.0 mg/kg p.o. in the haloperidol-induced catalepsy model in mice. Compound **24** suffered from poor solubility and it was found to be Ames positive, presumably due to metabolic liability of the furan. A variety of heterocyclic furan replacements were prepared that generally maintained good in vitro potency for both A_{2A} and A_1, but were not active in the mouse catalepsy model. Replacing the furan with phenyl gave **25** that had good in vitro potency and comparable in vivo activity ($ED_{50} = 8.0$ mg/kg p.o.) reversing catalepsy in mice. Compound **25** was negative in the Ames screen which somewhat confirmed that the furan was responsible for the positive Ames, but still suffered from poor solubility. The phenyl ring seemed to be a suitable replacement for the furan and was devoid of the Ames liability, so a variety of amines were

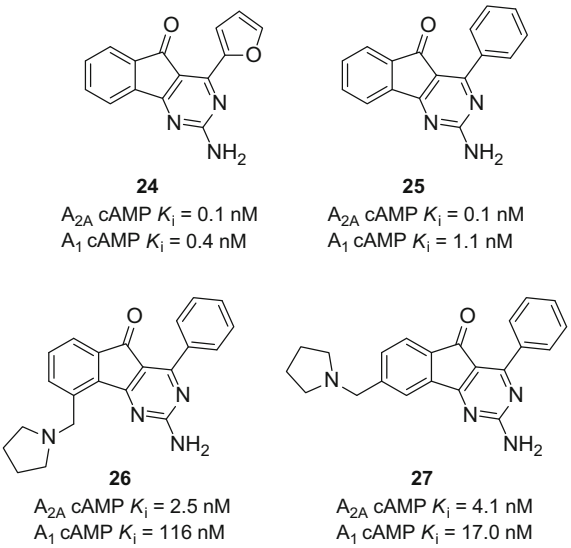
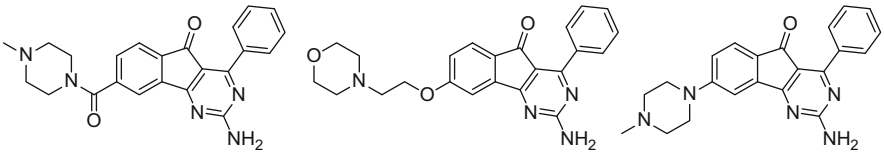


Fig. 9 Arylindenopyrimidines

incorporated at the 9-position of the scaffold to increase solubility. These efforts resulted in the synthesis of **26** that had good in vitro potency and had an ED_{50} of 3.8 mg/kg p.o. in the mouse catalepsy model. Moving the pyrrolidine to the 8-position gave compound **27** that was equipotent at A_{2A}, more potent at A_1, and significantly increased potency in vivo ($ED_{50} = 0.2$ mg/kg p.o.) in the mouse catalepsy model. Compound **27** had good bioavailability and exposure in mice, rats, and monkeys and had a brain-to-plasma ratio of 3 in rats. Further characterization of **27** showed that it was active in rat catalepsy ($ED_{50} = 0.5$ mg/kg p.o.) and had minimum effective doses of 1.0, 1.0, and 10 mg/kg for reserpine-induced akinesia model in mice, 6-OHDA lesion model in rats, and reversing motor disability in MPTP-treated marmosets, respectively [83].

Further evaluation revealed that metabolism of **27** resulted in the formation of reactive metabolites that were attributed to some adverse events seen in the 28-day GLP toxicology studies in nonhuman primates [84]. Metabolite identification studies showed that two reactive metabolites were formed, an endocyclic iminium ion in the pyrrolidine ring and an arylaldehyde formed by oxidation at the benzylic position [85]. The development of **27** was discontinued based on these findings, and the focus was to identify a suitable backup compound devoid of the metabolic liabilities [86]. Compound **28** is a representative amide that had good functional activity at both A_{2A} and A_1 receptors and reversed haloperidol-induced catalepsy in mice ($ED_{50} = 0.4$ mg/kg p.o.) (Fig. 10). PK studies showed that **28** had good exposures in mice, rats, and monkeys and had a brain-to-plasma ratio of ~0.5. Further characterization showed that **28** effectively reversed catalepsy in rats ($ED_{50} = 0.6$ mg/kg p.o.), had efficacy in mouse and rat catalepsy models for at



28
A_{2A} cAMP K_i = 8.2 nM
A_1 cAMP K_i = 58.4 nM

29
A_{2A} cAMP K_i = 6.5 nM
A_1 cAMP K_i = 48.2 nM

30
A_{2A} cAMP K_i = 2.7 nM
A_1 cAMP K_i = 35.6 nM

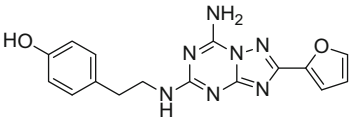
Fig. 10 Substituted arylindenopyrimidines

least 4 h after a 10 mg/kg dose, and had minimum effective doses of 1 mg/kg p.o. in the mouse and rat reserpine models and in the 6-OHDA lesion model in rats. The ether-linked compound **29** was very potent in vivo with an $ED_{50} < 0.1$ mg/kg p.o. in the mouse catalepsy model. A tissue distribution study in rats (10 mg/kg p.o.) showed that **29** had a brain C_{max} of 4.1 μM and a brain-to-plasma ratio of 3. Good exposures were also reported in mice and monkeys. Like **28**, compound **29** exhibited excellent in vivo activity (minimum effective dose in each model was 1 mg/kg p.o.) in a variety of PD models including rat catalepsy ($ED_{50} = 0.3$ mg/kg p.o.), mouse and rat reserpine models, and the 6-OHDA model in rats. The amino-linked compound **30** was potent in vitro and reversed catalepsy in mice with an $ED_{50} < 1.0$ mg/kg p.o. All of the compounds were devoid of the metabolic liabilities associated with compound **27**.

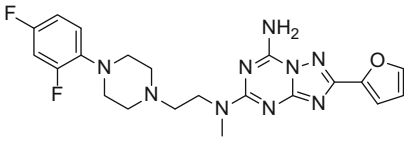
4 Bicyclic A_{2A} Antagonists

ZM241385 (**31**) is a potent A_{2A} antagonist that has good selectivity versus A_1 and has been widely used as a research tool (Fig. 11) [87]. This compound has favorable aqueous solubility but is not orally active in rodent models of PD. In 2008, a co-crystal structure of ZM241385 bound to the A_{2A} receptor was reported [88]. The structure revealed that the commonly featured furan moiety sits deep in the ligand-binding cavity where the oxygen forms a hydrogen bond to an asparagine residue. Compound **32** is a selective A_{2A} antagonist that reverses catalepsy in mice at 3 mg/kg p.o [89]. Interestingly, the des-methyl version has a similar in vitro profile, but is significantly less active in the mouse catalepsy model. One additional methylene between the NMe and piperazine resulted in reduced potency at A_{2A}. Only fluoro substituted phenyls were reported, so it is unclear how other substitution might affect activity and selectivity.

Substituted 1,2,4-triazolo[1,5-*c*]pyrimidines are a bicyclic system related to preladenant without the fused pyrazole ring (Fig. 12) [90]. A variety of linkers were explored including O, S, NH, NMe, and NEt corresponding to compounds **33–37** while keeping the optimized aryl piperazine, present in preladenant, fixed. In general, all of the linkers maintained good affinity for A_{2A} and exhibited good

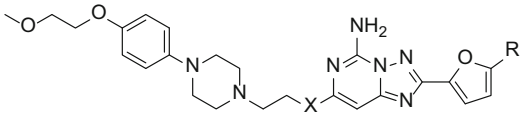


ZM-241385 (**31**)
A_{2A} K_i = 0.8 nM
A_1 K_i = 255 nM

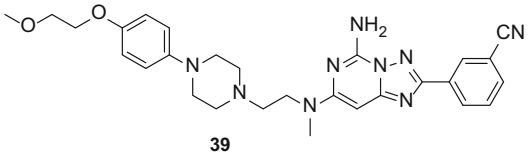


32
rat A_{2A} K_i = 4 nM
rat A_1 K_i = 820 nM

Fig. 11 Triazolotriazines



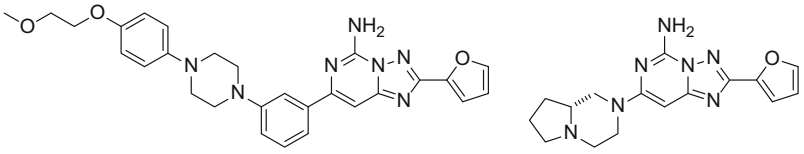
Compound	R	X	A_{2A} K_i (nM)	A_1 K_i (nM)	rat plasma AUC at 3 mg/kg, po, ng h/mL
33	H	O	2.8	1134	943
34	H	S	1.5	1448	965
35	H	NH	9.5	1376	103
36	H	NMe	2.5	1735	521
37	H	NEt	1.5	153	230
38	Cl	O	40	1600	6



39
A_{2A} K_i = 0.7 nM
A_1 K_i = 750 nM
Rat AUC (3 mg/kg) = 546 ng h/mL

Fig. 12 Substituted 1,2,4-triazolo[1,5-*c*]pyrimidines

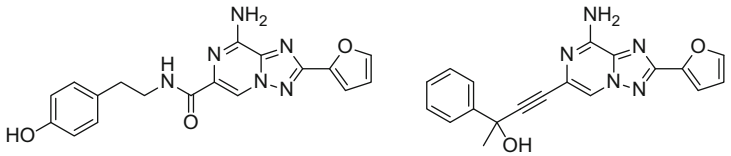
selectivity versus A_1, but the O and S linkages gave the best plasma exposures in rats compared to any of the nitrogen-linked analogs. Interestingly, a chlorine substituent (**38**) on the furan significantly reduced A_{2A} activity and dramatically reduced plasma exposure compared to the *des*-chloro analog **33**. Replacement of the furan in **33** with 3-cyanophenyl afforded compound **39** that had excellent binding affinity for A_{2A}, had good selectivity versus A_1, and had nearly identical exposure in rats. The compounds from this series were evaluated in the haloperidol-



40
A_{2A} K_i = 1.0 nM
A_1 K_i = 1059 nM

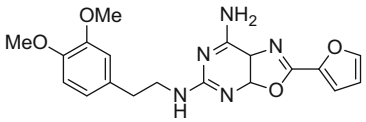
41
rat A_{2A} K_i = 63 nM
rat A_1 K_i = 1071 nM

Fig. 13 Triazolopyrimidines



42
rat A_{2A} K_i = 1.0 nM
rat A_1 K_i = 320 nM

43
rat A_{2A} K_i = 12 nM
rat A_1 K_i = 41 nM



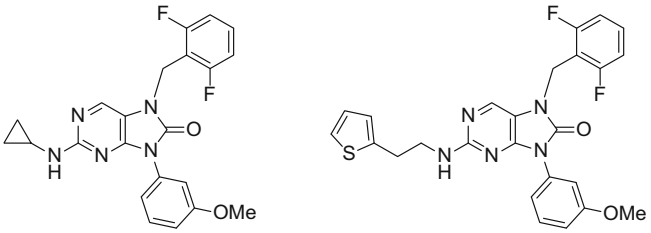
44
porcine A_{2A} K_i = 14 nM
porcine A_1 K_i = 1270 nM

Fig. 14 Triazolopyrazines and oxazolopyrimidines

induced catalepsy model in rats. Compound **34** gave the best results with 65% inhibition of catalepsy at both 1 and 4 h after 3 mg/kg p.o.

A related series of substituted 7-phenyl-[1,2,4]-triazolo[1,5-*c*]pyrimidine-5-amines were also reported (Fig. 13) [91]. Compound **40** was identified as having potent affinity for A_{2A} and good selectivity versus A_1. Further evaluation showed that **40** significantly reversed haloperidol-induced catalepsy at 3 mg/kg p.o. (55 and 40% inhibition at 1 and 4 h, respectively). It also reversed CGS-21680-induced hypolocomotion in rats by 59% after a 1 mg/kg p.o. The much smaller bicyclic substituted compound **41** and related analogs generally had reduced in vitro potency compared to larger substituents like those found in **40** [92]. However, **41** was able to reverse catalepsy in rats for at least 2 h after a 3 mg/kg oral dose. The *R*-stereochemistry of bicycle proved to be significant as it exhibited greater in vitro potency and in vivo efficacy than the corresponding *S*-enantiomer.

A series of related triazolopyrazines have been reported as potent A_{2A} antagonists having good selectivity versus A_1 (Fig. 14). Compound **42** incorporates the



45
A_{2A} K_i = 4 nM
A_1 K_i = 236 nM

46
A_{2A} K_i = 9 nM
A_1 K_i = 1967 nM

Fig. 15 Substituted purinones

amino-ethylphenol unit from ZM241385 that resulted in a very potent A_{2A} antagonist having >300-fold selectivity against A_1 [93]. Side chains containing cyclic amides like morpholine and piperazine resulted in steep decreases in A_{2A} activity. No in vivo characterization of this series of compounds was reported. A distinctly different series of alkyne substituted triazolopyrazines were reported and are generally represented by compound **43** [94]. Typically, compounds having this type of substitution exhibited good affinity for A_{2A} but were very modestly selective versus A_1. Interestingly the corresponding *des*-methyl analog of **43** had ~30-fold decrease in affinity for A_{2A}, and this trend was consistent for all tertiary alcohols compared to their corresponding secondary analogs. Compound **43** was effective in reversing catalepsy in both mice and rats, having an $ED_{50} = 3$ mg/kg p.o. for each. This compound also showed good activity in the 6-OHDA model in rat having a minimum effective dose of 3 mg/kg p.o. A related series of oxazolopyrimidines were prepared in an effort to find a suitable A_{2A} antagonist that could be used as a PET tracer [95]. Compound **44** was the most potent and selective A_{2A} antagonist reported from this series, but this series of compounds was not further developed as PET tracers due to their high degree of unspecific binding which was determined by autoradiography experiments.

A series of trisubstituted purinones was discovered and investigated from a high-throughput screening (Fig. 15) [96]. Substituting N-7 and N-9 with various aryl and benzyl substituents while keeping the cyclopropyl amine substitution at the 2-position constant exhibited some interesting SAR depending on the substitution of the aryl rings. Compound **45** was the most potent compound for A_{2A} and was >50-fold selective versus A_1 receptors. Replacement of the cyclopropyl amine with 2-(thiophen-2-yl)ethanamine afforded compound **46** that had comparable activity for A_{2A} but was considerably more selective (>200-fold) versus A_1 compared to compound **45**. The thiophene analog was the most potent compound reported and had good functional activity (cAMP) in rat ($K_i = 18$ nM). No PK or in vivo results were reported for any compounds within this series.

The (-)-(*R*,*S*)-enantiomer of antimalarial drug mefloquine (**47**) was found to have modest A_{2A} activity while researchers were investigating the compound's

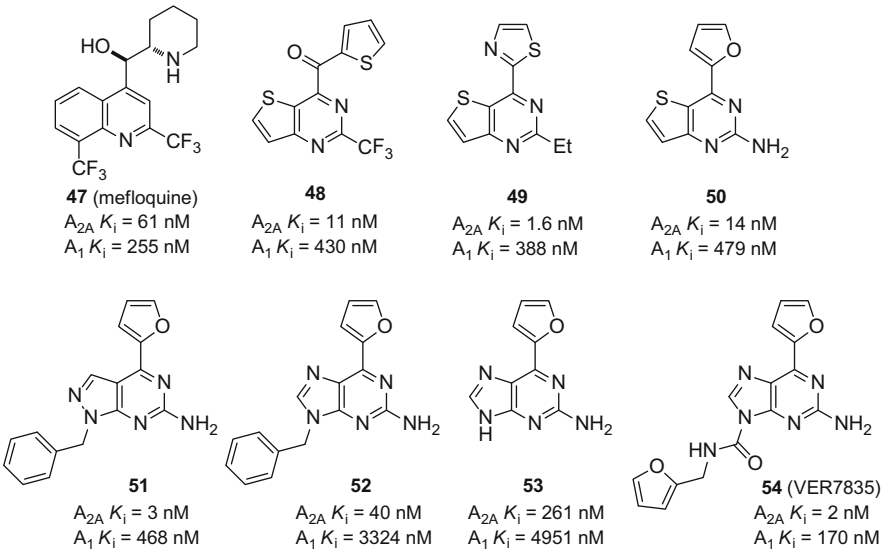


Fig. 16 Thieno[3,2-*d*]pyrimidines, pyrazolo[3,4-*d*]pyrimidines, and 6-arylpurines

side effects (Fig. 16) [97]. Compound **47** was not selective against A_1 and had no in vivo activity in rodent models of PD, but it did serve as an initial lead to develop novel A_{2A} antagonists. Optimization of this lead by changing the central scaffold resulted in the synthesis of a series of thieno[3,2-*d*]pyrimidines having good affinity for A_{2A} [98, 99]. Compound **48** represents one of the most potent compounds from this ketoaryl series and has modest selectivity versus A_1. In general, aryl and pyridyl keto substituents were significantly less potent compared to electron-rich 5-membered heterocycles. Direct attachment of the 2-thiazolyl ring in **49** resulted in significant increases in A_{2A} binding affinity ($K_i = 1.6$ nM) and selectivity over A_1. Compound **49** was also able to reverse hypolocomotion in mice at 30 mg/kg i.p. A similar aminopyrimidine **50** was identified that had good receptor binding for A_{2A} but was moderately selective versus A_1. Efforts to identify other bicyclic templates identified pyrazolo[3,4-*d*]pyrimidine **51** and 6-arylpurines **52** and **53** [100]. Compounds **51** and **52** both show good affinity for A_{2A} and were both able to reverse haloperidol-induced hypolocomotion in mice at 30 mg/kg i.p., but they were not active when dosed orally. Interestingly, the 6-arylpurine **53** had much weaker affinity for A_{2A} but was effective in reversing hypolocomotion in mice when dosed at 10 mg/kg i.p. and 1 mg/kg p.o. No plasma or brain exposures were reported to explain this unexpected result. Further optimization led to compound **54** (VER7835) which was very potent for A_{2A} and maintained modest selectivity versus A_1. Compound **54** was able to reverse haloperidol-induced hypolocomotion in mice after a 10 mg/kg oral dose, but low oral bioavailability and poor in vivo stability halted any further development of this series of compounds.

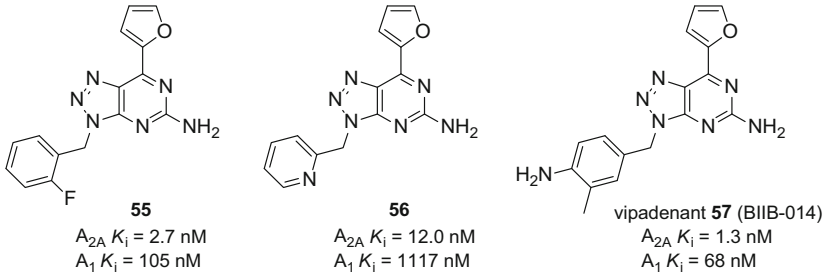


Fig. 17 Benzyl substituted triazolo[4,5-*d*]pyrimidines

The previous work led to an optimization effort to find more potent orally active compounds with good PK profiles (Fig. 17). A variety of substituted benzyl analogs were prepared that had good A_{2A} activity but were not able to reverse haloperidol-induced hypolocomotion when dosed orally [101]. Compound **55** had potent A_{2A} activity and exhibited moderate selectivity versus A_1, but its inactivity was attributed to low solubility and poor PK profiles and led to the synthesis of heterocyclic compounds like the 2-pyridyl analog **56**. Despite good in vitro activity and increased solubility, **56** was not active in vivo if given 30 mg/kg p.o. Further optimization of this scaffold led to the discovery of vipadenant (**57**) (V2006/BIIB014) which was evaluated in phase II clinical trials [102]. Vipadenant showed excellent binding affinity for A_{2A} and had modest selectivity against A_1 ($K_i = 68$ nM) and A_{2B} ($K_i = 63$ nM), while it showed good selectivity against A_3 ($K_i = 1{,}005$ nM). When tested in vivo in the mouse and rat haloperidol-induced hypolocomotion models, **57** had a minimum effective dose of 0.1 and 1 mg/kg, respectively. Vipadenant was able to increase contralateral rotations in 6-OHDA-lesioned rats when dosed orally in combination with apomorphine at 3 and 10 mg/kg [48]. The compound also showed reversal of motor disability in MPTP-treated marmosets without dyskinesias at a minimum effective dose of <5 mg/kg p.o [48]. Vipadenant also exhibited a favorable brain-to-plasma ratio as indicated by a 57% brain uptake in rats after a 2 mg/kg i.v. dose. The excellent in vivo data and a favorable safety profile helped progress this compound through phase II clinical trials. Despite positive phase II clinical results, in July 2010 Vernalis and Biogen have discontinued the development of vipadenant due to some preclinical toxicology findings that showed adverse side effects [103]. Vernalis is developing V81444 (structure not disclosed) which recently completed a phase I study in healthy male volunteers (clinicaltrials.gov identifier: NCT01634568). In December 2012, they reported positive results from a receptor occupancy study with this compound and that a phase IIa clinical trial was planned to begin by the second quarter of 2013 [104]. There are no reports indicating that this study is ongoing or has even started.

A series of triazolo-9*H*-purines were reported as potent A_{2A} antagonists having modest to low selectivity versus A_1 (Fig. 18) [105]. Compounds **58** and **59** (ST-1535) are representative examples of the series developed by Sigma-Tau.

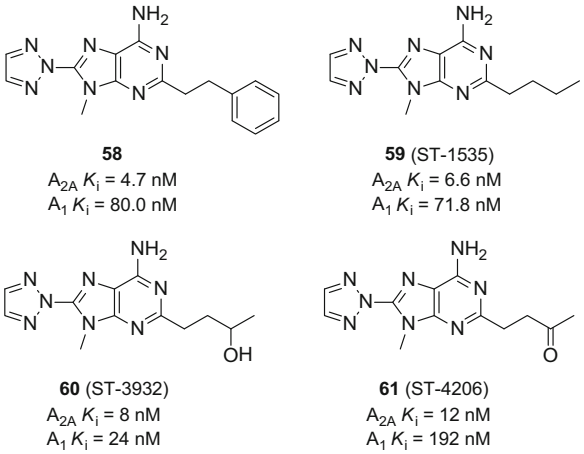


Fig. 18 Triazolo-9*H*-purines

ST-1535 has been characterized extensively in various animal models of PD. ST-1535 has minimum effective doses of 5.0 and 1.25 mg/kg in hypolocomotion and haloperidol-induced catalepsy models in mice, respectively [106]. Further studies show that ST-1535 potentiated L-DOPA activity in 6-OHDA-lesioned rats [107, 108]. In the MPTP-treated marmosets, ST-1535 dosed alone at 40 mg/kg significantly increased locomotor activity but did not improve motor disability [109]. However, when dosed at 20 mg/kg in combination with L-DOPA (2.5 mg/kg), a significant reversal of motor disability was seen compared to 20 mg/kg ST-1535 alone. The significance was not reported with respect to L-DOPA (2.5 mg/kg) alone. In addition, ST-1535 reduced tacrine-induced tremulous jaw movements in rats and was also studied for neuroprotective potential in rats. ST-1535 has been studied in phase I clinical trials, but it is no longer listed on Sigma-Tau's pipeline. A recent patent application revealed that compounds **60** and **61** are active metabolites of **59** and had similar A_{2A}, but had low selectivity versus A_1 [110]. Both metabolites were effective in reversing catalepsy in mice at 10 mg/kg p.o. and increased contralateral rotations in 6-OHDA-treated rats at 3 mg/kg p.o. It is not exactly clear why the development of these compounds was halted.

A series of aminomethyl substituted thieno[2,3-*d*]pyrimidines has been reported as A_{2A} antagonists having moderate selectivity versus A_1 (Fig. 19) [111]. The in vitro activities for A_{2A} and A_1 are reported as functional activity, not binding affinity. The unsubstituted furan proved to be the optimal substituent for A_{2A} potency and selectivity versus A_1. The basicity of amine substituent was the key to obtain in vivo activity in the haloperidol-induced catalepsy model in mice. Very basic amines like pyrrolidines and piperidines had good in vitro potency but were not able to reverse catalepsy in vivo at 10 mg/kg p.o. The morpholine compound **62** was much less basic (calcd $pK_a = 6.5$) than the corresponding pyrrolidines (calcd $pK_a = 8.2$) and piperidines (calcd $pK_a = 8.5$) and had a reported oral ED_{50} of

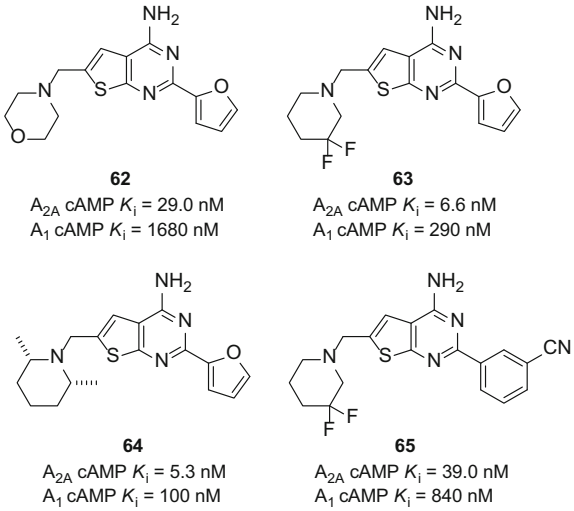


Fig. 19 Aminomethyl substituted thieno[2,3-*d*]pyrimidines

1.3 mg/kg in the mouse catalepsy model. A variety of less basic amines were explored to determine the scope and to identify if the basicity was indeed a real trend. Compounds **63** and **64** were even more potent in vitro than **62** and significantly reversed catalepsy at 10 mg/kg p.o. Further characterization of **63** showed that it had an ED_{50} of <1 mg/kg p.o. in mouse catalepsy, but suffered from a suboptimal PK profile that was attributed to metabolism of the furan. Efforts to substitute the 5-position of the furan with methyl, ethyl, isopropyl, cyclopropyl, $–CHF_2$, chlorine, and bromine generally resulted in slight decreases of in vitro potency, but dramatic decreases of in vivo activity. Replacing the furan with various 5-membered heterocycles resulted in a very large decrease of in vitro potency. Interestingly, the furan could be replaced with a 3-cyanophenyl group and maintain good A_{2A} activity. Compound **65** was ~6-fold less potent than the corresponding furan analog, but maintained good activity in vivo ($ED_{50} < 1$ mg/kg) and had a superior PK profile. Various donating and withdrawing substituents on the aryl ring were explored but only the 3-CN substituted compounds were able to maintain desirable A_{2A} activity.

A similar series of benzyl substituted thieno[2,3-*d*]pyrimidines has also been shown to have good A_{2A} activity and have varying degrees of selectivity versus A_1 (Fig. 20) [112]. Compound **66** was the representative example for this series having good A_{2A} activity and an ED_{50} of 2.2 mg/kg p.o. in the mouse catalepsy model. This compound had good oral exposures in rodent, but suffered from high clearance, a short half-life, and a short duration of action in mouse catalepsy. Compound **66** did, however, have a brain-to-plasma ratio of 1.2 in rats at 1 h post-dosing. The length of the aryl side chain was also investigated, and the benzyl length proved to be optimal as direct aryl attachment and phenethyl substituents significantly decrease A_{2A} activity. As with the previous series, the furan was replaced with various

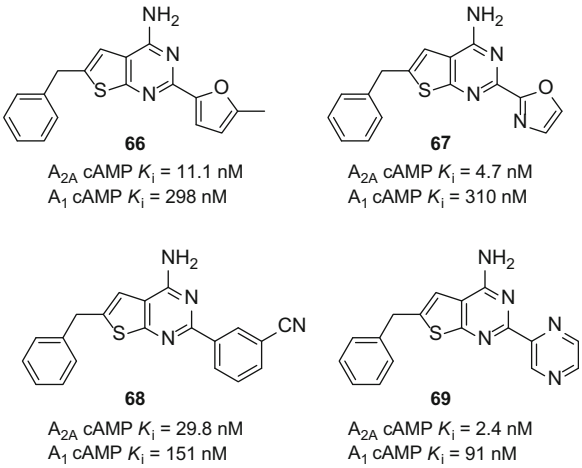


Fig. 20 Benzyl substituted thieno[2,3-*d*]pyrimidines

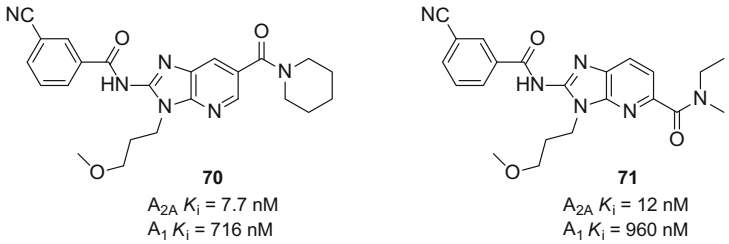


Fig. 21 2-Aminoimidazopyridines

5-membered heterocycles, but unlike the previous series, several oxazole and thiazole analogs maintained good in vitro activity particularly compound **67**. Unfortunately **67** was not active in mouse catalepsy at 3 mg/kg p.o. nor were any other 5-membered heterocyclic compounds that had good in vitro potency. Like the previous series, the 3-CN substituted phenyl was the ideal furan replacement, and compound **68** had good activity for A_{2A} and effectively reversed haloperidol-induced catalepsy in mice with an ED_{50} of <1.0 mg/kg p.o. Pyrazine was another furan replacement that maintained potent A_{2A} activity in compound **69**, but no in vivo efficacy or PK was reported. Substitution on the benzyl ring was investigated with this series of compounds and only *ortho* substituents like methoxy, chloro, and fluoro were tolerated.

A series of 2-aminoimidazopyridines **70** and **71** resulted from the optimization of a high-throughput screening hit (Fig. 21) [113]. Compound **70** showed good binding affinity for A_{2A} with 93-fold selectivity over A_1. A variety of amides were explored in the 6-position but generally suffered from poor microsomal stability. Moving the amide substituent to the 5-position resulted in compounds with similar binding affinities, but in general had better microsomal stability. Compound **71** was

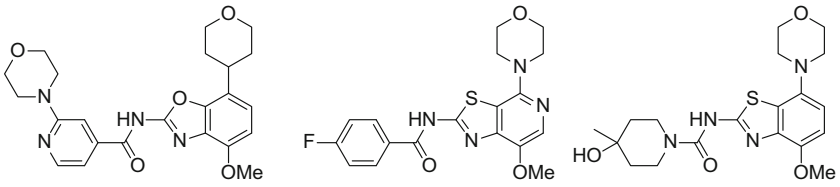


Fig. 22 Substituted 4-morpholino-benzothiazoles

a potent binder for A_{2A} and showed moderate plasma and brain exposures when dosed orally, but were not evaluated in animal models of PD.

Benzoxazole-, thiazolopyridine-, and benzothiazole-based series of compounds have been reported in the patent literature by Hoffmann-La Roche [114–116]. Compounds **72** and **73** are representative examples of the benzoxazole and thiazolopyridine series, respectively, but no efficacy in PD models was reported (Fig. 22) [117, 118]. Further optimization of this scaffold led to the urea compound **74** (tozadenant, SYN115) which had good potency for A_{2A} and was 270-fold and >4,000-fold selective versus A_1 in binding and functional assays, respectively. Tozadenant had an ED_{50} of 0.5 mg/kg when dosed orally in the (2-[(2-aminoethylamino)carbonylethyl-phenyl-ethylamino]-5′-ethylcarboxamidoadenosine) (APEC)-induced hypolocomotion model in rats [50, 119]. The compound also showed good PK parameters in both rats and dogs. In January 2007, Synosia acquired the rights from Roche to develop tozadenant for the treatment of PD [120]. Tozadenant recently completed a phase II/III clinical trials for PD (clinicaltrials.gov identifier: NCT01283594) and for a phase 0 clinical trial for cocaine dependence (clinicaltrials.gov identifier: NCT00783276). In February 2011, Synosia was acquired by Biotie Therapeutics. More recently Biotie has granted UCB Pharma a license for exclusive worldwide rights to tozadenant where they will be responsible for the ongoing/additional phase III trials and commercialization [121].

Domain Therapeutics is evaluating a selective A_{2A} antagonist DT1133, but no structure has been disclosed.[1] A recent patent application from the company claims a series of substituted imidazo[1,2-*a*]pyridines as A_{2A} antagonists (Fig. 23) [122]. In general, compounds had good binding affinity, but no A_1 activity was reported. Compound **75** had good affinity for A_{2A} and was able to reverse haloperidol-induced catalepsy at 10 mg/kg p.o. The related indazole **76** and a benzoxazole **77** were both very potent binders for A_{2A}, but no PK or in vivo data was reported for these compounds.

A series of benzofurans from Kissei Pharmaceuticals has been reported in a patent application (Fig. 24) [123]. Unfortunately, biological data was only reported

[1] DT-1133 was listed on the Doamin Therapeutics website http://www.domaintherapeutics.com/.

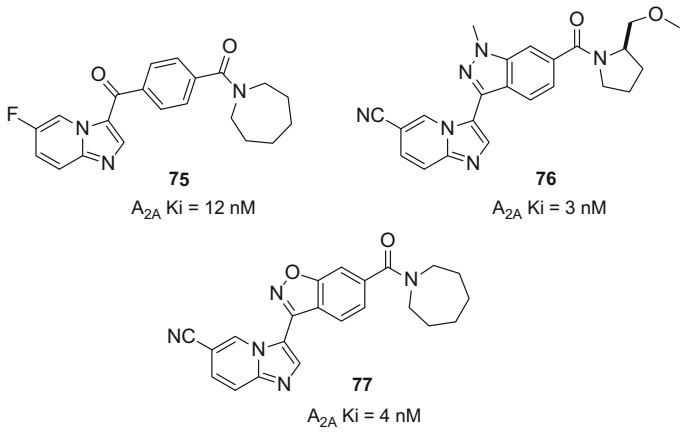


Fig. 23 Imidazopyridines

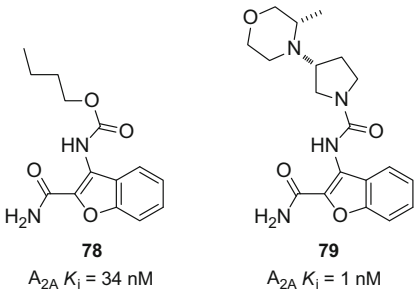
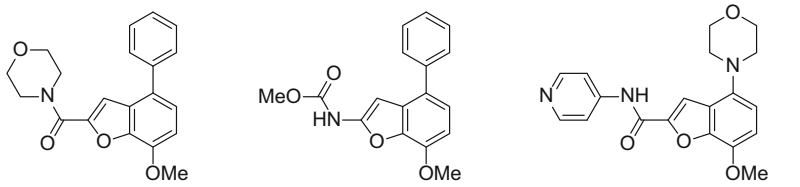


Fig. 24 Substituted benzofurans

for a limited number of compounds. The amino group in the 3-position of the benzofuran was substituted by various carbamate and urea linkages. The carbamate **78** was the only compound, of this type, with reported A_{2A} activity. The urea **79** was the most potent of the analogs that had A_{2A} activity reported.

A series of 4-aryl substituted benzofurans was identified and the 4-phenyl substituent was found to be the most consistent group for A_{2A} activity (Fig. 25) [124]. The morpholine amide **80** showed good affinity for A_{2A} and significantly reversed (78%) CGS-induced catalepsy in mice at 10 mg/kg p.o. Further exploration of this scaffold showed that the methyl carbamate **81** also had good A_{2A} activity and reversed catalepsy by 86% at 10 mg/kg p.o. A variety of amides was prepared from a related 4-morpholinobenzofuran series and were evaluated in vitro and in vivo [125]. The analogs in general exhibited similar in vitro potencies with respect to the 4-phenylbenzofurans, but also had increased aqueous solubility and improved PK properties. Compound **82** was identified having good in vitro activity for A_{2A} and excellent activity reversing (76% at 10 mg/kg p.o.) CGS-induced



80

A_{2A} % inh at 10 nM = 39%
A_{2A} % inh at 100 nM = 81%
A_1 % inh at 1 μM = 37%
A_1 % inh at 10 μM = 82%

81

A_{2A} % inh at 10 nM = 28%
A_{2A} % inh at 100 nM = 85%

82

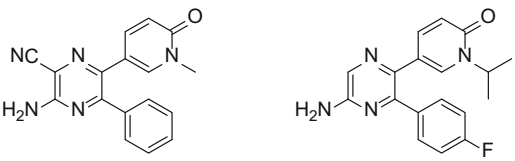
A_{2A} % inh at 10 nM = 30%
A_{2A} % inh at 100 nM = 73%

Fig. 25 4-Aryl and 4-morpholino substituted benzofurans

catalepsy in mice. The compound also showed significant reduction in motor disabilities and increased locomotor activity in MPTP-treated marmosets for 5 h after an oral dose of 10 mg/kg.

5 Monocyclic A_{2A} Antagonists

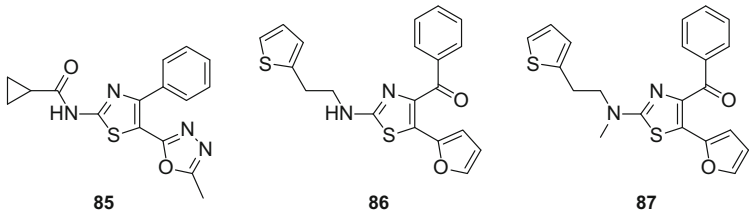
An aminopyrazine-based series of compounds was reported as dual A_{2A}/A_1 receptor antagonists (Fig. 26) [126]. Compound **83** had potent affinity for both A_{2A} and A_1 and significantly reversed haloperidol-induced catalepsy in mice at 3.2 mg/kg p.o. Further optimization of the pyrazine scaffold led to the identification of **84** (ASP-5854) that has been characterized extensively in numerous animal models of PD and cognition [127]. ASP-5854 reversed CGS-21680-induced and haloperidol-induced catalepsy in mice with ED_{50} values of 0.15 and 0.07 mg/kg p.o., respectively, and had a minimum effective dose of 0.1 mg/kg to reverse haloperidol-induced catalepsy in rats. In rhesus monkeys, ASP-5854 gave >85% occupancy of A_{2A} receptors in the striatum and reversed haloperidol-induced catalepsy with an ED_{50} of 0.1 mg/kg p.o [128]. ASP-5854 effectively potentiated L-DOPA-induced rotation in 6-OHDA-lesioned rats with a minimum effective dose of 0.03 mg/kg p.o. Statistical significance was reached in MPTP-treated mice and MPTP-treated marmoset models at 0.1 and 1 mg/kg p.o, respectively [129]. ASP-5854 produced positive results in scopolamine-induced memory deficits in the mouse Y-maze test and rat passive avoidance test, both models of cognition, with minimum effective doses of 0.3 and 0.1 mg/kg p.o., respectively. The selective A_{2A} antagonist KW-6002 (**6**) was also tested in parallel for comparison in these models and was significantly less effective having a minimum effective dose of 10 mg/kg p.o. in the mouse Y-maze test and no effect in the rat passive avoidance test up to 10 mg/kg p.o. These results suggest that a dual A_{2A}/A_1 may provide added benefit to PD patients to address the cognitive impairments associated with the disease.



83
A_{2A} K_i = 2.4 nM
A_1 K_i = 22.5 nM

84 (ASP-5854)
A_{2A} K_i = 1.8 nM
A_1 K_i = 9.0 nM

Fig. 26 Pyridone substituted pyrazines



85
A_{2A} K_i = 28 nM
A_1 K_i = 150 nM

86
A_{2A} K_i = 12 nM
A_1 K_i > 6700 nM

87
A_{2A} K_i = 8170 nM
A_1 K_i > 10000 nM

Fig. 27 Heterocyclic substituted 2-amino-thiazoles

A series of carboxamides of 2-amino-4-phenyl-thiazoles were prepared as adenosine A_{2A} receptor antagonists (Fig. 27) [130]. The optimization of this scaffold identified that the cyclopropyl amide was the optimal amide substituent. Further evaluation into the substitution at the 5-position of the thiazole led to the 5-methyl-[1,2,4]-oxadiazole compound **85** which had good affinity for A_{2A} but very modest selectivity versus A_1. Compound **85** also showed good oral activity in the haloperidol-induced hypolocomotion model in mice with an ED_{50} of 7 mg/kg. A series of related substituted 2-amino-5-benzoyl-4-(2-furyl)thiazoles were designed from a high-throughput screening hit [131]. Lead optimization led to compound **86** which had good affinity for A_{2A} and excellent selectivity versus A_1. Interestingly, a simple methyl substitution on the nitrogen (**87**) dramatically decreased the binding affinity for A_{2A}. No in vivo evaluation of **86** was reported.

A series of substituted 1,2,4-triazines were recently reported as potent A_{2A} antagonists (Fig. 28) [132]. The 5-position was substituted with a variety of phenyl and 4-pyridyl groups that had good A_{2A} activity but generally had low selectivity against A_1. They were claimed as dual A_{2A}/A_1 antagonists in a recent patent application [133]. Compound **88** had good oral exposure and bioavailability in rats and had a brain-to-plasma ratio of 3.2 at 0.5 h. This compound was able to reverse haloperidol-induced catalepsy in rats at 1 and 2 h with an $ED_{50} = 0.2$ mg/kg p.o. for both time points. A co-crystal structure was obtained with **88**, and it showed hydrogen bonding of the pyridyl nitrogen with water molecules and that generated a second series of compounds that replace the pyridine with cyclic amines [134]. Compound **89** and **90** are representative compounds containing a cyclic amine, and they each show increased selectivity versus A_1. The hydrogen bonding

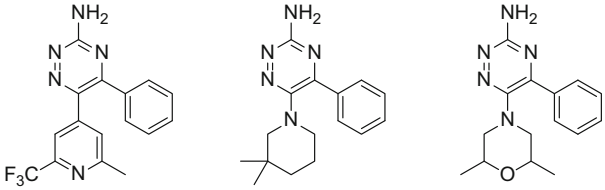


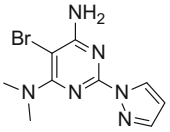
Fig. 28 Substituted 1,2,4-triazines

of the morpholine oxygen is presumed to be the reason for higher affinity compared to the piperidine compound **89**. No data was reported for their activity in animal models of PD.

Palobiofarma is currently investigating an A_{2A} antagonist PBF509 (structure not disclosed) for the treatment of PD.[2] A phase I trial to assess safety and tolerability has been completed (clinicaltrials.gov identifier: NCT01691924). A recent patent application claimed a series of substituted aminopyrimidines (Fig. 29) [135]. Limited biological data was reported for a select amount of compounds including **91** which has good binding affinity for A_{2A} and also good functional activity. A variety of substituents were explored in the 6-position, and it was shown that the pyrazine and thiazole analogs **92** and **93**, respectively, were two of the most potent compounds reported. No in vivo activity or PK data was given.

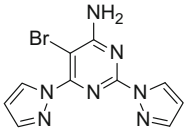
A series of trisubstituted pyrimidines was reported to have potent A_{2A} activity (Fig. 30) [136]. Compound **94** was a potent A_{2A} and A $_1$antagonist, but it had poor metabolic stability that was attributed to the unsubstituted furan moiety. SAR studies resulted in the synthesis of the thiazole compounds **95** and **96** that maintained potency for A_{2A}. Interestingly, replacing the benzylic methylene, present in **95**, with an oxygen atom (**96**) increased the selectivity against A_1. Compound **96** was further optimized by replacing the thiazole with the dimethylpyrazole to give compound **97** that was very potent for A_{2A} and had excellent selectivity versus A_1. Despite the increase in selectivity, **97** suffered from poor solubility, so basic amines were appended to the phenol ring to increase solubility and improve PK properties. A number of the amino alkyl compounds had good binding affinities for A_{2A} and good selectivity versus A_1, but had significant hERG liabilities. Compound **98** had the best hERG profile (patch clamp $IC_{50} = 1.2$ μM) and showed oral activity in the haloperidol-induced catalepsy in rats with a minimal effective dose of 10 mg/kg. Despite very high clearance in rats, **98** exhibited desirable brain levels up to 4 h post-dosing. Further optimization of this scaffold led to compound

[2] In June, 2012, on the pipeline website, PBF509 was listed as being in phase I development as an adenosine A_{2A} antagonist for the potential treatment of Parkinson's disease. http://www.palobiofarma.com.



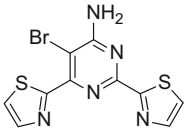
91

A_{2A} K_i = 35 nM
A_{2A} cAMP K_i = 50 nM



92

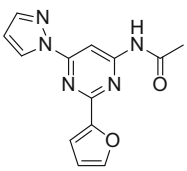
A_{2A} K_i = 12 nM
A_{2A} cAMP K_i = 25 nM



93

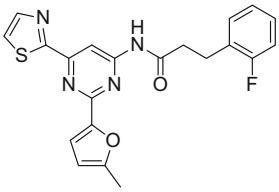
A_{2A} K_i = 1 nM
A_{2A} cAMP K_i = 12 nM

Fig. 29 Substituted bromoaminopyrimidines



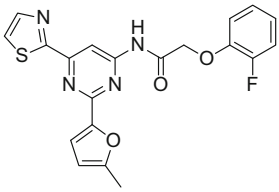
94

A_{2A} K_i = 0.6 nM
A_1 K_i = 9.2 nM



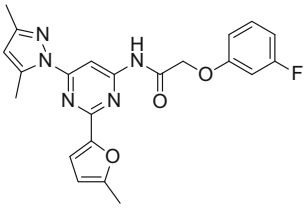
95

A_{2A} K_i = 16 nM
A_1 K_i = 410 nM



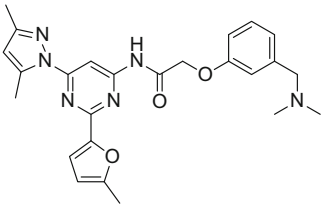
96

A_{2A} K_i = 7.5 nM
A_1 K_i = 650 nM



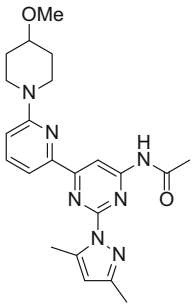
97

A_{2A} K_i = 3.7 nM
A_1 K_i = 9700 nM



98

A_{2A} K_i = 0.4 nM
A_1 K_i = 60 nM



99

A_{2A} K_i = 0.4 nM
A_1 K_i = 77 nM

Fig. 30 Various trisubstituted pyrimidines

99, which had potent affinity for A_{2A} and A_1. Compound **99** effectively reversed catalepsy in rats at 1 mg/kg p.o. and had a brain-to-plasma ratio of ~4 [137]. This compound also potentiated L-DOPA-induced rotation in the 6-OHDA lesion model in rats with a minimum effective dose of 3 mg/kg p.o. 4-Methoxypiperidine was replaced with a number of cyclic amines to increase solubility, but generally resulted in decreased A_{2A} activity and decreased selectivity against A_1 [138].

A series of 2-amino-*N*-pyrimidin-4-ylacetamides were prepared with compound **69** being the representative example (Fig. 31) [139]. Compound **100** had a high affinity for A_{2A} and was selective versus A_1. The compound exhibited very good exposure (2,700 ng/g) in rat brain at 2 h after a 10 mg/kg oral dose. The compound

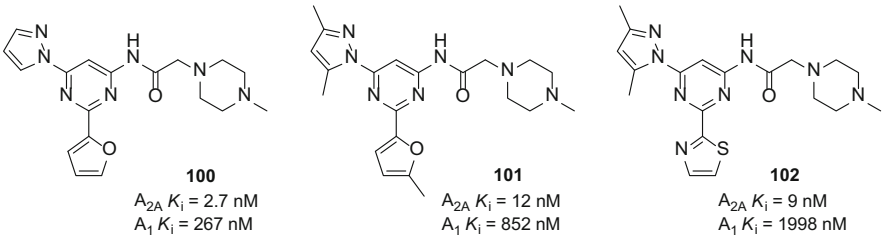


Fig. 31 Piperazine substituted pyrimidine acetamides

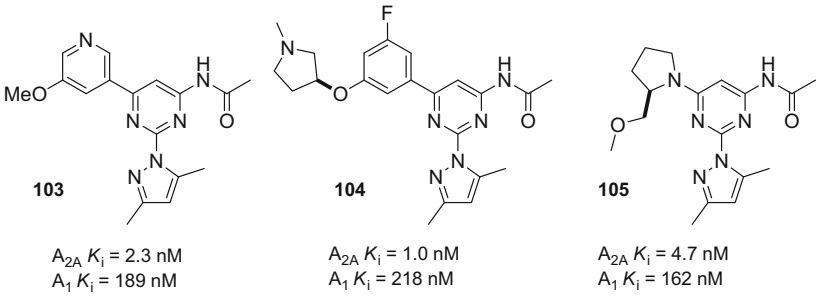


Fig. 32 Acyl amino pyrimidines

effectively reversed haloperidol-induced catalepsy in rats at 10 mg/kg p.o. Like **94**, the unsubstituted furan in compound **100** was being metabolized, so there was an effort to find a suitable furan replacement [140]. Several combinations consisting of 5- and 6-membered heterocycles were made to replace the furan and pyrazole substituents. Compounds **101** and **102** were identified as the most promising compounds which have good binding affinities and good selectivity versus A_1. Further evaluation of **101** showed it to be a potent inhibitor of hERG with and IC_{50} of 950 nM (patch clamp). The basicity of the piperazine was enhancing the hERG activity, so various replacements were prepared in effort to decrease the hERG liability. Although the hERG inhibition could not be completely eliminated, the removal of one of the basic amines decreased the hERG activity. Several compounds within the series showed activity in haloperidol-induced catalepsy in rats.

A related series of 2,6-diaryl-4-acylaminopyrimidines were developed with improved solubility and metabolic stability (Fig. 32) [141]. Compound **103** was a potent A_{2A} antagonist that had good plasma exposure in rats and reversed catalepsy at a dose of 3 mg/kg p.o. Unfortunately, **103** had poor solubility and low exposures in both dogs and monkeys. Compound **104** had high affinity for A_{2A}, was 218-fold selective against A_1, and was significantly more soluble than **103**. More importantly, the addition of the fluorine atom significantly increased metabolic stability, decreased intrinsic clearance, and reduced CYP inhibition compared to the corresponding *des*-fluoro analog, not shown. Further characterization of **104** showed that it significantly reversed haloperidol-induced catalepsy in rats at

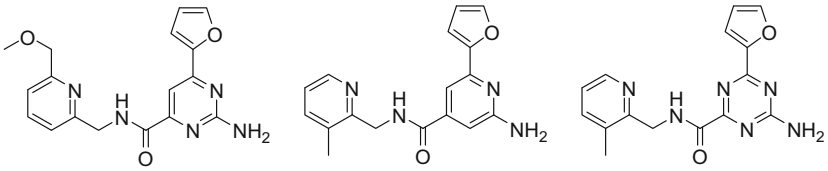


Fig. 33 Pyrimidine, pyridine, and triazine carboxamides

30 mg/kg p.o. The direct attachment of the pyrrolidine to the pyrimidine core gave **105** which has the ability to reverse catalepsy in rats at 10 mg/kg p.o. Compound **105** was able to potentiate L-DOPA-induced rotational behavior in the 6-OHDA-lesioned rat at 10 and 30 mg/kg oral doses.

Analogs of pyrimidine-4-carboxamides have been reported to display good potency for A_{2A} and with moderate to good selectivity over other adenosine receptor subtypes (Fig. 33) [142]. Compound **106** is a representative example having binding affinities of 1.7, 43, 460, 1,740 nM for A_{2A}, A_1, A_{2B}, A_3, respectively. Compound **106** also had a minimum effective dose of 0.1 mg/kg in the haloperidol-induced hypolocomotion model in mice. This compound had very good bioavailability ($F = 90\%$) in rats with very good brain uptake. Replacing the pyridyl with a variety of substituted aryls generally gave compounds with good affinity for A_{2A} but with very modest selectivity against A_1. Related pyridine **107** and triazine **108** have also been reported, but are significantly less potent than the corresponding pyrimidines [143].

6 Radiolabeled A_{2A} Antagonists

Several A_{2A} antagonists have been radiolabeled to study binding affinities and receptor occupancy in an effort to better understand the pharmacology of A_{2A} receptor and its relevance in treating PD patients. There have been many efforts to identify radio and PET ligands that bind specifically to A_{2A} receptors in the brain without the complication of nonspecific binding. The first generation of PET ligands used the xanthine core as a starting point and eventually discovered [^{11}C] TMSX (**109**) which was used to map A_{2A} receptors in the brains of PD patients (Fig. 34) [144, 145]. Like the parent compound, **109** is susceptible to photoisomerization which prompted efforts to identify non-xanthine-based PET tracers.

Several studies were reported using [^{125}I]ZM241385 and [^{3}H]ZM241385 (structures not shown), but their use has been limited because of its good affinity for A_{2B} ($K_i = 75$ nM). The first non-xanthine tracer to be used as a versatile research tool was [^{3}H]SCH58261 (**110**) which showed reversible binding in rat striatal membranes [146]. This tracer has been used in numerous studies including A_{2A} receptor

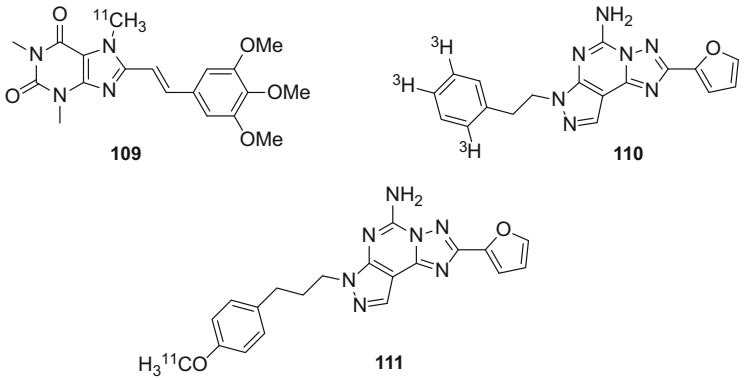


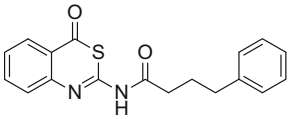
Fig. 34 Radioligands

labeling in human platelets and neutrophil membranes and autoradiography studies in rats [147–150].

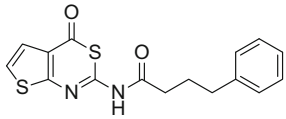
Perhaps the most significant and best radioligand is [^{11}C]SCH-442416 (**111**) which has high affinity for A_{2A} (h$K_i = 0.05$ nM) and is very selective against other receptor subtypes (A_1 h$K_i = 1{,}111$ nM, A_{2B} h$K_i > 10{,}000$ nM, A_3 h$K_i > 10{,}000$ nM) [151]. It also has good potency and selectivity in rat A_{2A} r$K_i = 0.5$ nM and A_1 r$K_i = 1{,}815$ nM. Compound **111** had good brain exposure and had a brain distribution that was four to fivefold higher in the striatum compared to other brain compartments 15 min post-dosing. This compound was successfully used as a PET tracer in healthy male volunteers to study the relationships between dose, plasma exposure, and receptor occupancy of vipadenant **57** [152].

7 Dual A_{2A} and MAO-B Inhibitors

A series of benzothiazinones and thienothiazinones have been reported as dual A_{2A}/MAO-B inhibitors (Fig. 35) [38, 39]. The activity and expression levels of MAO-B in the human brain increase with age while MAO-A remains constant [40]. Under L-DOPA treatment, the increased MAO-B activity is associated with increased hydrogen peroxide formation, which could contribute to neurodegeneration related to PD. As mentioned previously, it is known that inhibiting MAO-B improves symptoms of PD, and two MAO-B inhibitors, selegiline and rasagiline, are currently approved and used for treatment of PD patients as monotherapy or in combination with nn-DOPA [42, 43]. Therefore it was proposed that a compound having dual action, A_{2A} antagonist and MAO-B inhibitor, may have improved therapeutic and neuroprotective potential in the treatment of PD. The benzothiazinone **112** has good affinity for A_{2A} and is moderately selective versus A_1 [38, 39]. This compound also has good potency for MAO-B, one of the

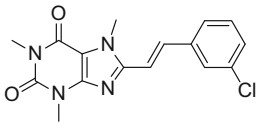


112
A_{2A} K_i = 39.5 nM
A_1 K_i = 2489 nM
MAO-B IC_{50} = 34.9 nM

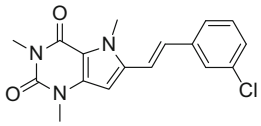


113
A_{2A} K_i = 82.5 nM
A_1 K_i = 330 nM
MAO-B IC_{50} = 69.7 nM

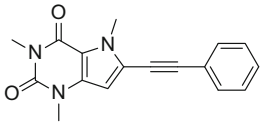
Fig. 35 Benzothiazinones and thienothiazinones



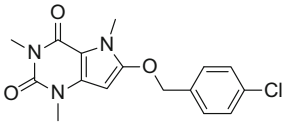
4 (CSC)
A_{2A} K_i = 54 nM
A_1 K_i = 28000 nM
MAO-B IC_{50} = 18.1



114
A_{2A} K_i = 260 nM
A_1 K_i = ND
MAO-B IC_{50} = 200 nM



115
A_{2A} K_i > 10000 nM
A_1 K_i = ND
MAO-B IC_{50} = 173 nM



116
A_{2A} K_i > 10000 nM
A_1 K_i = ND
MAO-B IC_{50} = 48 nM

Fig. 36 9-Deazaxanthines

most potent from this series, and is quite selective versus MAO-A. The length of the amide side chain was optimal, as in **112**, and substitution on the phenyl ring generally resulted in decreased A_{2A} activity. The related thienothiazinone **113** was approximately twofold less potent for both A_{2A} and MAO-B, but the biggest difference between **112** and **113** is that **113** is significantly less selective (<5-fold) against A_1 compared to **112** (>60-fold). No in vivo data was reported for either series of compounds.

CSC (**4**, Fig. 1) is a potent and selective A_{2A} antagonist from the xanthine series of compounds; it is also known to be a potent MAO-B inhibitor [39, 55] A recent series of 9-deazaxanthine derivatives have been reported as dual A_{2A} antagonists/MAO-B inhibitors with **114** being the most interesting of compounds (Fig. 36) [39]. It has a good balance of A_{2A} and MAO-B activity and was shown to dose-dependently reverse haloperidol-induced catalepsy with a minimum effective dose

of 30 mg/kg. Replacement of the chlorine with substituents like CF_3, OMe, and Br resulted in slight decreases in both A_{2A} and MAO-B activities. Replacing the olefin side chain with and alkyne (**115**) or alkoxyl (**116**) linker dramatically decreased A_{2A} activity but maintained comparable MAO-B potency.

8 A_{2A} Antagonists with $mGlu_4$ PAMs

Metabotropic glutamate receptor-4 ($mGlu_4$) positive allosteric modulators (PAM) have been reported to produce antiparkinsonian-like effects in preclinical models of PD [153]. It has also been reported that co-administration of a group III mGlu agonist with an A_{2A} antagonist produced enhanced reversal of haloperidol-induced catalepsy that was significantly better than the activity produced by either agent when dosed alone [47]. More recently it was shown that co-administration of VU0364770, an $mGlu_4$ PAM, significantly increased preladenant's (**14**) ability to reverse catalepsy in rats. VU0364770 was able to reverse catalepsy alone at a subcutaneous dose of 30 mg/kg but not at 10 mg/kg s.c [45]. In this study, it was also assessed that preladenant was able to reverse catalepsy in rats at 0.3 mg/kg p.o., but not at 0.1 mg/kg. It was shown that VU0364770 (10 mg/kg s.c.) given in combination with preladenant (0.1 mg/kg p.o.), not an effective dose of either by itself, produced significant reversal of catalepsy. Also, the dosing of VU0364770 increased the amount of reversal for all doses of preladenant (0.3 and 1 mg/k p.o.) compared to when preladenant was dosed alone. Although not completely understood, the observed pharmacologic interaction of these two receptors suggests that synergy may be occurring on the same and/or different neuronal pathways. There are two striatal GABAergic projection pathways, the direct pathway that projects directly to the substantia nigra pars reticulata and the internal globus pallidus, and the indirect pathway that projects from the striatum to the external globus pallidus [46]. Loss of striatal dopaminergic innervation results in overactivation of the indirect pathway which contributes to the motor deficits observed in PD. Adenosine A_{2A} and $mGlu_4$ receptors both act on the indirect pathway, and inhibiting A_{2A} receptors or activating $mGlu_4$ receptors has been shown to reverse motor impairments in animal models of PD. The roles of these receptors on the indirect pathway seem to complement one another and could thus explain the observed pharmacology and also suggest that targeting these two receptors could provide enhanced treatment for PD patients. It would be exciting to see if one could target both receptors within the same molecule.

9 Summary

Adenosine A_{2A} receptor antagonists continue to be a sought-after target for the treatment of PD. A variety of chemical scaffolds have been reported with interesting SAR trends that showed dramatic activity differences, both in vitro and in vivo, from subtle modifications. Despite the relentless research efforts to identify potent compounds, there still remains the challenge of achieving selectivity, solubility, and acceptable pharmacokinetic/pharmacodynamic properties to progress into the clinic. There have been a number of compounds that are in or have been in clinical trials including istradefylline (**6**), preladenant (**14**), vipadenant (**57**), ST-1535 (**59**), tozadenant (**74**), V81444, and PBF509. The progression through the clinic with positive trial results indicates the potential of A_{2A} antagonists to treat PD patients with potential advantages over the current standard treatments, but the lack of robust efficacy in phase III trials gives pause to whether this target is really beneficial to PD patients. The approval of istradefylline is exciting, but it will be interesting to monitor how PD patients will respond after continued treatment. Examples of dual A_{2A}/A_1 and A_{2A}/MAO-B were also described as having the potential to provide added benefit to PD patients, via A_1 antagonism or MAO-B inhibition. The dual activities, however, need to be tested in a clinical setting to completely understand possible synergies. Combination of A_{2A} antagonists with $mGlu_4$ PAMs is another interesting approach that will surely receive more interest and facilitate more studies due to the initial positive results in rodent models of PD. The ongoing clinical trials with tozadenant, V81444, and PBF509 will hopefully help validate this target to treat Parkinson's disease.

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Targeting α-Synuclein as a Parkinson's Disease Therapeutic

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Abstract α-Synuclein is a dynamic protein capable of assuming an ensemble of physiological and pathological structures. Its primary role in Parkinson's disease (PD) pathogenesis makes it an attractive though nonconventional therapeutic target. An understanding of α-synuclein intra- and intermolecular interactions and post-translational modifications that lead to its misfolding, aggregation, accumulation, and cell-to-cell prion-like spreading is necessary to inform the design of α-synuclein-directed therapeutics. Native α-synuclein interacts with membranes and plays an important role in synaptic transmission and vesicle trafficking at the presynaptic terminal, though aggregated α-synuclein may be compromised in its ability to perform these functions. In addition to discussing α-synuclein structural biology, this chapter explores the variety of experimental therapeutic approaches currently under investigation that aim to maintain physiological α-synuclein levels, limit toxic aggregates, and lessen effects of misfolded α-synuclein on cellular homeostasis. For example, oligonucleotide-based approaches limit α-synuclein gene expression. In addition, select small molecules and peptides inhibit α-synuclein aggregation at substoichiometric concentrations in favor of less structured, more soluble, and less toxic oligomers that may be better substrates for clearance. Some small molecules also remodel existing α-synuclein fibrils. Modest chemical changes to these small molecules can greatly impact their mechanism of action. Finally, α-synuclein-directed immunotherapy enhances the lysosomal clearance of α-synuclein in experimental models and is currently in clinical trials. Other therapeutic approaches target α-synuclein posttranslational modifications, aim to limit its impact on mitochondria or its ability to alter gene expression in the nucleus.

Keywords Amyloid, Autophagy, Disease-modifying, Immunotherapy, Lewy body, Natively unfolded, Nutraceutical, Off-pathway oligomers, Parkinson's disease, Protein misfolding, Reactive oxygen species, Small molecule, α-Synuclein

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1 Introduction

Novel approaches to treat PD, like those directed at many of the other eponymous neurodegenerative disorders characterized by pathogenic misfolded proteins, have rapidly evolved in recent years. Research and drug discovery efforts – initially aimed at understanding and treating the anatomical and neurochemical changes that cause the clinical syndrome – have expanded rapidly as a result of the full biochemical characterization of the pathological lesion and underlying molecular culprits. Hence, in the last 15 years, discoveries in molecular genetics have been a critical driving force behind the search for new medicines that target underlying disease etiology – so-called disease-modifying therapeutics.

It was within this new framework that intracellular lesions known as Lewy bodies first revealed the central role of α-synuclein in the pathogenesis and progression [1, 2] of PD and related "synucleinopathies" such as dementia with Lewy bodies (DLB) and multiple systems atrophy (MSA) (Fig. 1). Lewy bodies consist

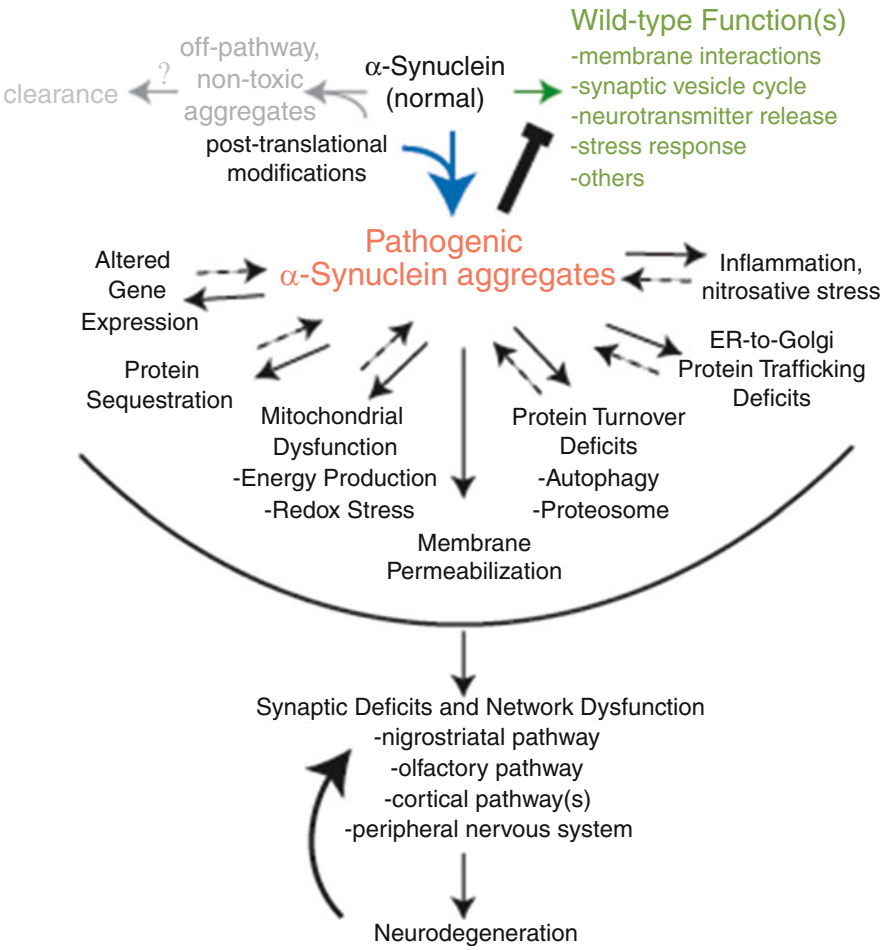

Fig. 1 Pathogenic mechanisms of aggregated α-synuclein. In its normal state, α-synuclein is a natively unfolded protein, helical tetramer, or another conformer whose wild-type functions (indicated by *green*) are not fully defined but likely involve a role in synaptic vesicle cycling, neurotransmitter release at the synapse, and stress response. Many of these wild-type functions are mediated by the ability of α-synuclein to bind membranes and populate a variety of structural conformations. However, when relatively rare misfolding events (*blue arrow*) occur under specific cellular conditions or as a result of posttranslational modifications (*blue curved line*) or genetic mutations (not shown), pathogenic aggregates form (indicated in *red*). These heterogeneous aggregates likely include toxic oligomeric species and β-sheet-rich fibrils that can disrupt α-synuclein wild-type function and/or confer toxic gain of function (*black arrows*). Moreover, most of the deleterious changes also increase steady-state levels of α-synuclein aggregates (*dashed lines* with *small arrowheads*), for example, by limiting their turnover or creating conditions (i.e., nitrosative stress, redox stress, protein sequestration, or decreased energy production) that promote their formation. Many of the deleterious effects of α-synuclein aggregates are interrelated, but the illustration of such relationships is omitted for simplicity. For example, α-synuclein aggregates can sequester transcription factors in the cytosol, resulting in decreased autophagic clearance. α-Synuclein-induced synaptic deficits and network dysfunction result from a collection of events

primarily of an eosinophilic dense core surrounded by a halo of radiating filamentous α-synuclein fibrils. Lewy bodies are reliably detected in the postmortem brains of PD patients in both subcortical and cortical regions, including the nigrostriatal pathway, the cortex, and the hippocampus [1, 3–5], with the extent of pathology depending on the disease stage [2]. The critical role of the small but dynamic α-synuclein was corroborated soon thereafter by extensive molecular genetic, biochemical, and cell biological studies. These studies revealed putative "gain-of-function" point mutations in α-synuclein that increase its propensity to misfold and aggregate [6–8] and result in autosomal-dominant familial PD [9]. Furthermore, duplication or triplication of the wild-type α-synuclein gene leads to severe, early onset forms of the disease [10–12], characterized by both classical motor features (bradykinesia, resting tremor, rigidity, and gait disturbances) and non-motor impairments, including dementia, with the severity of symptoms and rate of progression increasing with gene dosage [13]. Moreover, genome-wide association studies have identified polymorphisms at the α-synuclein locus that increase disease risk, possibly by altering levels of the protein [14]. It is worth noting the rare exception of PD lacking Lewy bodies in the substantia nigra resulting from mutations in parkin (reviewed in [15]), a protein with a direct role in Lewy body formation [16]. This observation suggests that PD is not one disease but rather a syndrome of multiple etiologies and pathological manifestations. Nevertheless, the overwhelming majority of PD cases, both idiopathic and inherited, exhibit extensive α-synuclein pathology. Indeed, misfolded α-synuclein exerts its deleterious effects in a number of cell types and neural networks, extending beyond just the dopaminergic nigrostriatal pathway to include the peripheral nervous system. Non-motor symptoms, including anosmia, gastrointestinal tract disorders, rapid eye movement (REM) sleep disorders, and psychiatric disorders such as depression are increasingly recognized among the earliest PD symptoms and critical determinants of quality of life for patients, in part because some are poorly responsive to dopaminergic therapies [17, 18]. Therefore, a therapeutic aimed at limiting α-synuclein toxicity may have benefits that extend beyond those gained from therapies that target specific neurotransmitter systems. Thus, a theme of this review is novel therapeutics that limit steady-state levels of specific toxic α-synuclein forms or block its downstream toxic effects.

Fig. 1 (continued) that over time lead to neurodegeneration in select brain regions, leading to clinical PD. Therefore, blocking the formation of pathogenic aggregates (*blue arrow*) or any of their downstream effectors (*black arrows* in top half of the figure) is the prime target for disease-modifying interventions. Alternatively, α-synuclein-directed therapeutics redirect α-synuclein to off-pathway, nontoxic aggregates (*gray arrow*) that may be good substrates for clearance

2 α-Synuclein Structure, Function, and Pathogenic Misfolding

2.1 α-Synuclein Populates an Ensemble of Native and Pathogenic Structures

α-Synuclein consists of 140 residues, lacking both Cys and Trp residues, and with a low predicted overall hydrophobicity. Its primary structural motifs consist of an N-terminal lipid-binding region (residues 1–60) capable of assuming helical character and containing imperfect 11-residue amphipathic repeats with the KTGEGV consensus sequence, a central hydrophobic region (residues 61–95) known as NAC (or non-amyloid component, originally discovered at autopsy in the brains of Alzheimer's disease patients) believed to be critical for amyloid formation, and a highly acidic C-terminal region (residues 96–140) that favors extended structures. As a result of the highly acidic C-terminal region, at neutral pH, α-synuclein has a large net negative charge of approximately –9 [19].

Unlike so many classic targets of drug discovery, including receptors, transporters, and ion channels, α-synuclein populates a wide variety of structures and conformations. In addition, its wild-type function is not fully understood, although it likely involves vesicle trafficking and modulation of dopamine release at the synapse. While considered by many to be a predominately natively unfolded monomeric protein [20–22], it has been recently asserted that α-synuclein in its native form is a helical tetramer [23–25] or that it populates a set of metastable multimeric conformers [26] distinct from the misfolded putatively toxic oligomers described in Sect. 2.5. In any case, it is generally accepted that the amyloidogenic precursor is a monomer (possibly a disassembled multimer or the true native form), and this free monomeric state of α-synuclein can populate a highly heterogeneous ensemble of natively unfolded or partially folded structures that may or may not coexist in vivo. α-Synuclein polymerizes through a nucleation-dependent process [27] and likely populates partially folded [20], β-like [28], monomeric, and metastable multimeric (oligomeric) states [29, 30], a subset of which seed (kinetically accelerate) further addition of monomer on the way to the thermodynamically stable, fibrillar form that resembles classic amyloid [31]. A corollary to this hypothesis is that a structural conversion from the initially formed unstructured oligomers to more stable, β-sheet-rich toxic oligomers is the key step in fibril formation [32]. Oligomeric forms of α-synuclein also include conformations that are incapable of nucleating or promoting further aggregation. These "off-pathway" oligomers are thus not precursors to large β-sheet-rich fibrils and are less likely to dissociate to monomers than transient oligomers but may instead coexist with fibrils in the steady-state phase [29]. For many, if not most of these structures, from monomer to oligomer to fibril, a dynamic equilibrium likely exists, the balance of which can depend on a number of conditions (discussed in greater detail below) and

ultimately determine whether α-synuclein behaves normally or assumes an ectopic deleterious structure/function.

More evidence of its chameleonic and multifaceted nature is found at the cell biological level. For example, α-synuclein is abundant in both brain and blood. It is typically found in both the cytosolic (soluble) and membrane-associated (particulate) fractions of the brain, and it may be present at both the synapse and the nucleus, as reflected in its name [33]. However, it localizes primarily to the presynaptic nerve terminal under normal conditions, and its pathogenic forms may be transmitted from cell to cell along neural networks (reviewed in [34]). α-Synuclein pathology may even originate outside the central nervous system in the enteric nervous system [35], consistent with constipation being one of the earliest PD symptoms, preceding motor dysfunction by many years [36]. With such a wide cellular and subcellular distribution, and spectrum of possible structures with potentially overlapping but separate pathogenic mechanisms, where does one start when attempting to target α-synuclein as a PD therapeutic?

Intensive research and drug discovery efforts are currently underway with the primary goal of targeting α-synuclein using a variety of approaches including those aimed at eliminating or lessening the abundance of the pathogenic forms directly and lessening their downstream deleterious effects. While great challenges exist in targeting a natively unfolded and dynamic target with a wild-type function, identification of a disease-modifying therapeutic will likely focus, at least in part, on the misfolded forms that are critical molecular determinants of disease etiology and progression.

2.2 *Natively "Unfolded" Monomers Are the Amyloidogenic Precursor*

Whether derived from a disassembled multimeric conformer or existing in its native state, monomeric α-synuclein is believed to be the amyloidogenic precursor, a critical part of the nucleation-dependent polymerization that gives rise to potentially toxic intermediates and amyloid fibrils. The monomeric form may also exert its wild-type function (see Sect. 2.4), thus providing further rationale for therapeutic approaches that favor monomeric α-synuclein.

2.3 *Intramolecular Interactions as Determinants of Aggregation*

Prior to its integration into amyloid, and its full adoption of structures that lead to amyloid formation, the monomeric state of α-synuclein populates a highly

heterogeneous ensemble of unfolded or partially folded structures that may or may not coexist in vivo.

As revealed by a variety of spectroscopic studies, including solution-state nuclear magnetic resonance (NMR), monomeric α-synuclein can be considered a natively unfolded protein, although it is more compact, on average, than pure random coil [37]. Molecular modeling experiments also predict structural characteristics of an unfolded protein, including greater surface area exposed to solvent and a less compact structure, comprised of conformations with greater radii of gyration, indicative of more intramolecular movement, relative to more compact globular states [38]. Why might an abundant and disease-relevant protein evolve to exist in a natively unfolded state, with the consequential increased propensity for aggregation? A natively unfolded state may provide functional benefits in the complex and crowded cellular milieu, for example, by creating greater surface area for binding and a more "flexible" functionality. These characteristics may, in turn, allow adaption to cellular conditions such as folding upon binding to the target (i.e., membranes in the case of α-synuclein [39, 40]) or service as a linker in the assembly of macromolecular arrays [41].

The amino acid composition of α-synuclein predicts secondary structure motifs. Indeed, even in its "unfolded" state, α-synuclein is not completely devoid of transient and/or more stable intramolecular interactions and secondary structure motifs. For example, the degree of signal broadening demonstrated in paramagnetic relaxation enhancement (PRE) NMR studies confirms that α-synuclein is, on average, more compact than expected for a random coil protein [42]. These studies also suggest the existence of long-range interactions between the N-terminus and C-terminus and between the C-terminus and NAC region. The relevance of such residual secondary structure and intramolecular interactions to the aggregation propensity of α-synuclein was first explored in an attempt to explain the accelerated aggregation rate of A30P mutant relative to wild-type α-synuclein. The A30P mutation [43] is the second of five α-synuclein point mutations that have been identified to date including A53T [9], E46K [44], and the recently discovered G51D and H50Q [45–47], all of which cluster in the N-terminal region of α-synuclein. Using high-resolution NMR, it was demonstrated that the A30P mutation disrupted residual helical content at the N-terminus. In wild-type α-synuclein, this helical region may provide a hydrophobic face that facilitates an interaction with the NAC region, thereby shielding the NAC region from intermolecular contacts. As a helix breaker, the proline substitution mutation relieves this interference and promotes oligomerization. The A53T mutation, however, has a more direct effect on the NAC region, favoring a slightly extended conformation that could facilitate intermolecular interactions [48]. Subsequent work demonstrated interactions between a hydrophobic cluster in the NAC region (amino acids 85–95) and the C-terminus (residues 110–130) and an electrostatic interaction between the C-terminal amino acids centered at residue 120 and the N-terminus, around residue 20 [49, 50]. These interactions could occlude the hydrophobic NAC region [50, 51] and thereby inhibit intermolecular interactions that involve this region, having the net effect of preventing misfolding into structures that give rise to higher-order,

potentially more toxic (i.e., oligomeric and fibrillar) forms. The notion of the C-terminus shielding the amyloidogenic NAC region is consistent with α-synuclein C-terminal truncations promoting misfolding [19, 52]. In addition, comparison of human α-synuclein with the more aggregation-prone mouse α-synuclein suggests that differences in intramolecular interactions could account for the different aggregation propensities, with mouse α-synuclein less prone to stabilizing intramolecular interactions relative to human α-synuclein [37].

More recently, however, the significance of intramolecular interactions on α-synuclein misfolding propensity has been challenged, with the assertion that long-range interactions shielding solvent exposure of the NAC region have only a minimal effect on oligomerization [53]. Instead, intermolecular electrostatic repulsion of the negatively charged C-terminus, hydrophobicity, and overall structural compactness may play a more prominent role in oligomerization [54–57]. For example, using single-molecule (sm) fluorescence resonance energy transfer (FRET), it was demonstrated that at low pH, charge neutralization at the C-terminus increases intramolecular contacts and allows inclusion into a hydrophobic cluster that is more aggregation prone [56]. Polycations, on the other hand, may shield the negative charges at the C-terminus, but not eliminate them, without causing significant conformational changes, thereby altering aggregation propensity by reducing electrostatic repulsion between protein molecules without requiring formation of the hydrophobic core found at low pH [56]. Notably, the C-terminus is phosphorylated in vivo at multiple sites [58], and this has been shown in some reports to increase aggregation (discussed in [59] and Sect. 3.3) consistent with the proper balance of electrostatic interactions involving the C-terminus being a determinant of aggregation propensity (although some phosphorylated forms, such as Ser87-P, may inhibit aggregation [60]).

Some of the differences in opinion about the role of intramolecular long-range interactions in α-synuclein aggregation should be viewed in the context of the limitations of the experimental methods used to draw conclusions, including the uncertainty associated with molecular modeling and the potential for structural changes resulting from introduction of a paramagnetic probe into the protein for PRE NMR analysis.

A related hypothesis focuses largely on internal protein dynamics as a determinant of aggregation. A key observation is the association between the reconfiguration rate of α-synuclein into its natively unfolded state and its aggregation rate. Accordingly, conditions that favor reconfiguration disfavor bimolecular interactions [61], whereas conditions such as high temperature or low pH that slow reconfiguration and reduce diffusibility allow sampling of aggregation-prone disordered conformations (i.e., where hydrophobes are solvent exposed) and thus favor bimolecular interactions and aggregation. Thus, the effect of disrupting interactions between the C-terminus and the NAC region at low pH is to allow the NAC region to instead collapse upon itself (presumably into a more aggregation-prone conformation). These findings provide more evidence that the type and extent of intramolecular α-synuclein interactions are important determinants of intermolecular interactions. These observations also imply that structural

probes of compaction, such as FRET and paramagnetic resonance enhancement, and measures of intramolecular diffusion, such as Trp-Cys contact quenching studies, are complementary methods for determining the composition of the unfolded ensemble [61]. Consistent with this hypothesis, it was shown that curcumin, a molecule known to have potent α-synuclein anti-aggregation properties [62, 63], favors α-synuclein reconfiguration to its native state(s) [64].

In summary, the amino acid sequence of α-synuclein dictates local aggregation-determining properties that can rapidly change and interconvert. The exact contribution of each local domain or secondary structure motif to overall aggregation propensity is dependent on multiple factors including, but not limited to, posttranslational modifications, solution conditions, and proximity to other biomolecules. Inhibition of the earliest steps of aggregation at the monomeric level is thus a logical point of therapeutic intervention.

2.4 Toward an Understanding of α-Synuclein Function

Despite intensive investigation, the wild-type function of α-synuclein is not fully understood. α-Synuclein was identified through a presumably unbiased biochemical screen as an inhibitor of phospholipase D. α-Synuclein has been shown to bind to lipid membranes [65, 66], consistent with the structural propensity of the N-terminus to form α-helical structures of the correct size and orientation to interact with phospholipids containing acidic head groups such as sphingomyelin, phosphatidylserine, and phosphatidylglycerol [67]. This interaction may affect the propensity to form aggregates (see Sect. 2.7) and is an example of α-synuclein misfolding causing the loss of wild-type function.

α-Synuclein lacks a transmembrane domain or lipid anchor, so its mechanism of localization to the synaptic bouton is uncertain, though the N-terminal amphipathic repeats likely play a role. α-Synuclein has been demonstrated to have moderate membrane mobility [68], although multiple pools of differing mobility may exist and aggregation may reduce its mobility [69]. Interestingly, the A30P mutation increases mobility, thus supporting the role for N-terminal α-helix in membrane binding that limits mobility [68]. α-Synuclein has a preference for membranes with high curvature, and α-synuclein itself has been shown to bend membranes [70, 71]. Synaptic vesicles are among the smallest biological membranes yet to be classified and therefore exhibit an intrinsic degree of curvature. α-Synuclein binds vesicles [72]. Upon redistribution from the synaptic vesicle to the relatively flat plasma membrane, for example, during exocytosis, α-synuclein disperses, suggesting that curvature of the membrane supports α-synuclein association [73]. α-Synuclein also interacts with membrane microdomains, such as lipid rafts, and this interaction may not be passive, with α-synuclein potentially contributing to raft formation [74].

Its membrane-associated, presynaptic localization and association with the distal reserve pool of synaptic vesicles strongly suggests a role in neurotransmitter release, synaptic function, and plasticity.

Some of the original studies describing α-synuclein function suggested an ability to promote neurotransmitter release [75, 76].

Other studies, however, using α-synuclein knockout mice, demonstrated an inhibitory role for α-synuclein in dopamine vesicle exocytosis (release) and neurotransmission [77, 78], but a supportive role in maintaining the readily releasable vesicle pool, as evidenced by a deficiency in refilling of vesicles and trafficking of vesicles from the reserve pool to the site of release [76, 77]. Further, siRNA knockdown of α-synuclein resulted in decreased availability of the reserve synaptic vesicle pool [75].

Overexpression of α-synuclein, at levels that do not cause neurotoxicity, has supported the assertion of an endogenous inhibitory role by causing decreased catecholamine release in cultured cells [79], including primary hippocampal and midbrain neurons by inducing a reduction in the size of the synaptic vesicle recycling pool [80] and by causing inhibition of intersynaptic vesicle trafficking and exocytosis [81]. Similarly, in PDGFβ-human α-synuclein transgenic mice, a model for DLB [82], the suppression of vesicle exocytosis by α-synuclein is evidenced at the ultrastructural level by enlarged synaptic boutons and convolution of internal membranes connected to the cell surface [83].

A better understanding of the complex role of α-synuclein at the presynaptic terminal resulted from the discovery of a biochemical association and functional interaction of the C-terminus of α-synuclein with vSNARE synaptobrevin 2 [84], the vesicle-bound component of the 4-membered complex that plays a critical role in membrane fusion and vesicle exocytosis leading to neurotransmitter release. As a result of this interaction with vSNARE, α-synuclein promotes assembly of the SNARE complex [84], although overexpression of α-synuclein may inhibit neurotransmitter release through a poorly understood alternative mechanism that involves the α-synuclein-vSNARE (or membrane-bound tSNARE) interaction. Alternatively, α-synuclein may inhibit SNARE-mediated vesicle fusion through direct binding to the adjacent membrane, without an interaction between α-synuclein and any of the SNARE proteins [85]. In any case, the net effect of α-synuclein at or near the SNARE complex is to alter its assembly and thereby modulate neurotransmitter release and vesicle recycling, and the biological effect likely depends on the levels and aggregation state of α-synuclein at the presynaptic terminal.

Genetic evidence links α-synuclein function and the presynaptic chaperone protein cysteine-string protein (CSPα). CSPα knockout mice show progressive synaptic degeneration and death by two months of age that is activity dependent and linked to a deficit in SNARE complex assembly. Interestingly, the CSPα phenotype is worsened by α-synuclein absence and, surprisingly, attenuated by α-synuclein overexpression [86]. By promoting SNARE complex assembly and dampening synaptic activity that contributes to the CSPα degenerative phenotype,

α-synuclein may partially compensate from the absence of CSPα [84, 86], suggesting a nonclassical chaperone role for α-synuclein.

Interestingly, knockout of α-synuclein protects against the toxicity of the mitochondrial toxin MPTP/MPP^+ [87], though overexpression may [88] or may not [89] enhance MPTP toxicity, and the role of α-synuclein in facilitating MPTP toxicity is poorly understood. Moreover, α-synuclein expression is upregulated by histone deacetylation, and this confers protection against glutamate-induced excitotoxicity [90]. Taken together, these results suggest that α-synuclein function may be most relevant under conditions of stress.

α-Synuclein also interacts with another synaptic protein, synphilin-1. Although the physiological consequence of this interaction remains uncertain, it appears to promote α-synuclein aggregation, making this interaction a potential therapeutic target (see Sect. 7.4).

α-Synuclein may also interact with the small GTPase3a, a protein known to play a role in synaptic vesicle trafficking and recycling [91, 92]. However, once again, the consequence of this interaction is uncertain given that the normal biological function of α-synuclein itself remains to be fully defined. Nonetheless, recent studies underscore the important role – biological, pathological, or both – that α-synuclein plays in endosomal transport events that depend on the E3 ubiquitin ligase Rsp5/Nedd4, including ER-to-Golgi vesicle trafficking [93].

Whatever the exact physiological function, α-synuclein appears to play an important role in synaptic transmission. Since α-synuclein accumulation, not depletion, is the more disease-relevant state, it is unlikely that therapeutics aimed at limiting overall α-synuclein levels will decrease α-synuclein levels below the critical threshold necessary for wild-type function. Nevertheless, appreciation of the role that α-synuclein plays in synaptic homeostasis should be considered when designing therapeutics that reduce overall α-synuclein levels.

2.5 Multimeric Intermediates: Defining the Identity and Pathogenicity of Oligomers

The IUPAC definition of an oligomer is a molecule of intermediate relative molecular mass, the structure of which essentially comprises a small plurality of units derived, actually or conceptually, from molecules of lower relative molecular mass. This definition as applied to the ensemble of α-synuclein oligomers would include not only their general size (i.e., small, medium, large) [94] but also their secondary structure characteristics including β-sheet content and arrangement [95, 96] and α-helical content [40], overall 3-dimensional shape (morphology) [30, 97–100], reactivity with conformation and epitope-specific antibodies [101, 102], and whether they are "on" [103] or "off" [32, 104–106] pathway to fibril formation. In addition, there are "operational definitions" that classify α-synuclein oligomers in terms of their biochemical characteristics or function,

including protease resistance [32], effect on cell viability [96, 107, 108], ability to generate reactive oxygen species [32], recruit or activate microglia [109, 110], bind to lipids [40], and form pores in lipid bilayers [103, 111–115]. In some cases, oligomers are formed as a result of an interaction with an exogenous small molecule [116, 117], and some of these oligomers are "off-pathway" to fibril formation, but yet they may share some characteristics with "on-pathway" oligomers (see Sect. 6.2).

Given their transient nature, how has the in vivo toxicity of α-synuclein oligomers been studied and defined? A study using viral expression of α-synuclein in rat brain and human mesencephalic cultured cells demonstrated that oligomers are toxic in vivo [102], likely as a result of aberrant permeabilizing membrane interactions. Interestingly, wild-type α-synuclein demonstrated only modest toxicity, whereas mutant forms, including both naturally occurring (A30P) and engineered mutations (E57K, E35K) that disrupt intramolecular salt bridges and promote oligomerization over fibrillization, were more toxic. These studies demonstrate the superior toxicity of oligomeric (~100 nm, A11 immunoreactive α-synuclein) versus fibrillar α-synuclein, though they also raise some questions about the direct applicability to idiopathic PD where wild-type α-synuclein is the main culprit.

Colla et al. [118] showed that high molecular weight, detergent-insoluble toxic oligomers (defined by reactivity with A11 and FILA-1 antibody) accumulate in the ER and precede full disease pathology in A53T mutant α-synuclein transgenic mice. Interestingly, these toxic oligomers are biochemically heterogeneous, are likely on-pathway to fibril formation, and are also enriched in human PD brains. Salubrinal, an anti-ER stress compound, when administered to the transgenic mice [118], or recombinant adeno-associated virus (rAAV)-A53T α-synuclein-injected rats [119], lessened ER-associated α-synuclein oligomers and disease manifestations [119] including Golgi fragmentation. Dopaminergic neuronal loss in the rats was not prevented by salubrinal treatment, however, consistent with evidence that α-synuclein can activate multiple cell death pathways in addition to ER stress, including mitochondrial dysfunction [120, 121] and changes in gene expression.

Additional evidence pointing to the pathogenicity of oligomeric species comes from a recent report showing that α-synuclein oligomers were toxic when injected into the brains of wild-type mice. These oligomers were morphologically higher-order aggregates of heterogeneous size, such as annular and amorphous protofibrillar oligomers [122], consistent with the heterogeneity described in other studies.

Despite the structural heterogeneity, some features, such as specific β-sheet and β-like structures, may be sufficient, though not necessary for toxicity. For example, rigid β-sheet structure is lacking in engineered mutants with proline mutations in the fibril core. These mutations reduce α-synuclein fibril formation, increase oligomer formation, and increase toxicity in a variety of model systems [123], though the direct relevance of the engineered mutants is uncertain.

α-Synuclein oligomers may also oppose monomeric α-synuclein wild-type function, for example, by opposing the α-synuclein-dependent assembly of SNARE complexes [124]. In addition, aberrant membrane interactions and

aggregation at the membrane under certain conditions (see Sect. 2.7) may oppose α-synuclein wild-type function in this critical compartment. Thus, the pathogenic impact of α-synuclein aggregates may be compounded by the interference with its wild-type function.

2.6 α-Synuclein Fibrils: The Pathological Hallmark

Polymerized α-synuclein fibrils, either detected in vivo in Lewy bodies and Lewy neurites, where they are associated with >70 additional cellular components [125], or formed in vitro from purified protein [126], have a remarkably consistent size and morphology, especially when compared to heterogeneous, smaller oligomers. How different are oligomers from fibrils? α-Synuclein fibrils average 10 nm in diameter (range of 8–12 nm), though are much longer, typically greater than 1 μM in length as revealed by X-ray fiber diffraction studies and electron and atomic force microscopy. Fibrils also have a highly ordered cross β-sheet structure, demonstrated by circular dichroism (CD), Fourier transform infrared (FTIR) spectroscopy [31, 126, 127], and NMR, with the core of the fibrils comprised of five β-strands, reminiscent of a five-layered β-sandwich [127], and that run orthogonal to the fiber axis [126]. Critical intermolecular interactions that result in fibril formation likely involve the 100 amino-terminal residues [128]. Fibrils bind characteristically to Thioflavin T/S and Congo red dyes and are microscopically detectable in situ. For example, it is reported that in vivo, α-synuclein filaments are 14–16 nm thick [129] and have lengths ≥1 μM. In contrast, on-pathway (to fibril formation) oligomers are reported to have a Stokes hydrodynamic radius of 7 nm [130, 131] and are much shorter. Spherical oligomers, which may be among the toxic species [107], are even smaller at 2–6 nm in diameter, whereas annular oligomers appear under AFM as a dense, remarkably homogeneous population of globular, mildly flattened oligomers with a narrow size distribution (average diameter of 21 ± 3 nm) and with a less dense interior or a pore-like toroid [106, 112].

Formation of α-synuclein fibrils is an energetically favorable reaction, especially once critical low-frequency nucleation events have occurred. Thus, the process may be kinetically regulated in vivo. For example, an interaction between α-synuclein and a molecule (SERF1a) with a binding constant (K_D) of approximately 10 μM markedly accelerates α-synuclein fibril formation by promoting early nucleation events critical for fibril formation. This is as an example of a biologically relevant, low-affinity interaction [132] that has a profound impact on α-synuclein aggregation and suggests that intervening at the nucleation step is a viable way to limit on-pathway α-synuclein aggregation.

Despite the abundant evidence that various oligomeric species are toxic in vivo, it is highly likely that α-synuclein fibrils are also toxic [133], thereby providing rationale for therapeutic approaches that can prevent their formation and/or lead to their clearance.

2.7 α-Synuclein Membrane Association

The association with biological membranes is a central feature of α-synuclein activity, aggregation propensity, and pathogenicity. As discussed in a recent review [59], α-synuclein binding to membranes as a result of its N-terminal helical propensity can have many consequences, some physiological, such as facilitating membrane-to-membrane interactions or inhibiting aggregation, and some pathological, including promotion of α-synuclein aggregation and pore formation.

Despite its clearly important physiological role at the membrane, many studies [134–140] have revealed a paradoxical effect of membrane interaction by showing that membrane-bound α-synuclein is more aggregation prone relative to the unbound form. Other studies [67], however, showed that helical α-synuclein bound to lipid membranes is less aggregation prone. Whether aggregation occurs may depend on the ratio of lipid or detergent to protein, with higher cytosolic protein concentrations promoting membrane-dependent aggregation and the low protein concentration in artificial vesicles allowing membrane interactions to inhibit aggregation [67, 141]. One mechanism by which lipids or lipid-like molecules may facilitate α-synuclein aggregation is simply by confining the protein to a small and/or two-dimensional surface (the surface of a vesicle or micelle), thereby increasing the effective concentration [142]. Alternatively, certain membranes may induce a partially folded membrane-bound intermediate, similar to what is seen in the presence of intermediate concentrations of fluorinated alcohols such as trifluoroethanol (TFE) [140], where the N-terminal part of the lipid-binding domain is helical and the C-terminal part of the lipid-binding domain is unfolded and unbound [139]. This form may still interact with the membrane, although with low affinity, and it may be that this partially helical intermediate drives aggregation by favoring intermolecular interactions between multiple nearby disordered C-terminal regions of the lipid-binding domain of α-synuclein leading to β-sheet formation [59].

Alternative helical forms of α-synuclein may also promote intermembrane associations that are part of its wild-type function. This may occur as the N-terminal lipid-binding region forms an imperfect “broken” helix upon binding to certain lipids, with amino acids Ala30 to Val37 in a non-helical, more mobile state. Thus, the two separate intramolecular helices within the same N-terminal domain may each bind to a different membrane and allow α-synuclein to serve as a bridge. For example, the membrane topology of fusing or budding vesicles could provide a scaffold for the broken-helix conformation of α-synuclein, thereby bridging these membranes with the plasma membrane [143, 144]. The protein could thus function to anchor the vesicle to the membrane or even possibly to sense the extent of budding/fusion. This model was inspired in part by observations that synuclein expression can lead to a clustering of vesicles in yeast [92] and to a stabilization of docked vesicles in chromaffin cells [79].

Posttranslational modifications (see Sect. 3) also affect α-synuclein membrane interactions. For example, N-terminal acetylation affects membrane binding by increasing helicity of the first 12 residues [145]. Nitration disrupts membrane

interactions [146], perhaps because of decreased helicity [146] and/or electrostatic repulsion of the negatively charged nitrotyrosine and the negatively charged phospholipids [147]. This could result in an increase in free α-synuclein concentration and increased propensity to aggregate [146].

3 Posttranslational Modifications of α-Synuclein Affect Its Aggregation

3.1 Oxidation and Nitration

Aging is a primary risk factor for PD. According to the free radical theory of aging, irreversible oxidative changes to critical biomolecules result in decreased cellular function over time, particularly in postmitotic cells with limited capacity for regeneration. Oxidative and nitrative damage to biomolecules accumulates with aging. Therefore, it follows that oxidized α-synuclein may be more prone to forming toxic aggregates. However, while oxidation of α-synuclein does modify the rate, extent, and character of the α-synuclein aggregates, it is not simply that all oxidation results in toxic forms of α-synuclein. In fact, under certain circumstances, oxidation may be protective, by directing α-synuclein to nontoxic, off-pathway stable intermediates. It has even been hypothesized that α-synuclein itself, through its reversible methionine oxidation and reduction driven by methionine sulfoxide reductase (Msr), may serve as a cellular antioxidant [148] by transferring oxygen-based radicals that are generated in modest amounts at cell membranes (i.e., lipid peroxidation products) to more stable and easily contained carbon-centered radicals [149]. This notion is consistent with findings that increased expression of MsrA in *Drosophila* prevents α-synuclein-dependent locomotor and circadian rhythm deficits [150] and MsrA protects against mutant α-synuclein-induced toxicity in cultured cells [151].

A thorough review of the oxidative and nitrative changes that occur in α-synuclein and their effects on aggregation and toxicity is the subject of a recent review [147]. Here, I highlight some topics consistent with the theme that modifications to the α-synuclein monomer have far-reaching effects on its aggregation by affecting the ensemble of intra- and intermolecular interactions that direct its aggregation. As such, these changes may be points of intervention for antioxidants, which are proposed as therapeutic agents for PD, but whose success so far for other neurodegenerative diseases such as Alzheimer's disease is limited [152].

It is proposed that dopaminergic neurons in the substantia nigra are particularly susceptible to neurodegeneration because dopamine is a redox-active molecule. Its enzymatic and nonenzymatic oxidation may yield superoxide, semiquinone radicals, H_2O_2, and other oxidants. Combined with the high iron content in these cells, the environment is favorable for Fenton chemistry that produces the highly reactive hydroxyl radical. Indeed, early reports demonstrated that oxidative conditions

in vitro [153] and in cultured cells [154] promote α-synuclein aggregation to amyloid-like structures. Moreover, using primary neurons from transgenic *Drosophila* expressing A30P mutant α-synuclein, it was shown that lessening dopamine-dependent oxidative stress with glutathione treatment or by sequestering dopamine in vesicles reduced toxicity [155]. In addition to iron, metals such as and copper, calcium, aluminum, and zinc have been shown to promote α-synuclein aggregation, perhaps by neutralizing the negative C-terminal charge, thereby promoting a partially folded amyloidogenic intermediate [156, 157], or through other redox-dependent interactions.

Moreover, inflammation, as occurs in PD, can cause oxidative and nitrative stress. Nitrated/oxidized α-synuclein and nigrostriatal neurodegeneration was detected as a result of inflammogen injection into the brains of mice that express human wild-type α-synuclein, but not in α-synuclein knockout mice [158], suggesting that α-synuclein can amplify inflammation into neurodegeneration.

Furthermore, nitrosative stress emanating from the endoplasmic reticulum is shown to be a critical α-synuclein-dependent pathogenic determinant in a cross-platform system comprised of yeast, primary rat neurons, and human neurons differentiated from the induced pluripotent stem cells derived from an A53T mutant α-synuclein PD patient [159].

Nitrated α-synuclein is detected in Lewy bodies [160] and is proposed as a PD biomarker [161]. It is also shown to accumulate with age in the substantia nigra of nonhuman primates [162] and in toxin models of PD [163]. Thus, nitrated α-synuclein may not only serve as a marker of neurodegeneration but may be causally involved in α-synuclein pathogenesis.

Lacking cysteine, α-synuclein is most vulnerable to oxidative and nitrative modification at methionine and tyrosine. Interestingly, oxidation at each of the four α-synuclein methionines to methionine sulfoxide has been shown to retard fibrillization and instead promote unstructured, off-pathway oligomers [148, 164, 165] that are nontoxic to primary cultures of dopaminergic and GABAergic mesencephalic neurons [108].

Similarly, nitration at tyrosine (of which there are four) alters the structural conformation of the monomer in a way that promotes oligomerization and inhibits α-synuclein fibril formation [166], but only in a pure system [167]. On the other hand, when mixed at substoichiometric proportions with unmodified α-synuclein, perhaps more representative of the in vivo situation, nitrated α-synuclein monomers and dimers, with a partially folded conformation, can seed the formation of α-synuclein fibrils [146].

Tyrosine oxidation that alters aggregation also occurs. For example, two α-synuclein-based tyrosyl radicals, formed through reaction with hydroxyl radical, may encounter each other, particularly at high-protein concentration, and form a dityrosine-linked α-synuclein dimer. When a nitrating agent (NO_2–) is included in the reaction, oxidatively cross-linked dimers become nitrated, and this augments the formation of very stable higher molecular weight soluble oligomers. Moreover, preformed α-synuclein fibrils are also greatly stabilized by tyrosine nitration [168]. Nitrative stress increased aggregation of α-synuclein in cultured cells, with

a correlation between the number of cells containing elevated peroxynitrite and perinuclear, fibril-like, nitrotyrosine-modified α-synuclein aggregates [169]. Thus, oxidation and nitration are cooperative events that dramatically affect α-synuclein aggregation depending on the degree of stress, the type of modification, and the presence or absence of cellular defenses.

Lipid peroxidation is another common form of oxidative damage with potential pleiotropic effects. Direct modification of α-synuclein by the product of lipid peroxidation, hydroxynonenal (HNE), results in increased production of off-pathway, β-sheet-rich oligomers that are toxic when applied to cultured cells [170], perhaps due in part to their increased capacity to seed further aggregation both within and between cells [171]. These oligomers form following covalent modification of α-synuclein by HNE, likely by Michael addition to Lys and His, although α-synuclein is not cross-linked to itself. The stable intermolecular interactions may result from a combination of hydrophobic, electrostatic, and hydrogen bonding interactions that exemplify the complexity of α-synuclein intermolecular interactions.

Other lipids, for example, docosahexaenoic acid (DHA), affect α-synuclein differently. For example, chemical and biochemical characterization of α-synuclein using ESI-MS in the presence of 5–10-fold excess DHA shows that DHA promotes oxidation of α-synuclein and promotes toxic oligomers containing DHA covalent adducts. At 10:1 molar excess, the oil droplets recruit α-synuclein and promote intermolecular interactions leading to amyloid-like fibrils, though the fibrils appear different than those formed from α-synuclein alone (structurally and chemically). At 50:1, the DHA locks all of the α-synuclein into annular oligomeric forms that are toxic to SH-SY5Y cells. Thus, the type of lipid-promoted oligomers formed depends on the conditions, types of chemical interactions, and the stoichiometry of the interaction [172].

3.2 Dopamine-Modified α-Synuclein: Pathogen or Innocent Metabolic By-Product

α-Synuclein aggregates, including oligomers, that are promoted in vivo under pro-oxidative or nitrative conditions, represent a heterogeneous mixture with different properties, aggregation propensities, and toxicities. This complicates the design of potential therapeutics that aim to limit oxidant-induced aggregates and underscores the importance of case-by-base candidate assessment. Indeed, not all molecules with redox activity will affect α-synuclein in the same way. For example, it is possible that redox-active dopamine, with its electron sink amino group, may be particularly prone to forming reactive quinones, whereas more conjugated compounds may generate less reactive intermediates.

It was initially demonstrated that dopamine modifies α-synuclein through a covalent interaction, likely at Tyr residues, thereby stabilizing off-pathway

protofibrils [173] and preventing fibrillization. Recent work, using cyclic voltammetry to demonstrate this dopamine–tyrosine interaction, supports this finding [174]. A similar covalent interaction was proposed for an oxidized quinone form of dopamine and α-synuclein, though the linkage instead occurs at α-synuclein amino groups. The dopamine quinone adducts blocked fibril formation in favor of toxic protofibrillar intermediates [175]. Interestingly, although DA-quinone-induced species were toxic to cultured PC12 cells, toxicity was no greater than that seen with unmodified aged α-synuclein, and sometimes less toxic, depending on incubation time.

Most recently, Nakaso et al. used PC12 cells overexpressing α-synuclein and modulation of TH levels to effect changes in dopamine levels. Decreasing intracellular dopamine blocked vulnerability to α-synuclein, and α-synuclein mutated at oxidizable C-terminal methionines and the YEMPS region was less toxic relative to wild-type α-synuclein, suggesting that dopamine, or its oxidized product, interacts with α-synuclein in the YEMPS region through an oxidative mechanism to promote toxicity. Met127 may be the critical target of oxidative modification, and Tyr125 and Ser129 may enhance that modification. The question of how α-synuclein binds to DA was not directly addressed, although the authors discussed how methionine sulfoxide reductase might reduce oxidized methionines in α-synuclein and lessen its toxicity [176].

Covalent interaction between α-synuclein and dopamine, or a dopamine oxidation product, however, may not be necessary to inhibit α-synuclein aggregation and result in stable oligomers. For example, Zhou et al. assessed the dopamine oxidation product DOPAC and found that at roughly equimolar ratios, its inhibitory effects on α-synuclein fibrillation could be mediated through non-covalent interaction. At high molar excess of DOPAC, concentrations at which DOPAC itself may polymerize, however, the interaction was covalent, as a result of Michael additions [177]. As with dopamine quinone, the DOPAC quinone can lead to α-synuclein Met oxidation, probably as a result of H_2O_2 production as a by-product of DOPAC oxidation. Notably, Met oxidation occurred after the non-covalent interaction between oxidized DOPAC and α-synuclein, suggesting Met oxidation is not a determinant of the initial interaction. Interestingly, of the compounds studied, those compounds with 1,2 dihydroxy groups (vicinal) were not subject to Michael addition at low concentration, whereas compounds containing 1,4 dihydroxy (para OH) were susceptible [177].

How might dopamine-dependent redox reactions affect α-synuclein aggregation? One possibility is that methionine oxidation at the N- and C-termini induced by dopamine inhibits end-to-end association and the resulting oligomers [178]. By using complementary methods, including sedimentation velocity analysis, small-angle X-ray scattering (SAXS), and circular dichroism spectroscopy to study the characteristics of α-synuclein oligomers promoted by DA, these authors demonstrate trimers that consist of overlapping *wormlike monomers*, with no end-to-end associations, and that lack extensive β-sheet structure. They propose that oxidation of the four methionine residues at the C- and N-terminal ends of α-synuclein prevents their end-to-end association and stabilizes oligomers formed by

cross-linking with DA-quinone/DA-melanin, which are formed as a result of the redox process, thus inhibiting formation of the β-sheet structure.

Other studies also suggest that non-covalent interactions are responsible for dopamine oxidation products' demonstrated inhibition of α-synuclein aggregation. For example, dopaminochrome was demonstrated to react reversibly with α-synuclein [179], and the ability of dopamine to inhibit wild-type α-synuclein aggregation was demonstrated at substoichiometric concentrations. Furthermore, aged, oxidized dopamine inhibits aggregation of α-synuclein that has been mutated at potential sites of covalent linkage (Met, Tyr, His). These authors propose that dopaminochrome interactions at the YEMPS region induce conformational changes that lessen abundance of classic β-sheet formation in favor of alternative β-pleated sheet-like structures that differ from the β-pleated sheet characteristic of filamentous α-synuclein. Consistent with this finding, Herrera et al. used molecular modeling and in vitro aggregation reactions to show that dopamine interacts non-covalently and nonspecifically through hydrophobic interactions with the YEMPS region to inhibit α-synuclein aggregation [180]. A subsequent study using molecular modeling supports the notion of non-covalent interactions between dopamine and α-synuclein [181], though the in vitro fibrillization experiments in this study did not assess this directly.

In summary, covalent interactions between α-synuclein and dopamine (or its oxidized forms) may be sufficient but not necessary to modify α-synuclein aggregation. In either case, inhibition of fibrillization by dopamine or its metabolites may involve oxidation of α-synuclein Met residues, and dopamine modification of α-synuclein is not necessarily toxic, although this is an ongoing debate.

Importantly, the study of dopamine/α-synuclein interactions has provided valuable information while also eliciting caution in the search for a pharmacophore that could inhibit α-synuclein aggregation. Given the impact of minor structural changes on the activity and mechanism of action, and the range of interactions that dictate α-synuclein aggregation and toxicity, molecules with structures similar to dopamine must be evaluated individually to assess their interaction with α-synuclein and impact on α-synuclein toxicity in vivo.

3.3 Phosphorylation

α-Synuclein contains multiple putative phosphorylation sites, consistent with its extensive phosphorylation in Lewy bodies [58, 60, 182, 183] and the cerebrospinal fluid of PD patients [184]. Phosphorylated α-synuclein is proposed as an early indicator of progressing pathology in specific brain regions [185] and a PD plasma biomarker [186], though plasma levels of phosphorylated α-synuclein vary greatly between individuals.

Despite these observations, the question of whether phosphorylation of α-synuclein plays a causal role in its aggregation and neurotoxicity is a controversial topic that is complicated by a number of factors. First, phosphorylation in vivo

occurs primarily at three sites, including Ser129, Ser87, and Tyr125, with some reports indicating that phosphorylation at different sites has opposing effects. Second, reported in vivo outcomes of even a single phosphorylation event, for example, at Ser129, are contradictory. Third, it is not clear if α-synuclein phosphorylation occurs before (early event) or after Lewy body formation (later event), or both. Finally, an extensive network of kinases and phosphatases control α-synuclein phosphorylation.

While a thorough examination of all of these topics is beyond the scope of the review, it is worth addressing some highlights since drug discovery efforts targeting phosphorylation have proven successful for other indications (i.e., cancer) and are a highly active area of PD research.

The consequence of α-synuclein phosphorylation appears to depend on the residue that is phosphorylated and the model system in which it is studied. For example, phosphorylation at Ser87 [60] but not Ser129 [187] interferes with membrane binding.

Some studies conclude that Ser129 phosphorylation of α-synuclein increases aggregation and toxicity in *Drosophila* [188] and toxicity (but not aggregation per se) in cultured cells [189] and rat brains expressing α-synuclein from rAAV vectors [190]. However, the effects of promoting and blocking phosphorylation are not always reciprocal. For example, in the cell-based study [189], blocking α-synuclein phosphorylation with the S129A mutation did not reduce toxicity relative to wild-type α-synuclein, whereas in the rat study [190], the S129A mutant was less toxic but formed larger aggregates. Recently, Sato et al. [191] reported toxicity from viral co-expression of A53T α-synuclein and the GRK6 kinase in rats. These authors report increased Ser129 phosphorylated α-synuclein in vivo without an increase in putatively toxic oligomers, another example of uncoupling of aggregation state and toxicity.

Consistent with the pathogenicity of Ser129 phosphorylation, phosphatase activators that reduced levels of Ser129 phosphorylated α-synuclein also improved motor performance in wild-type α-synuclein transgenic mice [192]. Furthermore, pramipexole, a dopamine agonist with demonstrated neuroprotective properties in model systems, has been shown to decrease phosphorylation at Ser129 by eliciting an inhibitory effect on casein kinase 2, one of the kinases known to phosphorylate α-synuclein [193], although the mechanistic relationship between the multiple activities of pramipexole is not fully understood.

In contrast to the phospho-Ser129-dependent toxicity described in the studies above, others indicate non-phosphorylated forms of α-synuclein may be more deleterious. For example, blocking Ser129 phosphorylation (by using S129A mutant α-synuclein) increases toxicity in vivo in the nigral pathway of rats transduced with rAAV-α-synuclein constructs [194, 195] and causes motor deficits and synaptic deficits in transgenic *C. elegans* (worms). This toxicity may result from increased cytosolic protease-resistant β-sheet-rich aggregates [195] that promote deleterious membrane interactions [196]. In two of these studies [194, 196], mimicking Ser129 phosphorylation (by using the S129D mutant form) was protective, although recent studies [187, 197–199] have cast some doubt on the ability of

the S129D and S129E substitutions to reproduce the effect of Ser129 phosphorylation on α-synuclein structure and aggregation in vitro. This disconnect may apply to Ser87 as well, since S87-P but not the phosphomimic S87E induced significant changes in the structure of membrane-bound α-synuclein, suggesting that only authentically phosphorylated α-synuclein may have reduced ability to form α-helical structure when binding to membranes. In the third study, [195] S129D α-synuclein still resulted in some neuronal loss, though it caused less toxicity relative to wild-type (intermediate) and S129A mutant (most toxic) α-synuclein, providing additional evidence that under some conditions, phosphorylation and neurotoxicity of α-synuclein aggregates may not be tightly coupled.

Consistent with non-phosphorylated forms being more deleterious, some reports indicate that α-synuclein phosphorylation may, instead, be protective. For example, Ser87 phosphorylation is reported to inhibit fibrillization by increasing conformation flexibility [60], whereas blocking phosphorylation at this site with the S87A mutation is reported to increase the formation of insoluble protease-resistant, β-sheet-rich aggregates, enhance toxicity, and induce hemi-Parkinsonian motor dysfunction in rAAV-α-synuclein expressing rats [200]. These S87A-dependent deleterious effects are similar to those seen with nigral expression of wild-type α-synuclein and greater than those seen with expression of the phosphomimic S87D mutant α-synuclein [200]. In a similar fashion, Tyr125 phosphorylation may also be protective [201].

At what point in the aggregation process does phosphorylation occur? Detailed structural analysis revealed that Ser87 is inaccessible to casein kinase when α-synuclein is fibrillar, although Ser129 may be phosphorylated after fibril formation has occurred, raising more uncertainty about the cause-and-effect relationship between α-synuclein phosphorylation, aggregation, and neurotoxicity [60]. Moreover, A53T-α-synuclein expressing mice show Ser129 phosphorylated α-synuclein that presents in heterogeneous discrete but overlapping patterns, suggesting that phosphorylation is not a prerequisite for α-synuclein aggregation and may be a marker of advanced pathology [202]. Perhaps phosphorylated α-synuclein is cleared less effectively, consistent with a secondary effect of phosphorylation [189].

In any case, a valid therapeutic approach may involve targeting specific kinases or phosphatases that modulate phosphorylation of specific amino acids. Dual specificity tyrosine-regulated kinase 1a (dyrk1a) is known to phosphorylate α-synuclein at Ser87 [203]. CK-1 and CK-II phosphorylate α-synuclein at Ser129 [204–206]. Polo-like kinases appear to be among the physiological kinases responsible for α-synuclein Ser129 phosphorylation [207, 208] and may play a role in its autophagic clearance [209].

Which kinases are possible therapeutic targets? Perhaps modulating the activity of the G protein-coupled receptor kinase (GRK) 2, another kinase known to phosphorylate α-synuclein at Ser129 [188, 191, 210], or AMP kinase, whose overexpression increases aggregation and causes decreased neurite outgrowth in primary neuronal cultures [211], may be beneficial. Interestingly, α-synuclein phosphorylation [212] and pathogenicity may be mediated, at least in part, by

LRRK2 [213], a kinase that when mutated causes inherited forms of PD and which is the target of extensive drug discovery efforts.

Recently, a cell-based siRNA screen of 711 known or predicted kinases and 206 phosphatases selected for changes in phosphorylated α-syn/total α-syn ratios [214]. This study confirmed that α-synuclein phosphorylation is controlled by an extensive network of kinases and phosphatases. In addition, this study identified a kinase, SMG1, whose knockdown in α-synuclein expressing neuroblastoma cells resulted in increased phosphorylated α-synuclein (and increased total α-synuclein). This counterintuitive negative regulation of α-synuclein phosphorylation by a kinase may be explained, at least in part, by the ability of SMG1 to promote α-synuclein-induced stress granule formation. Reducing the cellular response to α-synuclein-induced stress by knocking down SMG1 attenuates the buffer to further α-synuclein accumulation. Another counterintuitive finding in this study was that knockdown of protein phosphatase 2A, known to dephosphorylate Ser129, instead decreased the phosphorylated α-syn/total α-syn ratio, underscoring the challenge in therapeutic modulation of α-synuclein phosphorylation.

Translation of such findings into in vivo animal models and human proof of concept studies may provide compelling evidence that targeting α-synuclein phosphorylation is a bona fide PD therapeutic approach.

4 Defective Turnover and Clearance as a Consequence of α-Synuclein Aggregation

α-Synuclein has a relatively long half-life that increases with age in neurons [215]. It is normally cleared by both the ubiquitin proteasome system (UPS) [216] and lysosomal pathways such as autophagy [217, 218] (and reviewed in [219]). Even though both are likely important [220], the former may be more relevant under normal physiological conditions, whereas the latter may be more relevant to cellular attempts to clear aggregated pathogenic forms of α-synuclein [218, 221, 222]. Nevertheless, α-synuclein aggregates may resist degradation by both pathways, thereby fueling the vicious cycle that would result in greater aggregate accumulation and generalized defects in protein turnover. For example, α-synuclein aggregates may cause a general impairment of macroautophagy by reducing initiation of autophagy [223] or impairing autophagosome clearance [224]. In addition, mutant or modified (i.e., by dopamine) forms of α-synuclein are shown to impair chaperone-mediated autophagy (CMA) [225, 226]. Moreover, nitrated α-synuclein monomers and oligomers may resist degradation by the lysosome [169]. Additional evidence for a relationship between CMA and α-synuclein toxicity comes from the discovery that the pro-survival transcription factor MEF2D, normally active in the nucleus and degraded by Hsc-70-dependent CMA, instead accumulates in an inactive form in the cytosol when α-synuclein

inhibits CMA. Conversely, restoration of MEF2D function in the nucleus attenuates α-synuclein-induced neuronal death [227].

At the proteasome, α-synuclein aggregates are resistant to degradation and impair proteasomal function [228–230]. The net effect of inhibiting these clearance pathways is to increase steady-state levels of both monomeric and aggregated forms, thereby increasing aggregation propensity and neurotoxicity. Thus, it follows that therapeutic approaches aimed at increasing turnover of α-synuclein are actively pursued (see Sect. 7.4).

5 α-Synuclein "Strains" and Prion-Like Transmission

With α-synuclein monomers and oligomers capable of assuming such a heterogeneous collection of 3-dimensional structures of varying function and pathogenicity, it comes as little surprise that even in its most aggregated form, resembling classic amyloid- α-synuclein "strains" exist. While the concept of a "strain" was traditionally used to classify variability of heritable traits, including pathogenicity, at the organismal level, that result from genetic polymorphisms, the term is now commonly used in the context of protein misfolding and aggregation. A number of criteria define a protein aggregate "strain" including how the molecules pack within the aggregate, how well they seed new aggregates, their physical properties, their ability to cross the species barrier, pathophysiological characteristics, latency to disease, and differences in survival rates [231]. For misfolded proteins, this concept was first demonstrated for prions (infectious proteins), a corrupted version of an endogenous cellular protein that is the infectious pathogenic agent in rare but devastating neurological diseases like Creutzfeldt-Jakob disease [232, 233]. The concept has since been applied, in part, to other pathogenic misfolded proteins including Tau, α-synuclein, and Aβ, based partly on evidence that certain strains of one protein aggregate can seed others. For example, α-synuclein aggregates seed Tau aggregation [234], although the prion protein remains the only misfolded protein shown to be transmissible from one organism to another. The cell-to-cell spreading of α-synuclein pathology, in a "prion-like" fashion, was recently covered in two reviews [34, 235] and in a recent publication describing this phenomenon in the context of multiple systems atrophy [236]; hence, in-depth coverage of this topic is beyond the scope of the review. However, a summary and some recent studies are worth noting, particularly in the context of treating PD as a disease that spreads long distances along anatomically connected neural networks, thus raising the possibility that targeting pathogenic extracellular α-synuclein aggregates could be a viable strategy for preventing disease progression.

α-Synuclein pathology spreads in a stereotypical temporal and topological manner, likely starting in the olfactory bulb, the gastrointestinal tract, or both. In central nervous system (CNS), α-synuclein pathology, in general, spreads upward and in a caudo-rostral fashion through specifically affected brain regions, including the nigrostriatal pathway and eventually, in some cases, the cortex. [2, 237].

In vivo evidence for non-cell-autonomous effects of α-synuclein aggregates, i.e., cell-to-cell transmission of aggregates, comes from the demonstration that healthy fetal mesencephalic grafts placed in the diseased striatum of PD patients eventually show evidence of Lewy bodies [238–240].

Numerous in vitro and in vivo studies showed that α-synuclein can be released from one cell, taken up by another, and seed aggregation of endogenous α-synuclein in the recipient cell, with resulting deleterious pathological changes [129, 241–246]. It is likely that this transmission can occur without cell death or membrane leakage of the donor cell, perhaps through exocytosis [247], although cell death and membrane degeneration would accelerate the transmission by releasing a large amount of α-synuclein aggregates. Endocytosis likely plays a role in α-synuclein uptake in the recipient cell [241].

In one study, wild-type human α-synuclein preformed fibrils (pffs), but not monomer, seeded the formation of aggregates in adjacent cultured mouse hippocampal neurons that expressed endogenous levels of wild-type mouse α-synuclein [129]. Seeding was time and concentration dependent and resulted in recruitment of wild-type mouse α-synuclein away from the presynaptic terminal into Lewy neurite-like inclusions, with eventual intracellular propagation of α-synuclein aggregates from axon to cell body to dendrites. The effect of these inclusions was an impairment of coordinated network activity [129].

Most recently it was shown that specific forms of α-synuclein oligomers, including β-sheet-containing dimers [122] and HNE-modified [248] α-synuclein, seed the formation of oligomeric aggregates. For example, when injected into the hippocampi of wild-type mice, the dimer-derived oligomers, but not freshly dissolved α-synuclein or insoluble fibrils, promoted apoptosis [122].

Another study [231] demonstrated two different aggregated α-synuclein forms, depending on solution conditions, that fulfill many of the characteristics of strains, including differences in secondary and quaternary structure (Thioflavin T-reactive fibrils versus Thioflavin T-negative ribbons), the ability to cross-seed unfolded α-synuclein, the ability to bind membranes, and the degree of toxicity. Morphologically the two strains resemble ribbons and fibrils. Under conditions where α-synuclein ribbons form, the soluble precursor of α-synuclein fibrils does not appear to be populated. Thus, even when seeded with preformed fibrils, the result is unfaithful elongation of α-synuclein fibrils into ribbons. However, under solution conditions favorable to fibril formation, the soluble precursor will assume the shape that is consistent with the seeds that are present – i.e., ribbons recruit monomer to form more ribbons and fibrils recruit monomer to form more fibrils. This implies that strain stability is most dependent on the conformational ensembles that the precursor, soluble peptides populate [231] and is consistent with the hypothesis that factors that affect intramolecular compaction, diffusion, and reconfiguration of the monomer will profoundly affect the ultimate rate of aggregation and aggregation state.

6 Experimental Approaches Targeting Misfolded α-Synuclein

Despite the vast amount of knowledge gleaned from the studies described above, many critical questions still remain that are relevant to the development of a PD therapeutic targeting α-synuclein. (1) What is the desired cellular and subcellular concentration of α-synuclein? (2) What are the in vivo toxic species? (3) How does one effectively target a dynamic natively unfolded protein? (4) What are the critical downstream effectors of α-synuclein toxicity? (5) Are α-synuclein-dependent pathologies and functional deficits reversible? Despite having only partial answers to the above questions, a number of approaches are under investigation with the goal of maintaining α-synuclein at physiological levels, limiting toxic aggregates, and lessening downstream effects of misfolded α-synuclein. These approaches include targeting α-synuclein gene expression, targeting posttranslational modifications that affect its toxicity, inhibiting or reversing its aggregation, limiting its cell-to-cell transmission, and enhancing clearance of its toxic forms. The molecules that are being tested in model systems to achieve these aims include nucleic acids, small organic molecules, and biologics.

Despite extensive efforts, α-synuclein-directed therapeutics remain mostly in the discovery phase, with only one approach – the use of antibodies to enhance α-synuclein clearance and limit its transmission, having reached clinical trials (currently Phase 1).

Given the complexity of α-synuclein aggregation, one simple and direct approach that circumvents the nuances of its folding and misfolding is to limit its expression. Support for this approach comes from animal studies in which expression of A53T mutant α-synuclein is conditional and controlled through the use of the tetracycline-regulated "tet-off" promoter and doxycycline administration. In these mice, α-synuclein expression leads to pathology, synaptic structural defects and memory deficits that result from deposition of α-synuclein in the limbic system, including the hippocampus. However, when transgene expression is turned off in adult animals for 3 months (at 9 months of age), 12-month-old mice had cleared α-synuclein pathology in the hippocampus and arrested α-synuclein progression in other regions, and the mice recovered synaptic structural defects. These results support the design of therapeutics aimed at limiting the extent and degree of α-synuclein pathology. The limitation of the applicability of these studies to humans, however, is that intervention would have to occur early in the disease process since extensive α-synuclein pathology and neurodegeneration have already occurred by the time patients are symptomatic. An ideal therapeutic would both inhibit and reverse existing pathology and its downstream effects in patients in whom α-synuclein pathology is both present and progressing. More detailed descriptions of experimental α-synuclein-directed therapeutics are presented next.

6.1 Modulating SNCA (α-Synuclein) Gene Expression

Oligonucleotide-based approaches to modulate gene expression have generated great interest. Originally antisense oligonucleotides were a main focus, but more recently inhibitory RNAs (RNAi) including short hairpin (sh) RNA and related micro(mi)-RNAs have gained favor as means to control gene expression. For example, expression of the SNCA gene is normally modulated, in part, by microRNA-7 [249]. In cultured cells, increasing microRNA-7 decreased α-synuclein and protected against oxidative stress, whereas, conversely, knock-down of microRNA-7 increased α-synuclein levels. In vivo, the mitochondrial complex I inhibitor MPTP decreased microRNA-7, suggesting this could be the mechanism of how α-synuclein is increased in this PD model system. An obvious next step is in vivo enhancement of microRNA-7 as proof of concept that downregulation of α-synuclein elicits improvements in PD-relevant phenotypes.

Another approach to modulate α-synuclein gene expression is to use siRNA directed at the α-synuclein transcript. In this regard, naked (without viral vector) siRNA molecules chemically modified to prevent degradation by exo- and endo-nucleases were directly infused into the left substantia nigra of squirrel monkeys and resulted in reduced levels of α-synuclein mRNA and protein in the infused (left) but not the untreated (right) hemisphere. Quantitative analysis revealed a significant 40–50% suppression of α-synuclein expression in the absence of any adverse effects on dopaminergic neurons [250].

Similarly, α-synuclein-directed ribozymes [251] downregulate α-synuclein gene expression at the RNA level.

SIRT2 is a histone deacetylase (HDAC), and AGK2 is a small molecule that inhibits SIRT2 activity, increases acetylated proteins such as tubulin, and inhibits α-synuclein-induced toxicity in a dose-dependent manner in two in vitro models and transgenic *Drosophila*. Inhibition of SIRT2 promoted the formation of larger α-synuclein aggregates, suggesting the protective effect may result from minimizing aberrant interactions with cellular proteins as a result of decreased surface area [252]. Alternatively, α-synuclein may block protein acetylation, thereby attenuating the expression or activity of proteins that possess endogenous neuroprotective function. If so, blocking SIRT2-dependent deacetylation may compensate for this α-synuclein deleterious effect.

6.2 Small Molecules That Target α-Synuclein Aggregation

Initial discovery of molecules that inhibit α-synuclein aggregation was inspired by other molecules with anti-amyloid activity. It was hypothesized that since different pathogenic amyloidogenic proteins, such as α-synuclein and Aβ of Alzheimer's disease, contain similar structural motifs, such as cross β-sheet, they would respond similarly to many of these molecules. Based in part on this premise, it was

demonstrated that small molecules from a number of classes inhibit α-synuclein aggregation at substoichiometric concentrations [117]. Of the 79 compounds tested, belonging to 12 different classes, polyphenols were, as a class, the most potent inhibitors of α-synuclein aggregation as assessed by Thioflavin S fluorescence, electron microscopy, and detergent (sarkosyl) insolubility, consistent with potent inhibition by the polyphenol dopamine (see Sect. 3.2). In addition, compounds belonging to six other classes (phenothiazines, polyene macrolides, porphyrins, rifamycins, Congo red and its derivatives, and terpenoids) also inhibited filament assembly to some extent. Representative structures of compound classes with demonstrated α-synuclein anti-aggregation activity are presented in Fig. 2.

The interaction of many of the inhibitory compounds, including exifone (Fig. 2a), favored the formation of soluble low-to-middle molecular weight oligomeric species over fibrils. To explore binding sites, small molecule inhibition of aggregation was assessed on truncated α-synuclein and compared to full-length α-synuclein. These results showed that exifone and lacmoid (Fig. 2b) partially inhibited aggregation of α-synuclein 1–120, but other compounds were inactive, suggesting that exifone and related molecules may not require, but may still interact with, the C-terminus of α-synuclein. Dopamine, however, absolutely required an intact C-terminus to exert its inhibitory effects, demonstrating that not all polyhydroxylated compounds bind in the same fashion. In contrast to fibrils and oligomeric protofibrils, small-molecule-induced oligomers, purified by size-exclusion chromatography, were nontoxic to neuroblastoma cells, suggesting that these oligomers are off-pathway to fibril formation or that aggregation is halted at a stage preceding formation of a toxic intermediate.

EGCG, a polyphenolic component of green tea (Fig. 2a), is among the best-studied naturally occurring molecules with pharmaceutical-like properties (nutraceuticals) capable of disaggregating and detoxifying preformed α-synuclein aggregates. At equimolar concentrations, EGCG remodels α-synuclein fibrils into unstructured smaller oligomeric species that are seeding incompetent. These oligomers include benign amorphous aggregates that are off-pathway to fibril formation and nontoxic to cultured cells [253]. EGCG also prevents the aggregation of freshly dissolved α-synuclein by binding to the protein backbone at the C-terminus as demonstrated by NMR and quinoprotein staining [254] and diverting the aggregation pathway to nontoxic, detergent stable, unstructured oligomers. EGCG interactions with α-synuclein are complex and likely involve hydrogen bonding, hydrophobic interactions, and possibly covalent interactions and redox cycling. The specific contribution of each interaction is uncertain and likely depends on the species of α-synuclein and the stoichiometry of the reaction.

In addition to green tea components, the main polyphenolic compounds in black tea, theaflavins (Fig. 2a), are potent inhibitors of α-synuclein fibrillogenesis [255]. These polycyclic compounds are formed by oxidation and condensation of the catechin structure. Mixtures of α-synuclein with each of three different theaflavins caused inhibition of formation of toxic α-synuclein oligomers and fibrils in favor of a theaflavin-specific pattern of remodeled nontoxic oligomers, as detected by atomic force microscopy (AFM). The interaction between catechins

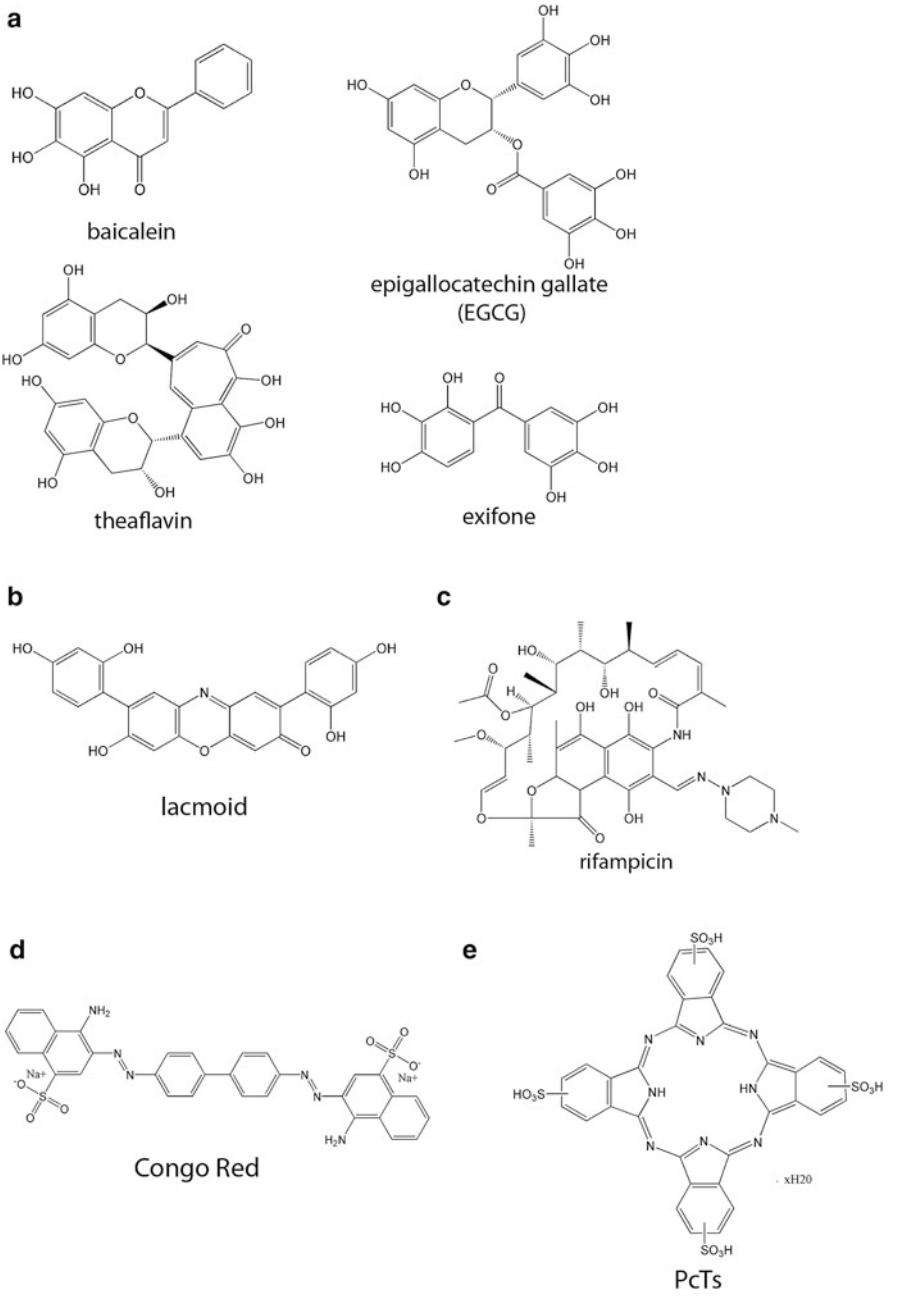

Fig. 2 (continued)

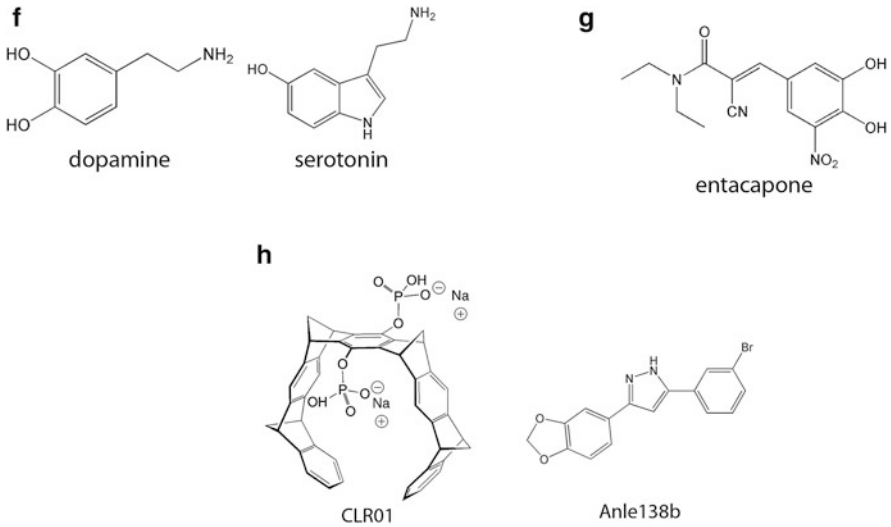

Fig. 2 Representative structures of compound classes with α-synuclein anti-aggregation activity. These structural classes include polyphenols (**a**), phenothiazines (**b**), rifamycin (**c**), Congo red derivatives (**d**), phthalocyanines (**e**), neurotransmitters (**f**), molecules used as symptomatic PD treatments (**g**), and others (**h**) that have demonstrated anti-aggregation activity in a variety of model systems

and amyloidogenic peptides may derive, in part, through presentation of at least one face of multiple phenolic groups in a planar orientation that interrupts amyloid intermolecular hydrogen bonding. These results are consistent with a recent report that polyphenols, including black tea, lessened the effects of exogenous α-synuclein on mitochondrial function [256].

In a related study, 14 polyphenolic compounds and black tea extract were screened using confocal single-molecule fluorescence techniques and short incubation times to assess inhibition and disaggregation of early (small) and intermediate-sized iron-induced on-pathway oligomers [257]. Interestingly, this study defined SAR similar to those in studies assessing fibril formation and disaggregation. For example, multiple compounds, including baicalein (Fig. 2a), EGCG, tannic acid, and NDGA, plus black tea extract, were identified as potent inhibitors and disaggregators of α-synuclein oligomerization, with IC_{50} values in the low μM range. Structurally, aromatic recognition elements are necessary for non-covalent binding to the amyloid core. Moreover, the presence of hydroxyl groups, capable of interfering with intra- and intermolecular hydrogen bonding in the amyloidogenic target, is critical for activity. Although polyphenols commonly have low oral bioavailability, high metabolic turnover, and low blood–brain barrier permeability [258, 259], given their potency, the therapeutic potential is considerable [260].

Another well-studied polyphenol with potent α-synuclein anti-aggregation properties is baicalein, a member of the flavonoid class. Like many polyphenols, flavonoids are abundant in food and potential nutraceuticals. Originally identified

as a component of the Chinese medicine, *Scutellaria baicalensis*, baicalein is shown to both potently inhibit α-synuclein fibrillization and disaggregate preformed α-synuclein fibrils, though likely by different mechanisms [116]. Baicalein binds freshly dissolved α-synuclein with a K_D of ~500 nM, most likely at one site, thereby stabilizing a restricted soluble conformation. By electron microscopy and AFM, in time-course experiments, individual oligomers of 15–20 nm in diameter and globular oligomers 32–45 nm in diameter were formed first, followed by larger amorphous protein aggregates. The authors also note the formation of annular oligomers 3.5–5.4 nm in height, 20–23 nm in outer diameter, and 11–14 nm in inner diameter, indicating a diverse oligomeric array. It is proposed that inhibition of aggregation occurs as a result of a covalent interaction between oxidized baicalein quinone and α-synuclein, possibly by Schiff base formation at a lysine side chain, although this adduct is not abundant, requiring purification by RP-HPLC for ESI-MS detection [261]. Interestingly, baicalin, with a covalently attached glucuronide, is active too, suggesting that some forms of metabolized polyphenols, typically highly glucuronidated, methylated, and sulfated in vivo, may retain activity. Purified baicalein-induced oligomers are shown to be nontoxic to membranes, relative to fibrils [262], consistent with other polyphenol-induced α-synuclein oligomers being nontoxic to neuroblastoma cells [117]. Baicalein also decreased fibrillization of E46K-mutant α-synuclein and decreased its toxicity [263].

Baicalein-stabilized oligomers form via a partially folded monomeric intermediate, have β-sheet content between that of monomers and fibrils, and inhibit aggregation of freshly dissolved α-synuclein at substoichiometric proportions [262]. Baicalein also shows modest anti-aggregation activity in a cell-based assay by reducing levels of toxic high molecular weight α-synuclein oligomers and protecting against oligomer-induced toxicity [264]. How might baicalein promote nontoxic oligomers in vitro but show no evidence for such an effect in a cell-based assay? Among the possible explanations for this discrepancy is that the cellular reducing environment is unfavorable for this reaction or, alternatively, the baicalein-induced oligomers are readily cleared in cells.

Although a redox reaction that occurs during the small molecule/protein interaction may be part of its complex mechanism of action, the antioxidant activity of baicalein may not be sufficient for its anti-aggregation activity [261]. For example, following non-covalent binding, perhaps through hydrophobic interactions and pi-pi stacking interactions at specific aromatic amino acids, oxidation to the quinone favors a "reversible" covalent Schiff base formation at Lys 21 (and possibly Lys 23, 34, 43) or a Michael addition at Tyr 39. Consequently, H_2O_2 is produced that catalyzes the reaction between baicalein and α-synuclein, possibly driving Met oxidation. Interestingly, Met oxidation of the protein is not likely a prerequisite for inhibition by baicalein since the H_2O_2 scavenger catalase inhibits Met oxidation, but does not inhibit baicalein quinone formation nor baicalein's inhibitory effects. The authors note that the C-terminal tyrosines may catalyze the inhibitory reaction perhaps as a result of hydrogen bonding with baicalein that consequently alters α-synuclein intramolecular interactions, thereby restricting conformational changes and/or precluding fibril assembly.

The in vitro inhibitory effect of baicalein is consistent with that of several other related flavonoids. For example, 6-HP, myricetin, and eriodictoyl are potent inhibitors of α-synuclein fibrillization, with related but separate mechanisms since the oligomeric species promoted by the flavonoids differ by compound [265]. The most efficient inhibition occurred when select flavonoids were added early in the aggregation process, suggesting that intervention occurs at the nucleation phase and consistent with an elongated lag time detected for α-synuclein fibrillization in the presence of inhibitors. Structure-activity relationship (SAR) analysis confirmed independent findings [257] that vicinal dihydroxyl groups are tightly linked with anti-aggregation potency [265] and more vicinal dihydroxy substituents increase inhibitory potential (i.e., 3 vicinal OH groups are more potent than 2) [265]. Many of the good flavonoid inhibitors of α-synuclein aggregation also disaggregate preformed α-synuclein fibrils [265]. Given the dynamic equilibrium that exists between the different aggregation states, it could be argued that any potent inhibitor of aggregation will result in disaggregation by increasing the "off-rate" for the subunits of the polymerized form.

Another polyphenolic compound, the antibiotic rifampicin (Fig. 2c), is shown to inhibit α-synuclein aggregation and disaggregate preformed fibrils, with low μM potency, and its mechanism of action may be related to quinone formation [266]. Growing evidence suggests that quinone-modified α-synuclein is not necessarily deleterious. For example, a stretch of α-synuclein amino acids (residues 36–46) was covalently modified by pyrroloquinoline quinone (PQQ), EGCG, and baicalein, thereby inhibiting its aggregation. Moreover, PQQ modification of α-synuclein reduced the toxicity of C-terminally truncated α-synuclein (α-syn 119) when the two were coincubated and added to cultured cells. Interestingly, the modified α-synuclein fragment was selective and did not inhibit Aβ aggregation [267]. Another example of nontoxic α-synuclein adducts that promote unstructured, off-pathway oligomers is the consequence of α-synuclein cross-linking with tissue transglutaminase [268].

Zhou et al. [108] further investigated the role of Met oxidation in the formation of stable nontoxic α-synuclein oligomers and propose that the effect of Met oxidation is to strengthen the intramolecular long-range interactions that depend largely on a negatively charged C-terminus. These putative interactions result in a more compact globular structure and prevent formation of the amyloidogenic partially folded intermediate, consequently precluding aggregation, similar to small-molecule-induced oligomers (i.e., baicalein). It is also likely that the addition of more negative charge at the methionine sulfoxide increases electrostatic repulsive interactions. Consistent with the auto-inhibitory role of the C-terminus, the inhibitory effect of Met oxidation is released at low pH, likely by charge neutralization at the C-terminus. Importantly, Met-oxidized α-synuclein oligomers are nontoxic. For example, they do not affect neurotransmitter uptake in primary dopaminergic and GABAergic cells (exposed to 5 and 10 μM Met-oxidized oligomers), whereas positive control HNE-modified α-synuclein oligomers were markedly toxic in this assay [108].

Methionine oxidation, however, does not necessarily imply a covalent small molecule/α-synuclein interaction. For example, the polyphenolic compounds exifone, dopamine (Fig. 2f), and gossypetin inhibit α-synuclein aggregation by promoting stable dimers (and higher-order oligomers) that form through interactions at the α-synuclein N-terminus [269], though dopamine may also interact at the C-terminus [179]. The interactions were determined to be non-covalent, since there was no polyphenol: α-synuclein adducts or α-synuclein cross-linking detected by highly sensitive HPLC/MALDI MS techniques. However, exifone caused the oxidation of α-synuclein at all four methionines. The methionine sulfoxides had only a minor effect on the overall α-synuclein structure as detected by NMR but profoundly impacted aggregation by eliminating fibril formation. Interestingly, Met oxidation was not required for inhibition of fibrillization, since at low inhibitor concentrations, methionine sulfoxide was not detected even though inhibition of fibrillization still occurred. Similar results were found for dopamine and gossypetin.

Given the complex mechanisms of action described for the various molecules in this section, and the structural heterogeneity of the target, it is not surprising that different molecules in the same class may work through different mechanisms to inhibit aggregation of a specific protein. Conversely, a particular molecule may have opposing effects on structurally related targets, for example, by promoting off-pathway oligomers of one target but promoting fibrillization of another [131]. Thus, structure-based predictions regarding mechanism of binding and resulting α-synuclein species are difficult.

6.3 Additional Inhibitory Interactions Between Small Molecules and α-Synuclein

While a single pharmacophore targeting amyloid is unlikely, recently it was proposed that there are similarities in the way small molecules inhibit and disaggregate amyloid fibrils or oligomers with similar structural motifs [270]. Using X-ray crystallography of the steric zipper-containing amyloid structures formed by KLVFFA from Aβ and VQIVYK from Tau, in the presence of small molecule inhibitors including orange G and curcumin, at least two common, class-specific binding mechanisms were identified. In the first, the negatively charged orange G is wedged between the paired β-sheets of the steric zipper, leading to partial opening. The binding is stabilized by packing of the aromatic rings of orange G against the apolar, partially aromatic spine of KLVFFA. Further stabilization arises from salt links between the negatively charged orange G and the positively charged lysines. This stabilization could be a general feature for charged molecules with electrostatic interactions at positively charged Arg and Lys residues. In the second mechanism, the apolar curcumin binds differently, though with a similar result of disrupting the dry interface of the steric zipper by shifting each member of the paired β-sheet. In this structure, curcumin likely lies along the void left by the

shifting steric zipper, with the long axis of the curcumin parallel to the fiber axis. The authors suggest that the lack of a site anchoring charge and the nature of the narrow tube binding site running along the β-sheets allows the small molecule freedom to "drift" along the fiber axis. Despite these differences, the common features of orange G and curcumin include binding to the fibers in a similar orientation, with their long axes parallel to the fibril axis and disruption of the β-sheet-paired structure.

Another example of a small molecule with a partially defined amyloid-binding mechanism is phthalocyanine tetrasulfonate (PcTS, Fig. 2e). PcTS when present at molar excess, traps early prefibrillar, amorphous (reduced β-sheet) detergent-sensitive insoluble species that are partially seeding competent. PcTS interacts with the N-terminus of α-synuclein, through aromatic interactions with amino acids including Tyr39 and also via electrostatic interactions. The phalocyanine aromatic system is capable of engaging in pi-pi stacking interactions, thus disrupting intra- and intermolecular interactions, likely involving aromatic residues that favor fibril formation [271]. Indeed, disruption of aromatic interactions may be a common feature of small molecule/amyloid interactions [272].

Despite a focus on the YEMPS region, near the C-terminus, the α-synuclein N-terminus may be a common interaction site for small molecule inhibitors. For example, interactions with chlorazole black E, Congo red (Fig. 2d), lacmoid, PcTS-Cu^{2+}, and rosmarinic acid were examined by NMR spectroscopy to identify α-synuclein sequence elements that are masked by these compounds. Surprisingly, similar α-synuclein interaction sites, encompassing residues 3–18 and 38–51, were detected for all molecules at equimolar small molecule: α-synuclein ratios. At higher ratios, virtually the entire amphiphilic region of α-synuclein (residues 2–92) is affected, revealing the presence of additional, lower affinity interaction sites [273] and consistent with the hypothesis that small organic molecules may drift along the fiber axis.

In addition to dopamine, other neurotransmitters and small molecules that represent symptomatic PD treatments such as selegiline, pergolide, and bromocriptine [274, 275] have α-synuclein anti-aggregation properties. For example, approximately equimolar or greater amounts of serotonin (Fig. 2f) inhibit fibrillization in favor of on-pathway, seeding competent, heterogeneously sized spherical (<50 nm), and granular (100–200 nm) intermediates with partial β-sheet secondary structure. The serotonin/α-synuclein reaction was only partially redox dependent since the reducing agent thiosulfate reduced, but did not eliminate, the potency of serotonin in vitro [276]. Serotonin likely binds directly to intermediates and not monomer, requiring a negatively charged intact α-synuclein C-terminus and a positively charged serotonin amino group.

Moreover, approximately equimolar amounts of norepinephrine, epinephrine, or dopamine or substoichiometric amounts of L-DOPA or baicalein caused sustained inhibition of α-synuclein fibrillization in favor of soluble oligomers as demonstrated by complementary fluorescence-based techniques [277].

Selegiline, a monoamine oxidase B inhibitor and a symptomatic PD treatment, inhibited α-synuclein aggregation at substoichiometric proportions as assessed by fluorescence-based and direct microscopic techniques and resulted in

disaggregation of preformed α-synuclein fibrils, consistent with reported neuroprotective effects for this molecule [278, 279].

Entacapone (Fig. 2g) and tolcapone, two nitrocatechol compounds and catechol-*O*-methyltransferase inhibitors, are used with L-DOPA to attenuate its metabolism and prolong its exposure. These two molecules also inhibit the formation of toxic α-synuclein aggregates at substoichiometric proportions in favor of off-pathway nontoxic oligomers [280].

CLR01 (Fig. 2h), described as a molecular tweezer, interferes with α-synuclein aggregation and toxicity in vitro, in cultured cells, and an in vivo α-synuclein zebrafish model [281]. Like other positively charged inhibitors, CLR01 interacts non-covalently with α-synuclein lysine residues. CLR01 interferes with fibril-promoting hydrophobic and electrostatic interactions, likely, in part, by promoting the reconfiguration rate of the monomer [282]. CLR01 likely acts at nucleation and extension and inhibits at stoichiometric proportions, though disaggregation requires molar excess. CLR01 promotes small nontoxic oligomers that migrate as larger oligomers on native gels. CLR01 restores function of the UPS, likely by limiting large aggregates that inhibit its activity. The authors suggest that small molecule inhibitors that bind with moderate affinity are effective because molecular interactions leading to the formation of oligomers are weak; hence, nucleus formation is rare and oligomers are metastable [281].

Curcumin is a primary component in the curry spice turmeric and is widely consumed. Curcumin inhibits α-synuclein aggregation in favor of SDS-resistant oligomers and disaggregates preformed fibrils, both in vitro and in cultured cells with potency in the low μM range [62]. The inhibitory activity of curcumin may be a result of its ability to favor α-synuclein reconfiguration to its native state (s) [64]. Despite low oral bioavailability, due in part to its low aqueous solubility and extensive metabolism, curcumin may pass the blood–brain barrier. Thus, advanced formulations aimed at increasing oral bioavailability such as solid lipid nanoparticles, dendrimers, nanogels (reviewed in [283]), or prodrugs [284] may allow curcumin or a derivative thereof to be used clinically.

Another small molecule with potent anti-aggregation effects was discovered as part of a high-throughput screen, followed by medicinal chemistry optimization of the diphenylpyrazole (DPP) lead to screen for substoichiometric inhibition of prion protein oligomer formation. The lead molecule Anle138b [3-(1,3-benzodioxol-5-yl)-5-(3-bromophenyl)-1*H*-pyrazole] (Fig. 2h) inhibited α-synuclein oligomer accumulation in Thy1-A30P human α-synuclein transgenic mice [285] following oral administration in food starting at 8 weeks of age, using ascending doses, up to 2×5 mg per day out to ~69 weeks of age. The authors report good oral bioavailability and brain exposure (up to 125 μM at 10 h post-5 mg dose), modest delays (~10 weeks over 69-week period) in disease onset, modest reductions in α-synuclein aggregates in brain sections, and reductions in oligomers. The molecule does not enhance clearance since overall α-synuclein levels were the same. The authors state that they are advancing this molecule into clinical trials.

Mannitol is an FDA-approved, non-metabolized osmotic diuretic agent. It also has blood–brain-barrier disrupting properties and could possibly be used in

combination with other less brain-penetrant drugs to target CNS disorders. Because of its effect on protein stabilization and activity at high concentration that result in molecular crowding effects, it is considered a chemical chaperone. Mannitol demonstrates low potency against α-synuclein aggregation in vitro, with 225 mM as the most effective dose, and a U-shaped dose response curve, with very high concentrations failing to inhibit fibril formation [286]. Despite inhibiting fibril formation, 225 mM did not prevent oligomerization, though very high doses had a modest effect, suggesting different low potency effects on the alternative aggregation pathways. The efficacy of mannitol was tested in vivo using A53T mutant α-synuclein transgenic *Drosophila* (where it reduced brain α-synuclein by 70% and improved behavioral deficits) and in heterozygous transgenic mice overexpressing human wild-type α-synuclein under the murine Thy-1 promoter (mThy1-α-syn/Line 61) [287]. This model has well-characterized α-synuclein pathology in the nigrostriatal pathway, hippocampus and cortex, and behavioral deficits in tests of sensorimotor function. It is widely used to screen potential α-synuclein-directed PD therapeutics. Daily intraperitoneal mannitol injections at 1,000 mg/kg for 4 weeks resulted in beneficial changes in a number of α-synuclein transgene-dependent readouts, including a reduction in detergent-insoluble α-synuclein aggregates in PD-relevant brain regions, reductions in the modest loss of TH-positive projections in the basal ganglia, and decreased α-synuclein-dependent inflammation. The in vivo effects on α-synuclein may result from its demonstrated induction of Hsp-70 rather than direct effects on α-synuclein [286].

Mannosyl glycerate is another putative chemical chaperone. It was shown to inhibit α-synuclein aggregation, in vitro and protect against α-synuclein accumulation, reactive oxygen species production, and toxicity in yeast [288].

Initially discovered for their ability to protect against the toxicity of the amyotrophic lateral sclerosis (ALS)-related protein TDP-43, 8-hydroxyquinolines, specifically HQ-415 and CQ, are shown to have potent rescue activity against α-synuclein foci accumulation and toxicity in yeast and *C. elegans* model systems with potency in the low μM range [289]. The mechanism of action likely involves intracellular metal chelation, though the activity is not likely mediated by reducing metal-dependent reactive oxygen species. Future studies should elucidate if there are in vivo effects in higher-order species.

Chlorogenic acid, a major polyphenol found in coffee, was originally identified as a weak in vitro α-synuclein aggregation inhibitor [117]. Subsequent work showed that chlorogenic acid prevents oxidized dopamine from promoting toxic α-synuclein oligomers and also directly protects against α-synuclein toxicity in cultured cells at low μM concentrations [290].

6.4 Biologics: Peptides and the Promise of Immunotherapy

Therapeutic peptides have gained attention in recent years as a result of successes in treating a variety of conditions, particularly those involving hormonal deficiencies

or imbalances. Despite potential shortcomings, such as instability and difficult in delivery, peptides may offer advantages for treating CNS disorders such as PD including target specificity, low toxicity, and limited drug–drug interactions.

Initially, peptides targeting α-synuclein incorporated the hydrophobic central region of α-synuclein and contained *N*-methylated amino acids. These peptides were designed such that when incorporated into growing aggregates, intermolecular hydrogen bonding, and thus subsequent self-association, would be interrupted [291]. Another peptide-based approach to inhibiting α-synuclein fibrils in favor of unstructured, nontoxic states is the use of inhibitory hairpin peptides that bear no sequence similarity to α-synuclein [292]. These ~16 amino acid long hairpin peptides contain exposed hydrophobic sites such as Tyr and Trp substitutions that facilitate intramolecular folding. The mechanism of action in the presence of α-synuclein is that the prestructured hairpin-containing strands facilitate intermolecular association in α-synuclein that precludes formation of mature oligomers and fibrils in favor of insoluble amorphous precipitates that stain less extensively with Congo red and do not exhibit red-green birefringence when viewed under polarized light.

The synuclein family member β-synuclein is shown to have potent α-synuclein anti-aggregation activity in vivo [99, 293] (reviewed in [294]). These results are consistent with substoichiometric inhibition of α-synuclein aggregation in vitro [295], likely through the formation of stable non-propagating heterodimers [99]. These results lead to the generation of a β-synuclein-derived peptide library, though peptides derived from this library did not result in beneficial effects on α-synuclein in vivo (reviewed in [294]).

Additional peptide-based approaches have been developed to interfere with specific aspects of the fibrillization process. For example, peptides and peptidomimetics such as KKDQLGK and peptidomimetics that bind to the C-terminus of α-synuclein and prevent head-to-head propagation at membranes are being tested [296]. While overall drugability characteristics may require optimization for peripheral administration, proof of concept in targeting brain α-synuclein has been established by intraventricular injections.

Immunotherapy for neurodegenerative disease was once thought to be applicable primarily to Alzheimer's disease, in which the key pathogenic determinant, Aβ, is excreted into the extracellular space and thus a more accessible target for antibody-mediated clearance. Aβ-directed immunotherapies are currently in Phase 3 clinical trials. Discoveries in the last 5 years, however, have established α-synuclein as both an intracellular and extracellular target that is secreted via exocytosis and can penetrate recipient cells and accumulate at the plasma membrane. These findings provide rationale for the development of immunotherapies for synucleinopathies (reviewed in [297]).

α-Synuclein-directed immunotherapy approaches were first tested preclinically in PDGFβ-human α-synuclein transgenic mice [298]. Following immunization with recombinant human α-synuclein, these mice displayed reductions in α-synuclein in the neuronal cell body and synapse, including reductions in membrane-associated oligomeric α-synuclein species and attenuated neurodegeneration. The antibodies

generated in response to immunization were shown to interact with α-synuclein in the lysosome, suggesting that immunization stimulated α-synuclein clearance by lysosomal activation. Importantly, immunization did not stimulate a neuroinflammatory response in these mice.

Given the concerns about potential side effects associated with active vaccination, such as vasculitis and autoimmune responses that have been noted in humans during active immunization clinical trials, emphasis is currently being placed on passive immunization paradigms that utilize administration of antibodies instead of antigens.

In active immunization paradigms, it was noted that many of the high affinity α-synuclein antibodies were directed against the C-terminus, perhaps because this portion of the protein is exposed to the extracellular space upon α-synuclein oligomer binding to and partial penetration of the plasma membrane [299]. Passive immunization with antibody 9E4, directed against amino acids 118–126 of α-synuclein, results in clearance of α-synuclein in the neocortex and hippocampus of PDGFβ-human α-synuclein transgenic mice. These mice also displayed improvement in α-synuclein transgene-dependent deficits in the Morris water maze, improved synaptic pathology, and reductions in calpain-cleaved α-synuclein. Importantly, 9E4 immunization did not perturb the microvasculature [300].

How do peripherally administered α-synuclein-directed antibodies lead to its clearance? Circulating antibodies may recognize aggregated α-synuclein at the plasma membrane. Antibody binding may then, in turn, stimulate receptor-mediated endocytosis and degradation [299, 300] likely by enhancing autophagy in the lysosome [300].

In addition to cell surface recognition, antibodies may recognize oligomeric α-synuclein in the extracellular space and block transmission to neighboring cells [248], including trans-synaptic spreading, and extracellular propagation from cell body to cell body, as well as transmission to grafted neural stem cells and endogenous astrocytes. α-Synuclein uptake by the latter is associated with the release of cytokines, including IL-1α, IL-1β, and IL-6 [243], suggesting that α-synuclein antibodies can block an α-synuclein-dependent proinflammatory cascade. Moreover, antibodies may stimulate physiological microglial activation leading to increased clearance and, in turn, attenuate the proinflammatory response initiated by these cells.

Preventive immunization approaches may be more efficacious and associated with fewer side effects than therapeutic immunization. Nonetheless, the first PD Phase 1 clinical trial utilizing active immunization against α-synuclein was initiated in 2012 by Austria-based AFFiRiS AG. The vaccine, AFFITOPE PD01, consists of a peptide-carrier conjugate and is formulated with aluminum hydroxide as the immunological adjuvant [301]. Though the sequence of the peptide is not published, it is reported that the peptides used in the vaccine are small enough to avoid an α-synuclein-specific T-cell-mediated autoimmune response and also designed to exclude cross-reactivity with β-synuclein. Immunization with AFFITOPE PD01 reduced the level of cerebral α-synuclein in synucleinopathy

mouse models and ameliorated α-synuclein-triggered neuropathological alterations such as neuronal cell loss and dendritic density loss [297]. This represents the first active immunization study to be tested clinically in PD.

The use of single-chain variable domain antibody fragments (scFv) is another antibody-based approach to inhibit α-synuclein aggregation, toxicity, and extracellular propagation. One such antibody, D5 scFv was shown to be a potent inhibitor of α-synuclein aggregation in vitro, likely by binding to an early oligomeric form. This antibody also protects cells from α-synuclein-dependent toxicity, possibly by interfering with deleterious oligomeric α-synuclein/cell membrane interactions [302]. Another antibody, syn-10H scFv, was shown to bind to a later stage oligomer, inhibit further aggregation in vitro, and neutralize α-synuclein-induced toxicity in cultured cells [30]. It is proposed that the therapeutic potential of these antibodies can be asserted either extracellularly or via intracellular expression (known as intrabodies). Intrabodies have been used to neutralize toxic effects of different pathogenic agents, including α-synuclein [303].

7 Molecules That Inhibit Downstream Toxicity of α-Synuclein

7.1 ER-to-Golgi and Other Vesicular Trafficking Pathways

N-aryl benzimidazole (Fig. 3a) was shown to suppress α-synuclein toxicity in a multiplatform system including yeast, *C. elegans*, and rat primary neurons expressing α-synuclein from a viral vector with potency in the low μM range. These effects likely result from rescue of trafficking defects that are dependent on the E3 ubiquitin ligase Rsp5/Nedd4 [93] and are related to nitrosative stress [159]. Consistent with an earlier report [304], these studies further define the role of α-synuclein as a "hub" protein, with effects on multiple cellular trafficking-related pathways. These include blocking early ER-to-Golgi events, as well as later exocytic events, early endocytosis, endosomal transport, retrograde transport, autophagy, lysosome vacuole function, ER stress, and ER-associated protein degradation.

Most recently, the benzene sulfonamide-containing compound ELN484228 was shown to block α-synuclein-dependent vesicular dysfunction and the resulting impairments in phagocytosis in cellular models, possibly by blocking redistribution of α-synuclein to the phagocytic cup [305]. ELN484228 also improves survival of primary midbrain neurons overexpressing A53T mutant α-synuclein, with potency in the μM range. ELN484228 does not alter α-synuclein aggregation, although it is predicted in silico to interact with α-synuclein monomer in a putative binding pocket.

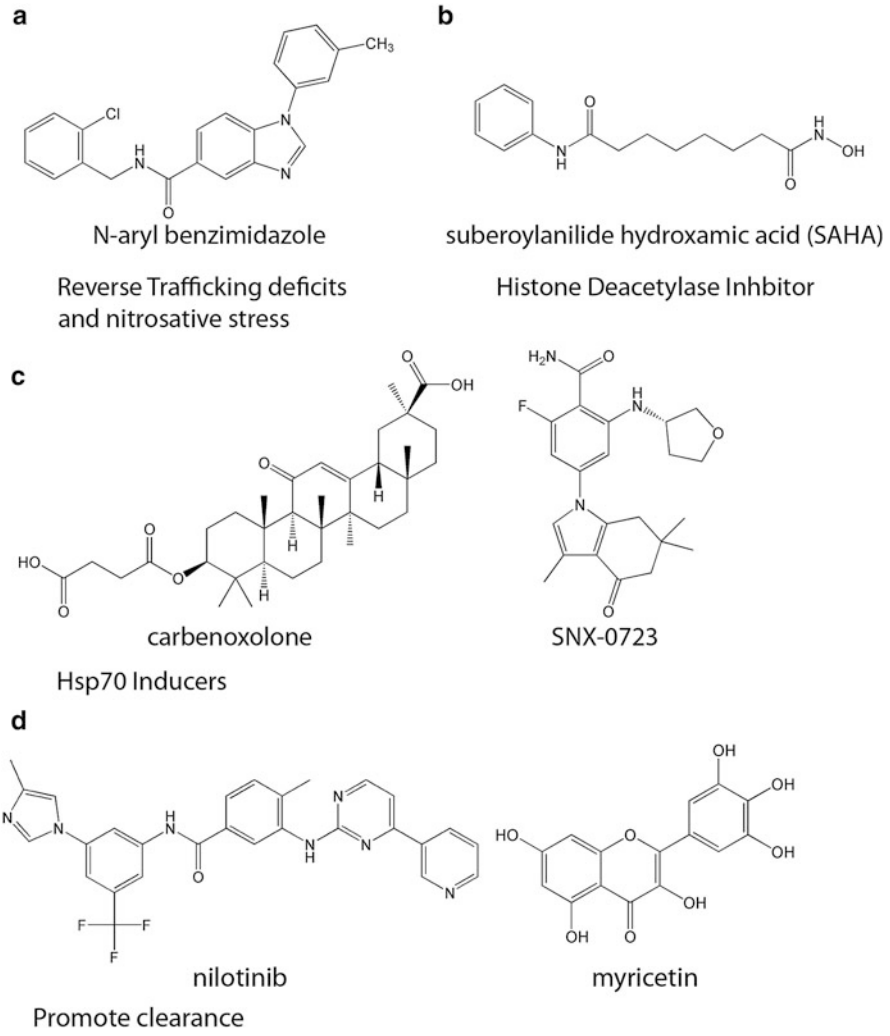

Fig. 3 Structures of select compounds that inhibit downstream toxicity of α-synuclein aggregates. These molecules down-modulate cellular dysfunction and/or stress in the ER/Golgi compartment (**a**), inhibit histone deacetylase activity (**b**), upregulate molecular chaperones such as Hsp70 (**c**), or promote clearance of toxic α-synuclein aggregates via the autophagy or proteasome pathways (**d**)

7.2 Blocking Deleterious Effects in the Nucleus

Using cultured cells and transgenic *Drosophila*, it was shown that α-synuclein that is targeted to the nucleus is more neurotoxic than α-synuclein that is excluded from the nucleus. α-Synuclein may act to modulate gene expression by interacting

directly with histones and blocking their acetylation. For example, dopaminergic loss induced by nuclear α-synuclein was reduced by the histone deacetylase inhibitors sodium butyrate and suberoylanilide hydroxamic acid (SAHA, Fig. 3b) [306]. HDAC inhibitors did not affect overall levels of α-synuclein. Valproic acid, another histone deacetylation inhibitor, was shown to protect against rotenone-induced monoubiquitinated α-synuclein nuclear localization and toxicity [307]. In contrast, another study [90] demonstrated that α-synuclein is induced by valproic acid in rat neurons and that this induction was protective against glutamate-induced neurotoxicity. Therefore, modulation of the deleterious effects of α-synuclein on histone acetylation-dependent gene expression alterations may prove challenging.

Other epigenetic factors such as DNA methylation may also mediate α-synuclein expression and toxicity and therefore be a potential therapeutic target. For example, human postmortem brains from PD and DLB patients showed reduction in levels of nuclear DNA methyltransferase 1 (Dnmt1) and consequential reduced DNA methylation [308]. Physical association of α-synuclein with Dnmt1 might mediate retention of Dnmt1 in the cytoplasm. Hypomethylation of DNA could in turn increase expression of α-synuclein, since the SNCA gene is shown to be regulated by methylation at intron 1, with decreased methylation detected in PD patients [309, 310]. Hypomethylation could also alter gene expression of factors that promote neurodegeneration directly or by counteracting other protective molecules. Such factors, if identified, could be therapeutic targets.

7.3 Mitochondria

Mitochondrial dysfunction, in particular electron transport chain complex I deficiency, is observed in a majority of PD patients [311]. Numerous recessively inherited forms of PD involve loss of function in proteins directly involved in mitochondrial maintenance, dynamics, or homeostasis, including Parkin, Pink1, and DJ-1 [312, 313]. α-Synuclein may have a normal role in the mitochondria, such as maintenance of mitochondrial calcium homeostasis [314], whereas pathogenic α-synuclein may target mitochondria [121, 315–318] through direct interactions in a conformation or aggregation state-dependent fashion [319–321] or indirectly through activation of cell death pathways in which mitochondria play a critical role [120, 228].

Mitochondrial oxidative stress is a potential link between α-synuclein aggregation, impaired neuronal function, and cell death [320, 322–325]. Recent studies showed that α-synuclein, like the mitochondrial toxin rotenone, directly inhibits complex I [316, 326] causing increased oxidative stress. In addition, α-synuclein may mediate the effects of mitochondrial complex I inhibitors such as MPTP and rotenone [87]. Interestingly, α-synuclein-dependent reactive oxygen species emanating from the mitochondria and activation of the mitochondrial permeability transition pore may play a critical role in generating a mitochondria to nucleus

pro-death signal via the release of the proapoptotic mitochondrial endonuclease G [325].

A53T mutant α-synuclein increases cell death by increasing autophagy of mitochondria [327], whereas another report demonstrated that wild-type α-synuclein promotes mitochondrial fission that results in a decline in mitochondrial function and cell death [328]. The A53T mutation may also impair a wild-type α-synuclein function at the mitochondria-associated ER membrane, thereby causing reduced apposition of mitochondria to ER and increased mitochondrial fragmentation [329].

Therefore, molecules that target α-synuclein/mitochondrial interactions, or lessen the effect of α-synuclein on the mitochondria, may be of therapeutic benefit.

7.4 Enhancing Clearance of Toxic α-Synuclein Aggregates

As described in Sect. 4, α-synuclein is cleared by both the UPS and autophagy. Thus, enhancing clearance of pathogenic α-synuclein species via modulation of these clearance mechanisms with small molecules and biologics is an active area of research.

For example, in addition to their potential direct effects on α-synuclein aggregation, the polyphenols myricetin (Fig. 3d) and purpurogallin blocked the α-synuclein-dependent proteasome inhibition [230], thereby potentially increasing clearance of toxic α-synuclein aggregates and raising the question of whether other common polyphenols have a similar mechanism of action, in addition to their direct anti-aggregation activities.

Molecular chaperones have evolved to provide quality control in the face of protein misfolding, either by recognizing misfolded proteins and preventing their aggregation or, in the case of protein-remodeling factors, by directly resolving protein aggregates that have already formed. Heat shock proteins (HSP) have been shown to fulfill both of these roles and thus may constitute promising therapeutic strategies.

The best understood of the molecular chaperones that modulate α-synuclein aggregation and toxicity is Hsp70, a protein that is upregulated as part of a protective stress response to α-synuclein misfolding and aggregation. Hsp70 inhibits α-synuclein aggregation in vitro at substoichiometric proportions through transient interactions with a pre-fibrillar form at the central NAC region that promotes monomer [330] and/or redirection to amorphous nontoxic aggregates [331]. In vivo, Hsp70 may direct misfolded species to degradation by the proteasome or the lysosome, although specific putatively toxic oligomeric forms of α-synuclein are shown to inhibit the Hsp70 chaperone system. Taken together, these results suggest that α-synuclein toxicity may result from reduced output from the chaperone machinery, thereby affecting α-synuclein and other improperly folded proteins and leading to cellular toxicity [106].

Hsp70 overexpression reduces α-synuclein pathology in transgenic mice [332] and reduces α-synuclein toxicity in transgenic *Drosophila* [333] and in cultured cells [252], including secreted α-synuclein oligomers that may be transmitted from cell to cell [334]. Most recently, chemical induction of Hsp70 levels with carbenoxolone (CBX, Fig. 3c), a glycyrrhizic acid derivative, was sufficient to reduce α-synuclein aggregation and related toxicity in cultured cells, thus favoring pharmacological manipulation of this pathway [335].

Despite having no mammalian ortholog, yeast Hsp 104 was shown to ameliorate α-synuclein expression-dependent neurodegenerative changes in the nigrostriatal pathway of A30P mutant α-synuclein expressing rats, possibly as a result of an Hsp104-dependent reduction in the percentage of cells containing phosphorylated α-synuclein aggregates [336]. Moreover, similar to small molecules such as EGCG described above, and unlike Hsp70, Hsp104 has both inhibitory and aggregate remodeling activity. For example, Hsp104 was able to inhibit α-synuclein aggregation in vitro, even at substoichiometric proportions, remodel preformed oligomers of A30P mutant α-synuclein, and remodel fibrils derived from wild-type, A30P, or A53T mutant α-synuclein. Furthermore, Hsp104 was shown to cooperate with Hsp70 in vitro [336].

Hsp90 is another chaperone studied in the context of α-synuclein aggregation, although it may promote rather than retard aggregation. For example, in one study, Hsp90 was shown to bind to mature monomeric α-synuclein in an ATP-dependent manner, abolish its interaction with small unilamellar vesicles, and promote fibril formation over oligomer formation [337]. In the absence of ATP, however, on-pathway oligomer formation was promoted. The toxicity of these oligomeric species was not assessed, though the results suggest that downregulation of Hsp90 activity could be beneficial. Consistent with this hypothesis, brain-permeable Hsp90 inhibitors such as SNX-0723 (Fig. 3c) were shown to upregulate Hsp70 expression, inhibit formation of high molecular weight toxic oligomers, and rescue α-synuclein toxicity in cultured cells [338].

In contrast, however, a potentially beneficial role for Hsp90 was recently put forth. In this study, Hsp90 was shown to inhibit the aggregation of the A53T mutant α-synuclein. Using a variety of biophysical techniques in vitro, these results demonstrated that Hsp90 redirects α-synuclein aggregation in an ATP-independent manner by forming a strong complex with transiently populated toxic oligomeric α-synuclein species and rendering the oligomers nontoxic to cultured cells [339].

Taken together, these results demonstrate that Hsp90 interactions with α-synuclein and the resulting effect on α-synuclein toxicity may depend on concomitant changes in Hsp70 levels and on ATP availability. Thus, additional studies will be necessary to determine the potential of inhibiting Hsp90 as a PD therapeutic.

α-Synuclein is believed to activate the protein tyrosine kinase cAbl through phosphorylation, and this activation is hypothesized to promote α-synuclein-dependent neurodegenerative changes, consistent with increased phosphorylated Abl levels in the striatum of PD patients and in mouse synucleinopathy models [340]. Conversely, injection of lentiviral vectors driving expression of Abl into

the substantia nigra of wild-type mice resulted in a marked increase in α-synuclein monomer and high molecular weight oligomers, supporting a positive association between Abl and α-synuclein levels in brain.

Nilotinib (Fig. 3d) is a brain-penetrant Abl inhibitor approved by the FDA for the treatment of leukemia. Daily intraperitoneal injections of nilotinib for 3 weeks at 10 mg/kg decreased total brain α-synuclein levels in prion promoter (Prp)-A53T human α-synuclein transgenic mice. Decreased α-synuclein was accompanied by an increase in the autophagy markers beclin-1 and Atg12 and a decrease in the autophagosome marker LC3, suggesting that nilotinib inhibition of Abl promotes autophagic clearance of α-synuclein. Injected nilotinib also markedly decreased α-synuclein accumulation in the substantia nigra of an α-synuclein lentiviral vector mouse model, attenuated the α-synuclein-dependent increase in proapoptotic caspase-3, and partially rescued the α-synuclein-dependent loss of tyrosine hydroxylase-positive neurons in the SN [340]. A follow-up study demonstrated that nilotinib and the related compound bosutinib promote parkin activity and its interaction with Beclin-1, thereby promoting proteasomal and autophagic clearance of α-synuclein [341]. These results, demonstrating that pharmacological activation of autophagy can mediate clearance of α-synuclein, are consistent with previous results demonstrating that genetically increasing autophagy via overexpression of beclin-1 [342] or Atg7 [223] in cultured cells and the brains of PDGFβ-human α-synuclein transgenic mice enhances clearance of brain α-synuclein. These results are also consistent with studies showing that rapamycin or rapamycin analogs are capable of reducing the accumulation of α-synuclein oligomers via the autophagy pathway [343]. Rapamycin inhibits phosphorylation of mTor (target of rapamycin) and activates autophagy. However, rapamycin, which is poorly brain penetrant, also downregulates protein synthesis and cell proliferation, potentially complicating its therapeutic use.

α-Synuclein aggregation may also have indirect effects on autophagy by sequestration of autophagy-related transcription factors. For example, overexpression and aggregation of α-synuclein in nigral neurons result in the cytoplasmic sequestration of the autophagy-regulating transcription factor EB (TFEB) and, as a consequence, impaired autophagy. Conversely, TFEB gene transfer, using intranigral injections of rAAV vectors driving TFEB expression, induced autophagy, similar to transfer of beclin-1. TFEB expression caused reductions in high molecular weight α-synuclein oligomers and protected against α-synuclein-dependent dopaminergic deficits and neurodegenerative changes [344].

UPS involvement in PD was supported by the discovery of mutations in parkin, a ubiquitin-protein ligase, and UCH-L1, a deubiquitinating enzyme involved in ubiquitin recycling. Mutations in parkin that result in partial or complete loss of parkin activity cause autosomal recessive juvenile onset PD. Parkin was shown to interact with glycosylated α-synuclein in Lewy bodies [345]. Parkin likely plays a role in α-synuclein clearance and Lewy body formation, consistent with a study demonstrating that lentiviral-mediated expression of parkin in the substantia nigra protects dopamine neurons against A30P α-synuclein-induced neurotoxicity and increases the number of phosphorylated α-synuclein inclusions [16]. In addition to

illustrating the role that parkin may play in α-synuclein-dependent toxicity, this study also exemplifies the potential benefit of sequestering α-synuclein in highly insoluble inclusions. Thus, parkin may detoxify proteins in both the soluble and aggregated forms, although parkin itself may not offer an obvious target for therapeutic intervention.

Parkin is also shown to interact with and ubiquitinate synphilin-1 [346]. Synphilin-1 binds to α-synuclein at its N-terminus [347] and prevents its degradation in the proteasome [348]. Aggresomes are formed by convergence of smaller aggregates via microtubule-based transport, cleared by autophagy and thus neuroprotective relative to smaller, diffusible aggregates. Consistent with this hypothesis, enhancing formation of α-synuclein containing aggresomes by modulating synphilin-1 [349] enhanced autophagic clearance [350] and was cytoprotective in cultured cells [351], *Drosophila* [352], and transgenic mice [353]. Other studies in yeast [354] and mice [355], however, have shown that synphilin-1 does not neutralize α-synuclein toxicity in vivo and may instead enhance it. Thus, while an intriguing target, the therapeutic potential of modulating synphilin-1 remains to be determined.

Neurosin is a serine protease that degrades monomeric and oligomeric α-synuclein, resulting in nontoxic fragments. Neurosin levels are reduced in the brains of both DLB patients and PDGFβ-human α-synuclein transgenic mice. Exogenous delivery of neurosin, using intracerebral injection of lentiviral vectors driving its expression, can reduce the brain accumulation of toxic α-synuclein in the mouse model. For example, neurosin delivery attenuated the α-synuclein-dependent reductions in microtubule-associated protein (MAP)-2 and synaptophysin at the synaptic terminal and decreased α-synuclein-induced microgliosis [356].

Mutations in the glucocerebrosidase (GCase) gene that cause the lysosomal storage disorder known as Gaucher's disease result in the highest genetic risk for developing PD and DLB, even among non-symptomatic heterozygous carriers [357, 358]. Indeed, a decrease in glucosylceramidase activity or the presence of a mutant gene can increase amyloid formation of purified α-synuclein by stabilizing soluble on-pathway oligomeric intermediates [359]. Moreover, transgenic mice with GCase mutations demonstrate α-synuclein pathology in the CNS [360]. In addition, PD patients without GBA1 gene mutations have decreased GCase activity [361] consistent with the finding that α-synuclein can inhibit glucocerebrosidase activity in the lysosome [359].

rAAV-mediated expression of glucocerebrosidase in the CNS of symptomatic homozygous mutant GCase mice rescued the aberrant accumulation of the toxic lipid glucosylsphingosine and reduced the levels of ubiquitin, tau, and Proteinase K-resistant α-synuclein aggregates. In addition, hippocampal expression of glucocerebrosidase in these mice also reversed their cognitive impairment even when expression is started at a postsymptomatic age. In regards to effects of GCase expression on α-synuclein, overexpression of GCase in A53T mutant α-synuclein transgenic mice reduced the levels of soluble α-synuclein, suggesting that increasing GCase activity can serve as a therapeutic strategy for both GBA1-related and unrelated α-synucleinopathies [360, 362]. Regardless of GBA1 genetic

background, it is postulated that increasing lysosomal GCase activity prevents the accumulation of toxic metabolites, thus improving lysosomal function leading to improved clearance of other toxic proteins including α-synuclein aggregates. Decreasing toxic α-synuclein aggregates may, in turn, allow proper trafficking of GCase to the lysosome and support its enzymatic activity [359, 362, 363]. Taken together these results suggest that augmenting glucocerebrosidase activity is a viable PD therapeutic approach.

Cathepsin D is another lysosomal enzyme that is now considered one of the main lysosomal enzymes involved in α-synuclein degradation and thus a potential therapeutic target [364]. For example, reduced Cathepsin D expression results in α-synuclein accumulation and degeneration of the dopaminergic system in experimental models and in patients with PD [365], whereas overexpression of Cathepsin D reduces the pathology associated with α-synuclein accumulation [366].

In addition to ubiquitination, α-synuclein undergoes posttranslational modification by addition of small ubiquitin-related modifier (SUMO) both in vivo and in vitro. Sumoylation of α-synuclein occurs primarily at Lys96 and Lys102 and results in substoichiometric inhibition of α-synuclein aggregation in vitro in favor of smaller, more soluble amorphous aggregates. Conversely, impairment of sumoylation by mutating Lys96 and Lys102 enhanced aggregation and exacerbated α-synuclein toxicity in cultured cells and in a rat model utilizing rAAV vector-driven expression of wild-type and Lys96/102 mutant α-synuclein [367]. Another study reported that proteasomal inhibition induces the accumulation of sumoylated α-synuclein aggregates in aggresome-like structures in cultured cells, consistent with the detection of sumoylated α-synuclein aggregates in PD and DLB brains and consistent with sumoylation promoting aggregation of other disease-related proteins including ataxin. The authors suggest that sumoylation may enhance aggregation since high molecular weight aggregates were absent in sumoylation-deficient cells [368]. However, it is possible that under normal conditions, sumoylation may promote α-synuclein solubility and clearance and that it may serve as a stress response in the face of accumulated insoluble proteins, perhaps even promoting the formation of nontoxic aggresomes. Distinguishing between these possibilities will be necessary to make informed decisions about therapeutic approaches designed to modulate sumoylation as a PD therapeutic.

8 Summary, Conclusions, Outlook

α-Synuclein is a logical target for a putative disease-modifying PD therapeutic. In its native form, α-synuclein plays an important role at cellular membranes, impacting vesicle trafficking and neurotransmitter release. In its aggregated and modified forms, α-synuclein resists clearance and assumes deleterious new functions, leading to neurodegeneration. α-Synuclein is a nonclassical target for pharmacological intervention due to its dynamic and heterogeneous structure. Thus, conventions regarding stoichiometry, occupancy, and pharmacodynamics may not apply. Initial success has been made in elucidating α-synuclein structure, function,

and in neutralizing its deleterious forms, particularly in preclinical models including transgenic mice, gene-transduced rats, and, most recently, human neurons derived from induced pluripotent stem cells of PD patients. Small molecules that engage, redirect, and detoxify misfolded forms show great promise, though their mechanisms of action are complex and not fully understood. Cell-to-cell spreading makes extracellular α-synuclein aggregates an attractive target and may obviate the need for cell penetration of molecules targeting this population. In addition, approaches that enhance α-synuclein clearance are rapidly advancing, including α-synuclein-directed immunotherapy, currently in clinical trials. Nevertheless, critical questions remain. For example, what is the composition of the unfolded ensemble that is capable of forming toxic aggregates and how can structures within this ensemble be safely and selectively redirected to functional or at least nontoxic forms? Moreover, what *are* the "moving targets" that comprise in vivo toxic aggregated species? Is lessening α-synuclein gene dosage or enhancing its clearance in humans safe, and if so how much and at what point in disease progression will these be necessary and sufficient to elicit therapeutic benefit? Further, can noninvasive imaging techniques detect α-synuclein pathology prior to motor dysfunction and herald the need for early intervention? Innovations that successfully address these questions should result in effective α-synuclein-directed PD therapeutics.

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Leucine-Rich Repeat Kinase 2 (LRRK2) Inhibitors

Paul Galatsis, Jaclyn L. Henderson, Bethany L. Kormos, and Warren D. Hirst

Abstract Mutations in the leucine-rich repeat kinase 2 (LRRK2) are the most common known cause of autosomal dominant Parkinson's disease (PD), accounting for approximately 1% of "sporadic" and 4% of familial cases. These mutations either lead directly to an increased kinase activity (G2019S and I2020T are in the kinase activation loop) or to a reduced GTPase activity (R1441C/G and Y1699C), that in turn positively regulate kinase activity. The physiological substrate of the LRRK2 kinase has yet to be definitively identified, yet autophosphorylation is emerging as a relatively robust measure of its activity. LRRK2 has been implicated in a number of diverse cellular processes such as vesicular trafficking, microtubule dynamics, protein translation control, inflammation, and immune function, all of which have been linked to PD. LRRK2 is a large, multi-domain protein; a thorough understanding of the protein domain organization and identification of interacting partners is important to determine the underlying mechanism of LRRK2. Substantial recent effort has been directed towards identifying potent LRRK2 kinase inhibitors, from the repurposed kinase inhibitors to the first through third generation of LRRK2-focused kinase inhibitors, from a range of chemotypes, which are now providing researchers with new tools to better interrogate LRRK2 function.

Keywords Inhibitors, Kinase, Leucine-rich repeat kinase 2, LRRK2

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Contents

Abbreviations

6-OHDA	6-Hydroxydopamine
ANK/AR	Ankyrin repeats
ARM	Armadillo
BA	Brain availability
BBB	Blood brain barrier
BI	Brain impairment
CNS	Central nervous system
COR	C-terminal of Roc
GPCR	G-protein coupled receptor
GWAS	Genome-wide association study
HB	hydrogen bond
HBD	hydrogen bond donor count
hERG	Human ether-a-go-go-related gene
HLM	Human liver microsome
KO	Knockout
LE	Ligand efficiency
LipE	Lipophilic ligand efficiency
logD	Natural logarithm of the distribution coefficient
logP	Natural logarithm of the water/octanol partition coefficient
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LRRK2	Leucine-rich repeat kinase 2
MDR1	Multidrug resistance
MPO	Multiparameter optimization
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MW	Molecular weight

NHP	Nonhuman primate
PBMC	Peripheral blood mononuclear cell
PD	Parkinson's disease
PSA	Polar surface area
RLM	Rat liver microsome
Roc	Ras of complex proteins
ROS	Reactive oxygen species
RRCK	Ralph Russ Canine Kidney
SAR	Structure–activity relationship
SN	Substantia nigra
THLE	Transformed human liver epithelial
VDW	Van der Waals
WD40	WD40 repeats
α-syn	α-Synuclein

1 Introduction

Parkinson's disease (PD) is the most common movement disorder and the second most common neurodegenerative disorder. The etiology of PD is complex but the most common phenotype is the loss of dopaminergic neurons of the substantia nigra (SN) leading to the clinical symptoms of bradykinesia, resting tremors, rigidity, and postural instability. Historically, at a pathological level, PD is characterized by inclusions containing the synaptic protein α-synuclein (α-syn) in the cell bodies and processes of surviving neurons (known as Lewy bodies and Lewy neurites, respectively). However, more recent observations describe not only a wider distribution of α-syn pathology, but also accumulation of tau and Aβ aggregates, suggesting a more complex pathology [1]. In addition, inflammation, including reactive gliosis, is observed in the striatum and substantia nigra of PD patients [2, 3].

The penetrance of the disease increases with age with 1% of the population over 65 being affected, rising to 5% by age 85 [4]. The past 17 years has led to considerable progress in both the identification of mutations that cause disease and in the mapping of common variants that alter risk for PD [5]. It is now clearly established that many, if not all, forms of Parkinson's disease (PD) contain a genetic component [5], and mutations in the leucine-rich repeat kinase 2 (LRRK2) are the most common known cause of autosomal dominant PD, accounting for approximately 1% of "sporadic" and 4% of familial cases [5–7]. In specific populations, notably Ashkenazi Jews and North African Berber Arabs, the prevalence of LRRK2 mutations can be as high as 40% [8]. Of the LRRK2 mutations, the most common is G2019S with penetrance ranging from ~30 to 70% by the age of 80 [8–10]. This mutation occurs in the kinase domain of the protein, increasing the kinase activity, and consequently, a great deal of effort has been directed towards identifying potent LRRK2 kinase inhibitors, with a biopharmaceutical profile congruent with clinical evaluation, as novel therapeutics for PD. This chapter describes the current state of the art in developing appropriate kinase inhibitors.

2 LRRK2 Biology and Pharmacology

2.1 LRRK2 Genetics and Human Biology

The LRRK2 gene has 51 exons, with multiple potential splice sites, which encodes a large, 2,527 amino acid protein containing two predicted enzymatic domains (GTPase and kinase) and multiple protein–protein interaction domains (Fig. 1). See below for a more detailed account of LRRK2 structural biology (Sect. 3). A number of novel variants have been identified in this gene in PD patients, but only seven of these (N1437H, R1441C, R1441G, S1761R, Y1699C, G2019S, and I2020T) can be considered as definitively disease causing, on the basis of co-segregation with disease in families, and an absence in controls [5, 11–13]. These mutations either lead to an increased kinase activity (G2019S and I2020T) or to a reduced GTPase activity (R1441C/G and Y1699C), which in turn regulates kinase activity [14–16] (*vide infra*).

The G2019S mutation is relatively frequent in some populations from Southern Europe and in certain populations, such as the Ashkenazi Jews and North African Berber Arabs, where the prevalence can be as high as 40% [8]. However, there is an incomplete but age-related penetrance, ranging from ~30 to 70% by 80 years old in different studies, that has been estimated for carriers of the G2019S mutation, and the associated range of PD onset age is broad, including patients with early and late disease onset [8–10]. Dopaminergic neuronal loss and gliosis in the substantia nigra are the common pathological features in patients with LRRK2 mutations, and classical Lewy bodies are found in the majority of them. However, in some cases α-synuclein-positive inclusions are not observed, and only tau-positive or ubiquitin-positive inclusions are seen [7]. Overall, the clinical characteristics of patients with LRRK2 mutations, particularly those with the more common G2019S mutation, are very similar to those with sporadic PD [8, 17].

The incomplete penetrance clearly indicates that other factors are involved in the pathogenesis (Fig. 1); these may include other genetic contributions, both known (e.g., α-synuclein, Tau, RAB7L1, GAK) [5, 18] and yet to be defined, and/or "environmental" factors such as inflammation or oxidative/nitrative/unfolded and/or misfolded protein stress [19–21], many of which are actively being investigated. For example, LRRK2 kinase levels are increased by inflammatory mediators, such as IFNγ and LPS, in vitro [22, 23] and in vivo [23], and two recent reports suggest that LRRK2 levels are increased in sporadic PD brains [24, 25], potentially regulated by microRNA-205 [24]. LRRK2 has been shown to exist as a dimeric protein [26–29] with this form having greater kinase activity [28, 30]. These data have led to a proposed model in which LRRK2 cycles from a cytoplasmic, low-activity monomer to a higher-activity plasma membrane-associated dimer driven by, for example, LPS [28, 31, 32]. However, such biochemical experiments, investigating these potentially more active conformations of LRRK2, have not yet been performed in postmortem patient brains. Furthermore, to date, there is no direct evidence of increased LRRK2 kinase activity in postmortem patient brains from either G2019S mutation carriers or patients with sporadic disease. This is partly due to the challenges of immunoprecipitating sufficient active LRRK2 for in vitro phosphorylation assays, compounded by

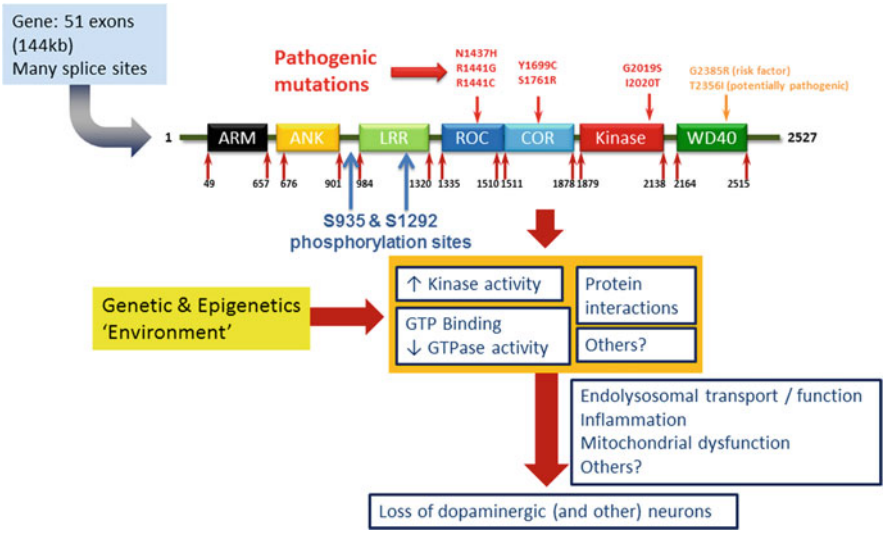


Fig. 1 LRRK2 protein domain structure and putative PD pathways

the potential effects of postmortem delay on the activity [33]. The lack of a validated substrate (vide infra) and the reliance on surrogate substrate, e.g., LRRKtide and NICtide [34, 35], also hampered such efforts. Despite this we, and others, are currently investigating these assays, and other end-points, to provide a critical link, which is currently missing, between LRRK2 and sporadic disease.

2.2 LRRK2 Substrates

The physiological substrate of the LRRK2 kinase has yet to be definitively identified. A number of potential substrates, other than LRRK2 (see next section), have been proposed (Table 1). However, full validation of these, including a clear demonstration of an increase in human PD patient cells and tissues and reduction in phosphorylation with multiple, structurally diverse inhibitors and by knocking out LRRK2, remains to be demonstrated.

2.3 Effects of Mutations on Kinase and GTPase Activity

The G2019S mutation, in the activation loop, has been consistently shown to result in increased kinase activity of LRRK2 [57, 58]. However, the functional consequences of the other mutations reported in the literature are conflicting. The other kinase domain mutation (I2020T) has been reported to either increase [59] or decrease kinase activity [34, 60]. Similarly, mutations in the GTPase domain have been demonstrated

Table 1 Putative LRRK2 kinase substrates

Protein	Phosphorylation site(s) – if determined	Identification and validation	References
4E-BP	T37/T46, T70	In vitro assay, drosophila, and LRRK2 overexpression in HEK293 cells	[36]
		Note: follow-up publications did not show robust phosphorylation	[37, 38]
α-Synuclein	S129	In vitro kinase assay (G2019S)	[39]
Akt1 (PKB), ARHGEF7, ARHGEF11 and others	S473, S153, S176 and S150	SH-SY5Y cells (endogenous and overexpressed LRRK2) +/− LRRK2-IN-1. Pathway analysis, microglial inflammatory responses and neurite outgrowth demonstrated off-target effects of LRRK2-IN-1	[40]
Akt1 (PKB)	S473	In vitro kinase assay and LRRK2 knockdown	[41]
ArfGAP1	S155, S246, S284, T189, T216, T292	Interaction studies and in vitro kinase assay	[42, 43]
β-tubulin	T107	In vitro assay and LRRK2 overexpression in HEK293 cells	[44]
Endophilin A	S75	In vitro assay and effects on synaptic vesicle trafficking	[45]
Ezrin/radixin/moesin proteins	Not determined	Primary neurons pERM increased with G2019S	[46]
FoxO	S259 (drosophila, corresponding human residue: S319)	In vitro assay and drosophila	[47]
MAP2	T1433	Consensus phosphorylation motif and peptide phosphorylation	[48]
MARKK	Not determined	In vitro kinase assay (G2019S)	[49]
MKK3/4/6/7	K149/S207 (MKK4 and 7)	In vitro kinase assay (G2019S)	[50, 51]
MKK3/6/7	Not determined	In vitro kinase assay	[52]
Moesin	T558	KESTREL screen and in vitro kinase assay	[34]
Praja	T74	Consensus phosphorylation motif and peptide phosphorylation	[48]
Ribosomal protein s15	T136	Interacting phosphoproteins identified by tandem affinity purification, LRRK2 overexpression in neurons	[53]
RIPK2	Not determined	Protein array with G2019S	[49]

(continued)

Table 1 (continued)

Protein	Phosphorylation site(s) – if determined	Identification and validation	References
Serine–protein kinase ATM (ataxia telangiectasia mutated)	T1769	Consensus phosphorylation motif and peptide phosphorylation	[48]
Snapin	T117	Interaction identified by yeast-2-hybrid. In vitro kinase assays with purified recombinant Snapin and LRRK2	[54]
Tau (tubulin associated)	T181	In vitro kinase assay and LRRK2 knockdown and overexpression in SH-SY5Y cells	[55]
	T149, T153, T205, and S199/S202/T205	In vitro tau phosphorylation; LRRK2/Tau_{P301L} transgenic mice	[56]
ULK1	T456	Consensus phosphorylation motif and peptide phosphorylation	[48]

to increase kinase activity [57, 61], whereas in other studies, they have had little or no effect [34, 58]. More recent data have shown that the R1441C/G and Y1699C mutations reduced the GTPase activity that in turn regulates kinase activity [14, 15]. The majority of studies have used either recombinantly expressed and purified LRRK2 [58] or immunoprecipitated LRRK2 from recombinant mammalian expression systems [34, 57, 61] and investigated autophosphorylation or the use of surrogate substrates (myelin basic protein, LRRKtide, NICtide), all which are relatively poor substrates under those assay conditions.

LRRK2's autophosphorylation has been characterized [30, 62, 63], and given the caveats of the validation of the exogenous substrate, perhaps this end-point is currently the most relevant. This is supported by recent data that demonstrated an increase in phosphorylation at S1292 by the pathological mutations [16]. Importantly, and in contrast to most of the previous results using purified enzyme, the magnitude of increase in phosphorylation of S1292 by, for example, G2019S is over 10-fold, when compared to wild-type LRRK2 [16] versus the 2–3-fold typically observed with the isolated enzyme [58]. This is clearly suggesting that the microenvironment of LRRK2 within the cell, including accessory proteins, interaction partners, and membrane association, is critical for the optimal kinase activity and that such a measure of kinase activity is a potentially valuable biomarker if it can be applied to patient samples. This will be a challenge as the stoichiometry of phosphorylation at S1292 is very low (<1%) ([16], unpublished observations), which may also reflect the tight control of LRRK2's activity in the cellular context.

There are additional phosphorylation sites on LRRK2, including S910 and S935 [64], which have been shown to interact with 14-3-3 proteins and regulate the subcellular localization of LRRK2 [65]. While not autophosphorylation sites, they

are clearly sensitive to LRRK2 kinase inhibitors, potentially due to conformational changes [66] exposing these residues to phosphatases, and have been used extensively in both cellular and in vivo studies (vide infra).

2.4 Role of LRRK2 in Normal and Pathological Biological Pathways

The understanding of LRRK2's role in normal and pathological biological pathways is still at a relatively early stage, with a number of key questions that remain to be answered. For example, the endogenous substrate for the kinase domain is not known (vide supra and Table 1), and despite recent progress identifying interaction partners [67, 68], the full understanding of LRRK2's function and interactome in different cell types remains an area of active research. The signaling pathways, through which LRRK2 elicits its actions and others that it potentially modulates, are emerging [69, 70] and its role in PD pathophysiology, while not definitive, is also developing.

LRRK2 has been implicated in a number of diverse cellular processes [19, 71, 72] including autophagy, vesicular trafficking, microtubule dynamics, neurite outgrowth, endosomal/synaptic dysregulation, protein translation control, mitochondrial pathology/ROS, WNT and MAPK/MEK/ERK/EIF2 signaling pathways, inflammation, and immune function, all of which have been linked to PD.

2.5 Animal Models to Understand LRRK2 Function

Studies in *Drosophila melanogaster* [73] and *Caenorhabditis elegans* [74] have provided important insights into LRRK2 toxicity in these species. The LRRK2 transgenic mouse models created to date do not completely recapitulate the hallmarks of PD (i.e., dopaminergic neuronal loss, α-synuclein accumulation, the development of Lewy bodies, and behavioral phenotype) [75–77], and with the exception of pharmacodynamic end-points, measuring reduction of LRRK2 phosphorylation at S935 and S1292 has not been used, to date, for any long-term LRRK2 inhibitor studies [16]. In contrast, viral overexpression of LRRK2 does result in dopaminergic neuronal loss [78, 79], but these models need to be fully validated with the more selective, brain-penetrant compounds described herein.

Toxins such as 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have been effective in acutely inducing DA cell loss [80]. However, the LRRK2 knockout mouse displays the same sensitivity to MPTP as wild-type mice [81] suggesting that LRRK2 may not be involved in the events downstream of the mitochondrial toxicity in the mouse brain. There are no published reports on 6-OHDA in in vivo models, with LRRK2 overexpression or with endogenous LRRK2. The use of other toxins, such as LPS, may provide a more relevant model as LRRK2 kinase levels are increased by LPS in vitro [22, 23] and in vivo [23].

The lack of validated mammalian preclinical models of LRRK2 has been an impediment to the development of LRRK2 kinase inhibitors. This has also been hampered by the lack of suitable tool compounds that possess the appropriate pharmacokinetic properties and safety profiles for long-term dosing studies that are likely to be required.

2.6 LRRK2 and Potential Safety Concerns

LRRK2 is widely expressed, with highest expression in kidney, lung, and peripheral blood monocytes and lower levels in the brain [82]. LRRK2 functions in the peripheral tissues are also not well understood, and transgenic animals, knock-ins and knockouts, have been generated in an attempt to gain greater insight into LRRK2 biology. In all cases, the animals are viable and exhibit normal life spans. Initial studies with LRRK2 knockout (KO) mice exhibited phenotypes which showed a lack of hypersensitivity to MPTP, caused impairment of protein degradation pathways, resulted in accumulation of α-synuclein in kidney, not brain, and resulted in apoptotic cell death [76, 80, 83]. A single report with conditional LRRK2 G2019S overexpression in rats showed impaired dopamine reuptake and improved locomotor activity without loss of SN dopaminergic neurons [84]. LRRK2 KO rats are now commercially available [85]. While these animals appear to not have functional impairment, they do exhibit two distinct peripheral phenotypes, similar to the LRRK2 KO mice: (1) the kidneys present with a dark color that show inclusion bodies upon microscopic examination, and (2) type II pneumocytes of the lungs show changes in lamellar body morphology with altered surfactant secretion, measured in vitro [86–89]. The changes in type 2 pneumocyte vacuolation/lamellar body enlargement have recently been observed with 7- and 29-day NHP toxicology studies [90]. It remains to be determined whether KO phenotypes are a consequence of a total lack of LRRK2 from the embryonic stage to adult and if the drug-mediated phenotypes are chemotype or mechanism based.

3 LRRK2 Structural Biology

3.1 LRRK2 Protein Domain Structure

The LRRK2 gene encodes a 286 kDa protein comprising 2,527 amino acids [6, 91]. LRRK2 protein belongs to the Roco family of proteins whose members contain multiple domains including a tandem Roc (Ras of complex proteins)–COR (C-terminal of Roc) motif [92, 93]. As with the other members of the Roco family, LRRK2 contains multiple domains (Fig. 1): the enzymatic Roc–COR and kinase domains, which are flanked by putative protein–protein interaction domains, including the N-terminal armadillo (ARM) repeats, ankyrin repeats (AR or ANK)

and leucine-rich repeats (LRR), and the C-terminal WD40 repeats [94, 95]. Currently, there is no unanimous agreement on the identity and number of armadillo, ankyrin, leucine-rich, and WD40 repeats in the LRRK2 protein [94]. A thorough understanding of the protein domain organization and identification of interacting partners is important to understand the function(s) of LRRK2.

3.1.1 Protein–Protein Interaction Domains

No X-ray crystal structures have yet been reported for any of the LRRK2 putative protein–protein interaction domains; therefore, its domain identification relies on bioinformatic analysis of the primary sequence to predict domain composition and boundaries.

The N-terminal LRRK2-specific repeats characterized by Marín [94] have been predicted to adopt the folds of 13 armadillo-type repeats from residues E49 to K657 [95]. Each ARM repeat is made up of ~42 amino acids that form three α-helices; tandem ARM repeats form a super helical structure that is involved in protein–protein interactions in other ARM repeat proteins [96]. This domain is predicted to be the largest LRRK2 domain containing ~600 amino acids.

Seven ANKs have been predicted to comprise the subsequent LRRK2 domain, made up of residues F676–S901 [95, 97]. Each AR is made up of 33 amino acids that form two antiparallel α-helices followed by a β-hairpin or a long loop, assembling to form a curved structure [98]. AR proteins are involved in a wide range of functions and typically mediate protein–protein interactions [99].

The next domain in sequence is the LRR domain, from where LRRK2 gets its name. As many as 14 LRRs have been predicted from residues I984 to R1320 [95, 100], though fewer have also been suggested [92, 94, 97, 101]. Each LRR is typically made up of 20–30 amino acids (~24 in LRRK2) and contains an unusually high number of leucine residues that form a β-strand followed by an α-helix or extended chain [102]. Tandem LRRs assemble into a horseshoe- or solenoid-type shape whose curvature depends on the specifics of the secondary structure. The framework of LRR domains makes them well suited for protein–protein interactions, and the concave surface of the LRR structure is usually, though not always, the site of macromolecular recognition or dimerization [103].

The C-terminal domain has been predicted to contain one [94], two [92], or seven [95, 97] WD40 repeats; the seven repeats are predicted to occur between residues N2164 and K2515 [95]. Each WD40 repeat contains a four-stranded, antiparallel β-sheet made up of 40 residues [97]. They usually occur in multiples of six or seven and assemble to form a propeller-like structure that serves as a rigid scaffold for protein–protein interactions [104].

3.1.2 Enzymatic Domains

The Roc domain of LRRK2 is a Ras-like GTPase domain that is followed immediately by the COR domain as in all members of the ROCO protein family

[93]. These domains approximately span residues M1335–N1510 and F1511–E1878, respectively [97]. Ras family GTPases act as molecular switches between an active GTP-bound state and an inactive GDP-bound state [105, 106]. Evolutionary analysis indicates that not all ROCO proteins contain a kinase domain; as such, it has been suggested that GTPase activity is the primary function of ROCO proteins and those with kinase domains evolved independently to regulate that function [92, 93, 95, 107].

Two X-ray crystal structures have been solved providing the ability to form hypotheses about the LRRK2 Roc and COR domains and their functions based on structural information. One is a structure of the human LRRK2 Roc domain bound to GDP-Mg^{2+}, which reveals a domain-swapped homodimer [108]. This structure suggests the Roc dimer acts as one functional unit with each nucleotide binding site formed by contributions from both monomers. The other relevant X-ray crystal structure contains both the Roc and COR domains from the bacteria *Chlorobium tepidum* and does not exhibit the domain swapping configuration [109]. This structure suggests that the COR domain is responsible for dimerization, that the Roc domain is stabilized by the COR domain, and that the Roc–COR domain is stabilized by nucleotide binding. Much discussion of the validity and relevance of these X-ray crystal structures has taken place [105, 107, 110–114]. Regardless, these structures both have implications for LRRK2 GTPase function that have spurred important discussions, and results from hypotheses generated based on these structures will only improve our understanding of the LRRK2 protein.

The kinase domain has the most straightforward therapeutic potential and thus has garnered the most interest of all of the domains in the LRRK2 protein. Characterized as a Ser/Thr kinase in the TKL group of kinases, it has the highest homology to LRRK1 and the receptor-interacting protein (RIP) kinases [94, 115], which are sensors of intracellular and extracellular stresses [116]. The LRRK2 kinase domain spans residues Q1879–V2138. Protein kinases catalyze the transfer of a phosphoryl group from ATP to serine, threonine, or tyrosine residues in protein substrates and mediate most cellular signaling processes [117]. The physiological substrate of the LRRK2 kinase has yet to be definitively identified (vide supra). The X-ray crystal structure of the kinase domain from the slime mold *Dictyostelium discoideum* Roco4 protein may provide insight into the structure of the LRRK2 kinase domain [118]. With a kinase domain that is ~30% identical and ~50% similar to that of LRRK2, as well as a similar domain structure that contains LRRs, Roc–COR, kinase, and WD40 repeat domains, the *D. discoideum* Roco4 kinase domain may be a suitable crystallography surrogate to help build an understanding of LRRK2 kinase structure and mechanism.

3.2 LRRK2 Pathogenic PD Mutations

To date, the proven pathogenic PD mutations (Fig. 1) segregate to the enzymatic Roc–COR and kinase domains of LRRK2 [13, 119, 120], though there are a number of variants that have been identified as PD risk factors that are distributed

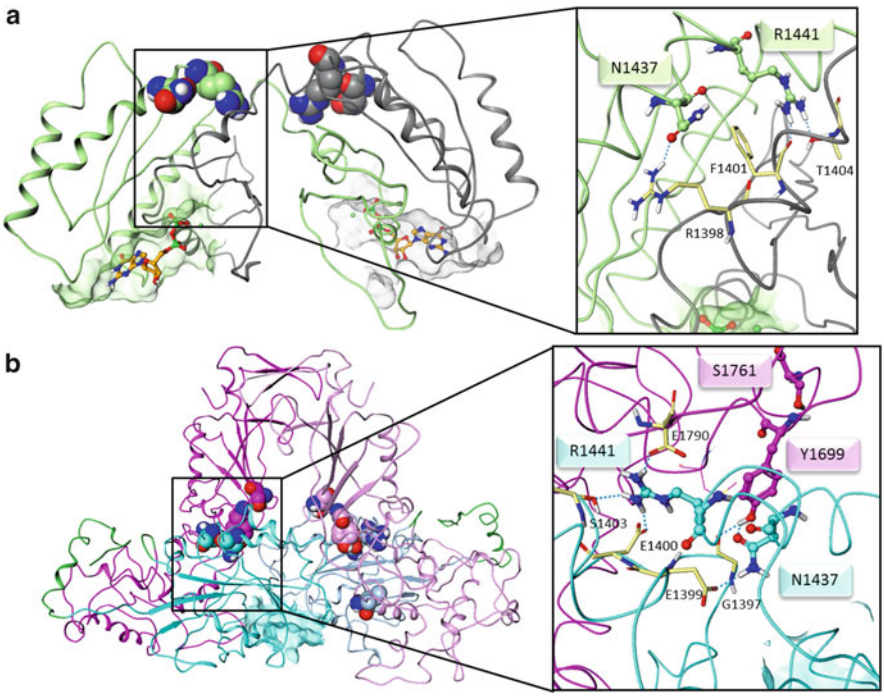


Fig. 2 Roc–COR structures with positions of pathogenic mutations and interacting residues. (**a**) Self-homology model of the domain-swapped LRRK2 Roc–Roc homodimer (PDB ID: 2ZEJ). *Orange*: GDP; *Green*: monomer A; *Gray*: monomer B; *Yellow*: interacting residues. (**b**) Homology model of LRRK2 Roc–COR dimer based on the *C. tepidum* structure (PDB ID: 3DPU). *Cyan*: Roc monomer A; *Pink*: COR monomer A; *Light blue*: Roc monomer B; *Light pink*: COR monomer B; *Yellow*: interacting residues

throughout the entire protein [121, 122]. As such, these mutations have the potential to disrupt not only LRRK2 enzymatic functions but also protein–protein interactions. In the absence of LRRK2 X-ray crystal structures, homology models [123, 124] have been valuable tools to help understand the position of pathogenic mutations and the effect(s) they may have on the function of the LRRK2 protein.

3.2.1 Roc–COR Pathogenic Mutations

The R1441C/G/H and N1437H pathogenic mutations are located in the Roc domain and the Y1699C and S1761R mutations are located in the COR domain. The two current models for the Roc domain suggest different roles for these residues (Fig. 2) [125]. In the domain-swapped homodimer X-ray crystal structure of the human LRRK2 Roc domain proposed by Cookson and coworkers [108], R1441 and N1437 are situated such that they stabilize the Roc–Roc dimer interface through interactions with the other monomer. R1441 forms HB interactions with the backbone carbonyl of

F1401 and the side chain of T1404 and forms a cation-π interaction with the side chain of F1401, while N1437 forms a HB interaction with the side chain of R1398.

Alternatively, in the X-ray crystal structure of the Roc–COR dimer from *C. tepidum* proposed by Wittinghofer and coworkers [109], the residues equivalent to R1441, N1437, and Y1699 ($Y558^{C.\ tepidum}$, $H554^{C.\ tepidum}$, and $Y804^{C.\ tepidum}$, respectively) are clustered together at the interface of the Roc and COR domains, involved in interactions within and between the Roc and COR domains. Based on the $Y558^{C.\ tepidum}$ interactions, it is hypothesized that the aliphatic side chain of R1441 is involved in structurally important hydrophobic interactions with the COR domain. A homology model based on this structure suggests that R1441 may also form HB interactions with the side chains of E1400 and S1403 in the Roc domain and that N1437 forms HB interaction with the side chains of E1399 in the Roc domain and Y1699 in the COR domain [126] (Fig. 2a). Based on the $Y804^{C.\ tepidum}$ interactions, Y1699 may form a hydrogen bond with N1437 ($H554^{C.\ tepidum}$). A homology model based on this structure suggests Y1699 may also form a HB with the N1437 side chain or the G1397 backbone carbonyl and a CH-π interaction with the C^{α} of N1437 [126] (Fig. 2b).

Neither model places these residues close enough to the binding site to interact with GTP or GDP, so the pathogenic mutations do not appear to disrupt nucleotide binding directly. In both models, mutation of R1441 to Cys, Gly, or His and N1437 mutation to His would disrupt HB and van der Waals (VDW) interactions, and in the *C. tepidum* model, mutation of Y1699 to Cys would disrupt a HB between the Roc and COR domains. Therefore, evidence from both models suggests that these residues play an important role in dimer formation or interactions between domains, affecting LRRK2 protein function (vide supra). In addition, these three residues form interactions with residues in the Switch II region of the Roc domain [109], a conserved region in Ras proteins that is important for GTPase function [127, 128]. This suggests that mutation of these residues could also affect LRRK2 GTPase function by altering interactions with the Switch II region.

The S1761 residue in the COR domain corresponds to $S852^{C.\ tepidum}$ in the *C. tepidum* structure. This residue is farther away from the Roc–COR interface and Switch II than the other pathogenic mutations and is situated on the loop between β9 and β10. As such, it may be involved in protein–protein interactions with other LRRK2 domains or other scaffolding proteins. Mutation to Arg could affect these protein–protein interactions or the flexibility of the loop.

3.2.2 Kinase Domain Pathogenic Mutations

The G2019S and I2020T pathogenic mutations are located in the kinase domain. In the absence of an X-ray crystal structure of the LRRK2 kinase domain, a number of homology models have been reported and used to understand the position of these residues and the implications of the pathogenic mutations. There is no consensus on a "best" template to build LRRK2 kinase domain homology models due to the fact that the kinases for which X-ray crystal structures have been published have at most ~30% sequence identity to LRRK2. The most common templates have been from

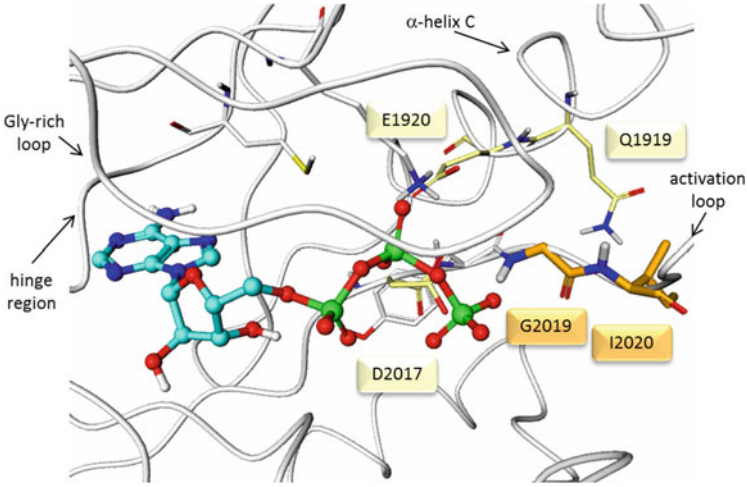


Fig. 3 LRRK2 ATP-binding site. ATP (*cyan*) is shown docked into a LRRK2 homology model based on the X-ray crystal structure of JAK3 with CP-690550 (PDB ID: 3LXK). *Orange*: location of the G2019S and I2020T mutations; *Yellow*: hypothesized interacting residues

B-Raf [94, 101, 129–131], JAK-2 [132–138], MLK1 [132, 136–139], ROCO4 [131, 140], and TAK1 [132, 141, 142] kinases, chosen based on overall kinase sequence identity, ATP-binding site identity, and/or crossover of inhibitor activity. Consensus from these alignments is that G2019 is the Gly in the conserved DFG motif (DYG in LRRK2) and I2020 is the subsequent residue in the activation loop (Fig. 3) [125].

Modeling of the G2019S mutation suggests that the change from a flexible Gly residue to a Ser causes the activation loop to be less flexible due to an increase in HB interactions with D2017, E1920, or other nearby residues, increasing the population of conformations in the active state [114, 131, 136, 141]. The X-ray crystal structure of Roco4 with the mutation that corresponds to LRRK2 G2019S, $G1179S^{Roco4}$, was solved to 2.04 Å resolution and reveals a HB interaction between $G1179S^{Roco4}$ and $R1077^{Roco4}$ in the αC-helix, which corresponds to Q1919 in LRRK2 [118]. This construct was shown to have increased kinase activity compared to WT. In addition, the double mutants $G1179S^{Roco4}/R1077A^{Roco4}$ in Roco4 and G2019S/Q1919A in LRRK2, in which the Ser is unable to make a HB interaction to the αC-helix to stabilize the active conformation, have nearly wild-type activity. Metadynamic simulations combined with kinetics studies suggested that the energy barrier to achieve the inactive DYG-out conformation in LRRK2 is much higher for the G2019S mutant than for WT [131]. Together, this evidence suggests that an additional hydrogen bond in the G2019S mutant stabilizes the active kinase conformation, causing an increase in kinase activity.

Modeling of the I2020T mutation, on the other hand, does not suggest a clear hypothesis for its pathogenicity. The X-ray crystal structure of Roco4 kinase with the corresponding mutation, $L1180T^{Roco4}$, was solved to 2.3 Å resolution and shows the side chain of the Thr interacting with solvent [118]. It was suggested that

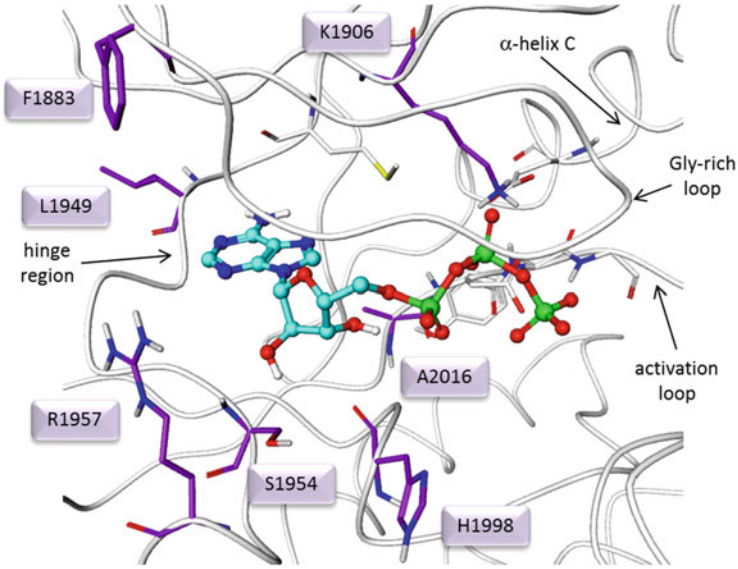


Fig. 4 LRRK2 ATP-binding site. ATP (*cyan*) is shown docked into a LRRK2 homology model based on the X-ray crystal structure of JAK3 with CP-690550 (PDB ID: 3LXK). *Purple*: residues that have been targeted for LRRK2 specificity

the effect of the mutation may only be evident upon LRRK2 dimerization or interactions with other domains, which cannot be discerned from the X-ray crystal structure of the kinase domain alone. Alternatively, molecular dynamics simulations suggest the Thr mutation may form a HB interaction with the backbone carbonyl of D2017, which is involved in substrate binding [60, 114]. Metadynamic simulations combined with kinetic studies suggested that the I2020T mutant stabilized the active DYG-in conformation in LRRK2 compared to WT [60].

3.3 Using LRRK2 Structure to Guide Kinase Inhibitor Design

Most of the LRRK2 kinase inhibitors that have been published to date are ATP-competitive inhibitors that contain hinge-binding motifs common to many kinase inhibitors (vide infra). Through the use of structure–activity relationships (SAR), X-ray crystal structures, and homology models, specific residues in the LRRK2 ATP-binding pocket have been targeted for inhibitor interactions to improve potency and selectivity for LRRK2 kinase (Fig. 4). Hydrophobic interactions with A2016 have been shown to be important for potency and selectivity of some LRRK2 kinase inhibitors [35, 140, 143, 144]. Selectivity of these compounds is especially improved over kinases that have a more polar residue at that position – 28% of kinases have a Ser or Thr at that position. Mutation of A2016 to Thr

abolishes activity in some inhibitors [35, 140, 143], but not others [145–147], which is indicative of the inhibitor binding modes.

The non-conserved residues L1949, S1954, R1957, and F1883 were identified as potential selectivity handles in the LRRK2 kinase domain, due to their proximity to the ATP-binding pocket for a series of diaminopyrimidine compounds [132–135]. L1949 near the hinge region was targeted for selectivity since it is a smaller residue than Phe or Tyr, which is found in this position in approximately 60% of the kinome. Inhibitors with a nitrogen lone pair or substituents or that extend into this region can be accommodated with the Leu residue, but sterically clash with the larger Phe or Tyr. This strategy was used to obtain selectivity for LRRK2 over the JAK family of kinases and JAK2 was found to be a useful surrogate for predicting general kinome selectivity. Interactions with S1954 were targeted to achieve both general kinase selectivity and to overcome specific selectivity issues with the kinase TTK. Approximately 55% of the kinome has a larger group at this position, and approximately 50% has a negatively charged Asp or Glu residue in this position. Strategies to mitigate crossover to TTK, which has an Asp residue in this position, have included the introduction of small groups that would cause unfavorable steric and/or electrostatic interactions with the Asp side chain [132, 133, 135]. Selectivity of a series of 7-aryl-substituted quinoline derivatives may also be due to the placement of polar groups in the vicinity of the unconserved S1954 and R1957 residues [138]. Interactions with the unconserved R1957 and H1998 LRRK2 residues may also contribute to selectivity in a series of indolinone compounds [144]. Optimizing interactions with H1998 and the catalytic K1906 in this series of compounds was hypothesized to improve kinase selectivity, especially over RET kinase.

Alternative strategies used to obtain selectivity in kinases are through Type II inhibitors, which stabilize an inactive DFG-out conformation, or Type III inhibitors, which are not ATP-competitive. Only a handful of LRRK2 inhibitors have been reported that fall into these categories [131, 148]. These inhibitors were shown to be more potent against WT LRRK2 kinase compared to G2019S, supporting the hypothesis that the pathogenic G2019S mutation stabilizes the active conformation of LRRK2 [60, 114, 131, 136, 141]. This suggests a Type II kinase inhibitor may not be the optimal approach for the treatment of PD due to the LRRK2 G2019S mutation. The X-ray crystal structure of Roco4 kinase domain co-crystallized with the inhibitor H1152 revealed two binding sites for this inhibitor: one in the expected ATP-binding site and the other close to the αC-helix [118]. The implications of this second binding site for LRRK2 are unclear, but suggest not completely ruling out the possibility of developing Type III inhibitors for LRRK2 kinase.

4 Medicinal Chemistry: LRRK2 Kinase Inhibition

Historically, kinase inhibitors have been a key target for oncology indications. The critical role of kinases in cell cycle or apoptotic signaling pathways made them a logical target. From a drug discovery perspective for non-oncology indications, pharmacologically promiscuous kinase inhibitors can potentially give rise to safety

Table 2 Physicochemical property comparison of CNS marketed drugs and Pfizer CNS clinical candidates (CANs) with marketed kinase drugs

	MW	clogP	tPSA	clogD	HBD	pK_a	CNS MPO
CNS drugs[a] (121 cmpds)	298.7	2.72	47.6	1.70	1	6.2	4.61
CNS CANs[a] (108 cmpds)	357.4	3.44	53.6	2.16	1	7.5	4.25
Kinases[a] (21 cmpds)	461.2	4.24	85.6	2.63	2	5.6	3.56

[a]Average values

concerns due to their varied roles in signaling pathways. These safety issues are particularly acute for the treatment of chronic diseases that require long-term treatment, typically experienced with neurodegenerative disorders. Recently, kinase inhibitors have been developed for non-oncology indications. Xeljanz® (tofacitinib), a JAK3 inhibitor, is a good example and was approved in 2012 for moderate to severe rheumatoid arthritis (http://www.xeljanz.com/).

For a central nervous system (CNS) indication, in addition to the off-target selectivity, one needs to contend with the blood–brain barrier (BBB) and designing compounds that have access to the central compartment. The medicinal chemistry challenge is readily apparent when one analyzes the current marketed kinase chemical space relative to the historical CNS chemical space. From a kinase inhibitor perspective, the potential pharmaceutical agent must contend with equilibria of active, inactive, and intermediate conformations (e.g., DFG-in vs. DFG-out). Most of the initially marketed kinases targeted the DFG-out conformation which generates a much larger active site pocket, thus giving rise to compounds with MW, logP, or PSA parameters outside the range of typical CNS space. Comparison of these physicochemical parameters is presented in Table 2. These data were generated from an analysis of marketed kinases relative to the marketed CNS drugs and a set of preclinical CNS candidates from the Pfizer pipeline. Additionally, employing the CNS multiparameter optimization (MPO) score [149], as a guide to the likelihood the compound may be CNS drug like, clearly shows the current chemical matter is outside this space. Furthermore, as a consequence to the high concentration of ATP present in the cell, any potential kinase inhibitor will need to have high potency to be able to compete at a mass balance level.

For a CNS indication, such as PD, clearly having free drug in the brain at the site of action is an absolute requirement. Moreover, to minimize the potential for off-target toxicology, the requisite concentration of free drug in the brain should be achieved with minimal exposure in the periphery. Combining all these attributes generates a CNS kinase design strategy that incorporates the selection of chemical matter that has low MW and is polar and neutral with high brain availability. These compounds additionally will need to have high ligand binding efficiencies (LE), lipophilic efficiencies (LipE), and low efficacious concentration (C_{eff}), all the while having high kinome selectivity.

The current public domain LRRK2 kinase inhibitor chemical matter will be summarized with an eye to how they compare to the design strategy articulated (vide supra).

4.1 LRRK2 Patent Space Analysis

As a target, LRRK2 was first identified in 2004 and its connection to PD was confirmed by genome-wide associate studies (GWAS) in 2010 (For a recent PD genetics review: [150]). This short-time frame coupled with the lack of its (patho) physiological role(s) (vide supra) has presented a challenge to the medicinal chemist as witnessed by a relatively small compound set compared to targets with a more rich medicinal chemistry history. Conversely, the highly conserved nature of the ATP-binding site of kinases does give rise to the great potential for crossing-over of chemical scaffolds from inhibitors designed for alternate kinases. Indeed, the first set of LRRK2 kinase inhibitors investigated were compounds repurposed from other kinase programs (vide infra). A handful of LRRK2 selective kinase inhibitors have been published in the primary literature; however, they are typically specific examples from a greater set of compounds claimed in the LRRK2 patent literature. The Markush structures for this set of LRRK2 kinase inhibitors are presented in Fig. 5. In general terms, the LRRK2 ATP-binding site can accommodate known core scaffolds that incorporate 1-, 2-, and potentially 3-point hinge-binding motifs. Addressing the safety, CNS design features, and chemical novelty will arise from how these scaffolds are adorned with requisite substituents.

The attributes that the medicinal chemist has the greatest design control over are the physicochemical properties. All subsequently measured compound properties are embedded once the structure has been fixed. Figure 6 provides an overview of the LRRK2 patented chemical matter as it relates to physicochemical space. The box plots provide a summary of each property showing the data distribution with the average (red line), the CNS drug set average (green line), and the marketed kinase set (black dashed line). MW shows a normal distribution with the average between those for the kinase and CNS drug sets. This tendency towards the CNS drug set is not observed for PSA with the LRRK2 and kinase sets being essentially the same. Measures of lipophilicity (clogP and logD) again show a normal distribution with the average closer to the CNS drug set. HB donor count (HBD) for the LRRK2 compounds will be skewed to the kinase set due to the required hinge interactions, and this was observed. The distribution for pK_a appears to be bimodal for the LRRK2 set, but the average tends closer to the CNS drug set.

Figure 7 provides an overview of the probability of in vivo toxicology findings (tox plot) [151]. The upper left quadrant ($clogP > 3$ and $PSA < 75$) was determined to have the greatest likelihood of in vivo safety findings. Conversely, the lower right quadrant ($clogP < 3$ and $PSA > 75$) was found to have the lowest probability of a safety finding. The pie charts are scaled to the number of compounds in each quadrant, and Fig. 7 is color coded for CNS MPO desirability. Clearly, most of the compounds reside in good CNS space ($MPO > 4$), but a significant number of these compounds reside in physicochemical space expected to give rise to safety findings.

Figure 8 illustrates the breakdown of chemical matter by the patent assignee. Again, most organizations are working in chemical space that is congruent with CNS drugs, and in several instances, the companies have leveraged existing chemotypes from marketed kinases (Fig. 5). John Hopkins University has patented the use of existing kinase inhibitors, e.g., staurosporine, damnacanthal, SP600125,

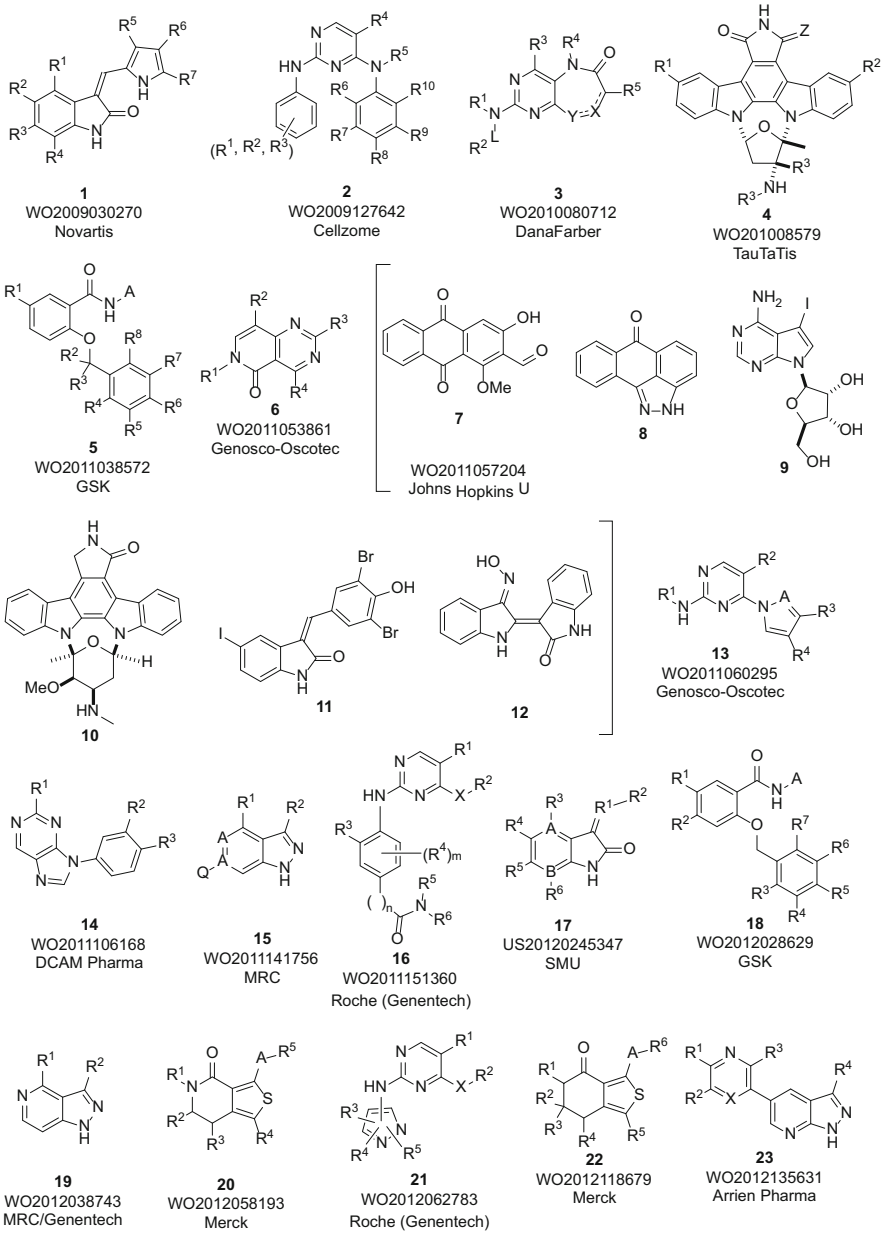


Fig. 5 (continued)

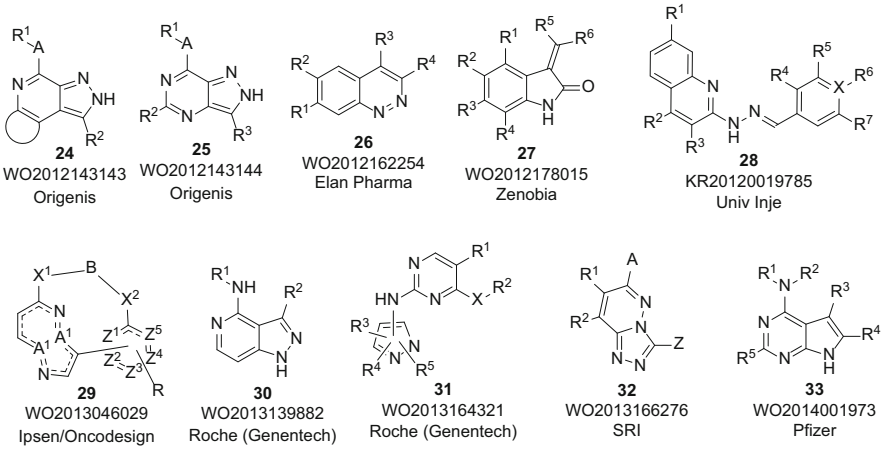


Fig. 5 LRRK2 kinase inhibitor Markush structures (chronological order)

5-iodotubercidin, GW5074, and indirubin-3′-monoxime as LRRK2 inhibitors, whereas Tautatis focused on modified versions of staurosporine analogues. Novartis, Southern Methodist University, and Zenobia focused on the oxindole framework as exemplified by sunitinib (Sutent®). The diaminopyrimidine core can be found in patents from Cellzome, Dana Farber, and Roche (Genentech). The Medical Research Council alone and in collaboration with Genentech, DCAM Pharma, Merck, Arrien, Origenis, Pfizer, and Southern Research Institute have all generated variations on a 6,5-fused bicyclic framework. Ipsen/Oncodesign extended the use of this scaffold by exemplifying macrocyclic variants of this core. GSK has disclosed a novel LRRK2 kinase inhibitor scaffold in the form of an aryl ether amide.

Comparing this set of compounds, as a whole, to the data for CNS drugs and marketed kinases (vide supra) provides potential insight as to the position in chemical space these compounds have relative to the space currently defined by the CNS drug and marketed kinase sets. All these compounds are ATP-competitive inhibitors presumably targeting the DYG-in conformation as the equilibrium distribution between the DYG-in and DYG-out (inactive conformation) appears to be skewed towards the active conformation by 5–6 kcal/mol [131]. Despite the lack of a LRRK2 crystal structure, one can leverage the knowledge from the kinase literature and predict that these compounds will interact with the hinge motif in a 1- or 2-point manner. Thus, to drive selectivity and ultimately safety, the substitution pattern from the core is crucial to accomplish this requisite attribute. From homology models, Genentech has identified two critical residues necessary to drive LRRK2 selectivity. These include the hinge residue Leu^{1949} and the activation loop residue Arg^{1957}. In the case of the diaminopyrimidines, Genentech was able to increase selectivity to off-targets (e.g., MST2 and JAK1) with substituents on the pendant aniline or aminopyrazole moieties.

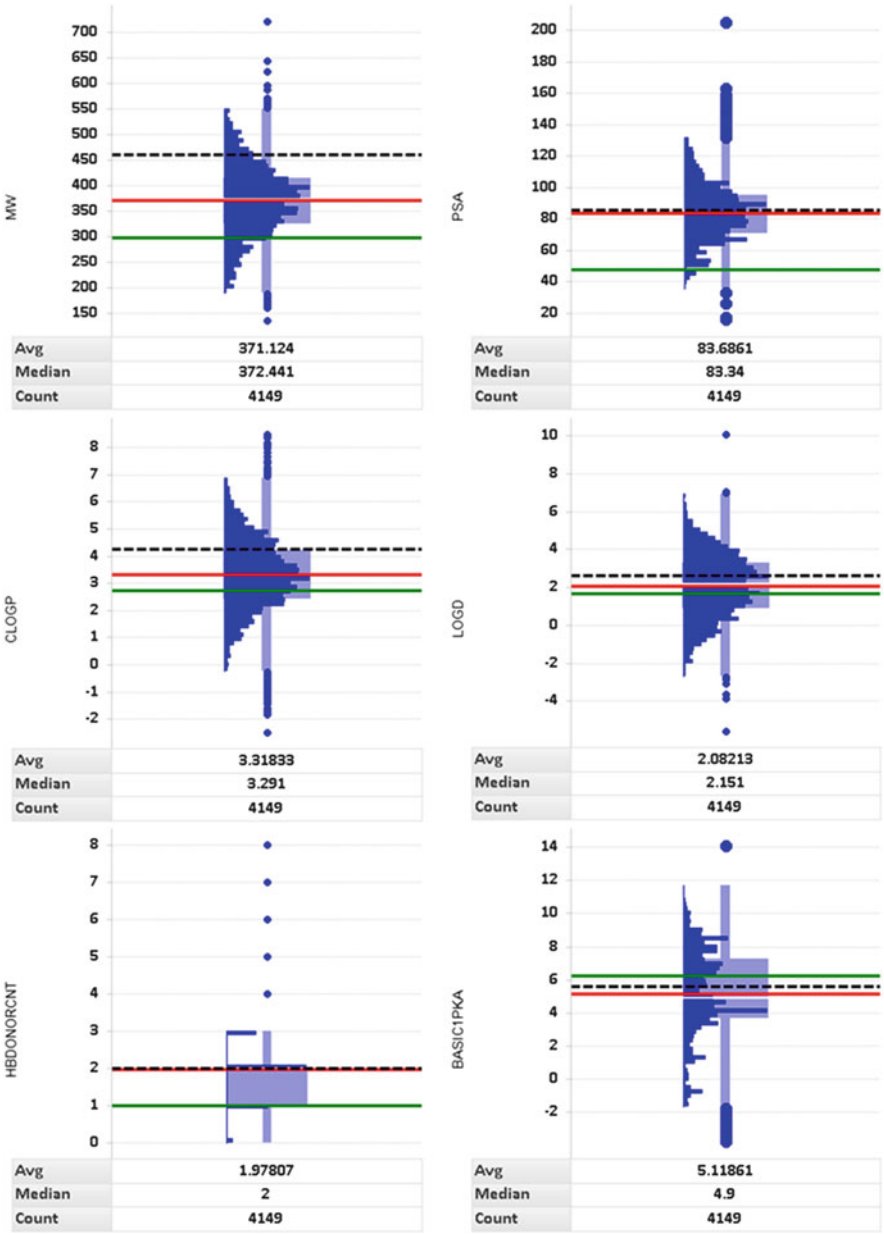


Fig. 6 Physicochemical analysis of LRRK2 patent chemical matter. Each box overlays the corresponding distribution for the descriptor on its box plots. The *dots* represent outliers and the *lines* denote LRRK2 data set average (*red*), CNS drug set average (*green*), and marketed kinase drugs average (*black dashed*)

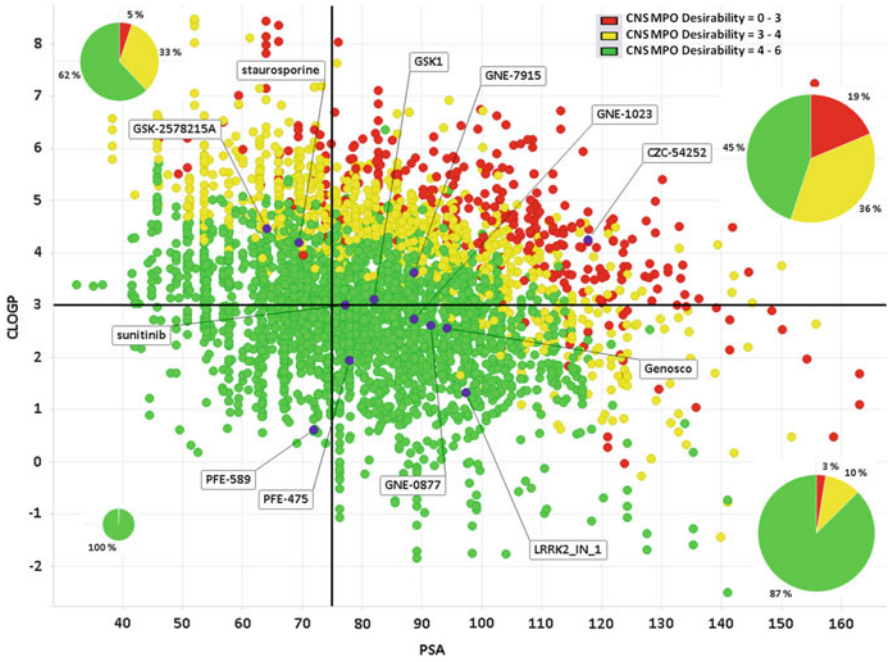


Fig. 7 Toxicity plot for LRRK2 patent chemical space (2009–2014)

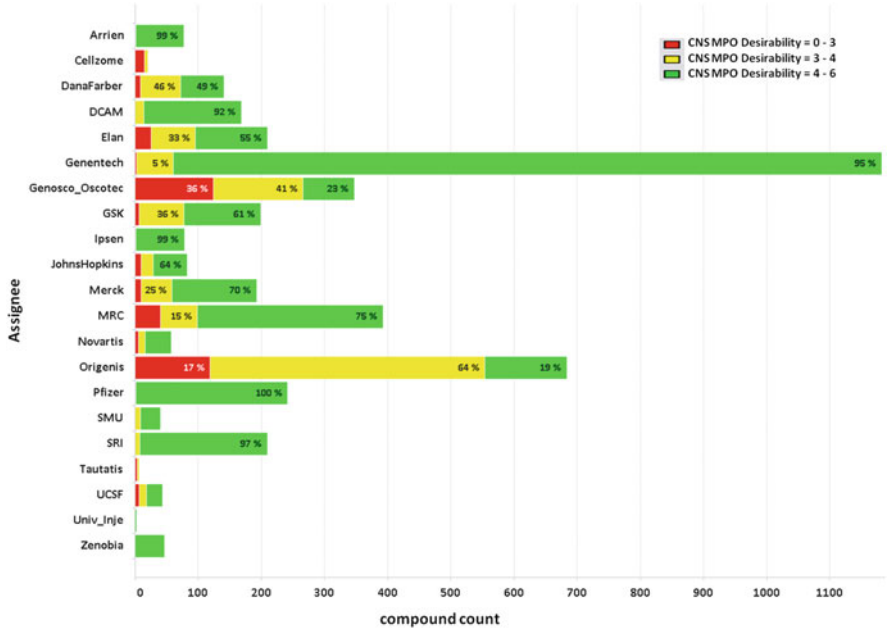


Fig. 8 CNS MPO desirability analysis for LRRK2 patents (2009–2014)

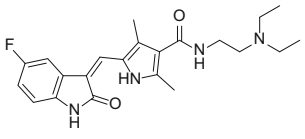
4.2 LRRK2 Chemical Matter Overview

It has been suggested that the ability to study a protein's function is enhanced when small molecule tools for the target exist [152]. Ideally a "compound toolbox" would be available, covering different chemical scaffolds and physicochemical and ADMET properties, and these compounds would be well characterized in terms of on- and off-target effects. With distinct scaffolds, overlap of off-target effects, for example, between the compounds is likely to be reduced, and consistent findings across the compounds would therefore be indicative of activity at the target in question.

The 28 patent applications of small molecules (Fig. 5), to date, represent roughly 10 unique chemical scaffolds. Through the use of SAR, X-ray crystal structures (surrogate crystallography), and homology models, specific residues in the LRRK2 ATP-binding pocket have been targeted for inhibitor interactions to improve potency and selectivity for LRRK2 kinase. Initial activities focused on LRRK2 potency and selectivity. As the compounds achieved these goals, focus shifted to address ADME and safety issues that were revealed as these compounds were used to expand LRRK2 biology. The diversity of the current chemical matter provides a toolbox of compounds that should enable the detailed elaboration of LRRK2 kinase function.

4.2.1 Repurposed Kinase Inhibitors

Early work in the field of LRRK2 kinase inhibitor development sought to compliment the range of biochemical tools that were becoming available [153] with small molecule kinase inhibitors. By screening panels of commercial compounds, groups began to identify known kinase inhibitors that also displayed activity against LRRK2 [35, 78, 154]. Initially identified compounds, using LanthaScreen detection and a surrogate peptide substrate (LRRKtide), included **7–9** and **11–12**. Staurosporine, **10**, was one of the most potent LRRK2 inhibitors reported (wt $IC_{50} = 6$ nM); however, it is also highly promiscuous across the kinome [155]. Capitalizing on this observation, **4** illustrates how modifications to the bis-indole core were pursued to potentially address the selectivity issue. Sunitinib, **34**, is a potent LRRK2 inhibitor (wt $IC_{50} = 37$ nM), but also displays similar promiscuity. Related indolidinones (**1**, **17**, and **27**) have been disclosed. Screening of a number of Rho-kinase inhibitors led to the identification of **35** [35]. Although it has a number of off-target effects, selectivity is much improved over **10** and **34**, although potency against LRRK2 is also reduced (wt $IC_{50} = 244$ nM).



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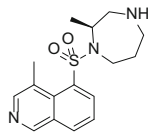

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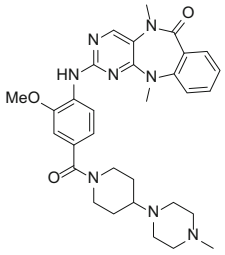
Although these early inhibitors are structurally different, they have in common potent activity across a range of kinases, and thus it is difficult to separate effects at LRRK2 from off-target effects, especially when examined in isolation. These compounds proved useful for initial study of the function of LRRK2; however, it was clear that the field could benefit from more selective compounds.



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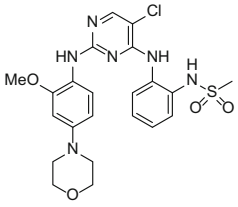

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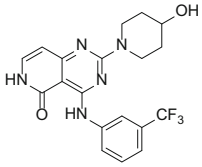

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4.2.2 First-Generation LRRK2-Focused Kinase Inhibitors

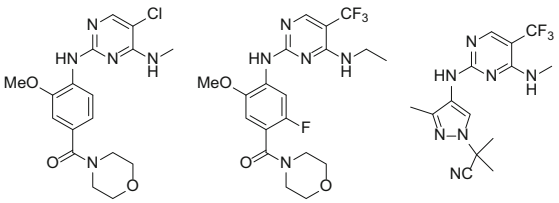
In 2011, the first LRRK2-optimized kinase inhibitor LRRK2-IN-1, **36**, was reported (derived from **3**) [143]. Due to its high potency against both wild-type and mutant LRRK2 (IC_{50} values of 13 and 6 nM, respectively, measured in cell-free enzymatic assays), and its improved selectivity across a range of kinases, it was clearly a vast improvement on previous compounds and was initially employed throughout the field as a selective LRRK2 inhibitor. While **36** is potent against both LRRK2 WT and G2019S mutant enzymes, it has been demonstrated to display moderate potency in a whole cell assay, 200–600 nM [40, 146]. Further, high doses are required to observe any in vivo effects due to its low permeability and high levels of plasma protein binding. Additionally, no CNS effects were observed as **36** had low brain availability (unbound C_{brain}/unbound C_{plasma}). Although **36** demonstrated improved selectivity in comparison to previous LRRK2 inhibitors, it has some significant off-target potency, most notably displaying equipotent activity against ERK5 (BMK1, MAPK7), DCAMKL, PLK1, and PLK4. This off-target activity confounded some of the biological assays in which the effects of **36** are attributed to LRRK2, in particular when assessing effects on neurite outgrowth and inflammation end-points [40]; consequently, use of **36** has diminished.

4.2.3 Second-Generation LRRK2-Focused Kinase Inhibitors

As intensive research into the function and dysfunction of LRRK2 and its mutants continued, a number of other tool compounds and inhibitor series have been published from both academic and industrial laboratories. The most potent of these derived from **2**, in both enzyme (5–7 nM in WT and G2019S, respectively)

and cell-based assays (attenuation of G2019S and R1441C-induced neuronal injury and death in a concentration-dependent manner with an EC_{50} of <10 nM), was CZC-54252 (**37**) reported in 2011 [156]. Unfortunately, **37** had disappointing selectivity across the kinome. Although CZC-54252 may prove a useful tool for cell-based assays, its poor CNS MPO score (2.12) suggested it was outside of CNS space which will limit its application in in vivo models (confirmed with a reported brain availability of 0.05).

The diaminopyrimidine core has been extensively examined by several groups. In addition to compounds derived from **2** and **3** (vide infra), variations including analogues from **6**, **13**, **16**, **21**, and **31** have been reported. An early example is exemplified by **38** (CNS MPO = 3.97), but more advanced analogues successfully balanced good physicochemical properties with high degrees of LRRK2 selectivity [157, 158]. Focusing on diaminopyrimidine HTS hits, due to high ligand efficiency and reasonable PK properties, optimization of the hits using a LRRK2 homology model to identify both key interactions for LRRK2 potency and sites that may offer enhanced kinase selectivity yielded **39** (MPO = 5.37) with improved kinase selectivity over their initial hit, suitable in vivo exposures, and similar cellular activity (IC_{50} = 389 nM) to **36**. While disclosed in a patent the previous year (WO2012062783; see Fig. 5, above), **39** was subsequently described as HG-10-102-01 and reported to exhibit in vivo mouse brain activity [132].



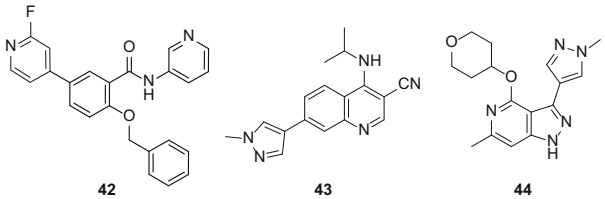
39 40 41

Having identified TTK (MPS1) as the major off-target interaction of this series, and significant safety concern, continued optimization by Genentech scientists, using structure-based drug design initially arrived at **40** (CNS MPO = 4.60), a compound having excellent selectivity for LRRK2 [145]. The in vivo activity of this compound and related analogues was shown using dephosphorylation of S1292 as a measure of LRRK2 kinase activity. Although **40** is an excellent tool compound for probing the effect of LRRK2 inhibition in vivo, it has some major liabilities, thus further optimization was needed for progression towards a potential clinical candidate. As the diaminopyrimidine forms the key hinge interactions with LRRK2, optimization was focused around the side chain. The aniline motif, which could lead to idiosyncratic toxicity findings, was replaced with an aminopyrazole, and side chains to enhance aqueous solubility were incorporated [133–135]. This optimization lead to GNE-0877, **41** (CNS MPO = 5.45), which has been progressed to preclinical safety studies (see Sect. 2.6, above).

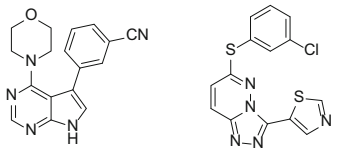
4.2.4 Third-Generation LRRK2-Focused Kinase Inhibitors

Moving away from diaminopyrimidines, several other chemotypes have been reported. A series defined by **5** and **18**, while likely to be ATP-competitive, do not contain a common hinge-binding motif [135]. This attribute may be responsible for the excellent kinase selectivity. GSK2578215A, **42**, is a representative analogue from this series and showed reasonable potency ($IC_{50} = 47$ nM). However, it has poor rodent PK, with a relatively low oral bioavailability ($\%F = 12$) and half life of 1.1 h [159]. The lack of LRRK2 inhibition observed in the brain (potentially predicted by its CNS MPO score of 3.82), in comparison to kidney and spleen, suggested low levels of unbound compound in brain potentially limiting the use of this series for in vivo models.

Quinoline derivatives, **26** and **28**, are known single-point hinge binders and have been found to be good LRRK2 kinase inhibitors [139, 147]. Although the cinnoline variants [141] were potent LRRK2 inhibitors (wt $IC_{50} = 7$ nM), they were found to be promiscuous in a small kinase panel. By focusing the core to quinoline [138], e.g., **43**, kinase selectivity was significantly improved and in vivo activity was demonstrated (CNS MPO = 5.05).



Pyrazolopyri(mi)dines, **15**, **19**, **23**–**25**, and **30**, have been well represented in the patent literature. For analogues, such as **44** (CNS MPO = 5.83), potency is comparable to **36** with overall good ADME [159]. Alternate 6,5-fused ring systems, **14**, **20**, and **22**, have been reported; however, the pyrrolopyrimidine **33** appears to have the best alignment of properties. Specifically, **45** (CNS MPO = 5.83) is one of the most active LRRK2 inhibitors in vivo (brain free drug wt $IC_{50} = 15$ nM) along with high kinase selectivity [160].



One final variation of the 6,5-fused ring system is illustrated by **32**. These compounds are also postulated to be single-point hinge binders, interacting through one of the triazole nitrogens. A representative compound from this series, **46** (CNS MPO = 4.88), displayed excellent in vitro potency (wt $IC_{50} = 31$ nM; G2019S $IC_{50} = 8$ nM) against LRRK2, but this did not translate to cellular assays resulting in a 100-fold right

shift [137]. As witnessed with other scaffolds (vide supra), compounds from this chemotype would make good tool compounds for probing LRRK2 in vitro biology.

4.2.5 Pharmacological Profiles for Key LRRK2-Focused Kinase Inhibitors

As the relatively young LRRK2 field of research has evolved, there have been frequent advances in the biochemical reagents available, as the biology has developed. In part, this has been facilitated by the improvement of the chemical tools (vide supra). However, the literature data documenting the pharmacological profiles of the tool compounds is often not consistent in terms of the assays or conditions used in reporting their potencies. Thus, Table 3 presents a head-to-head comparison of several of the top compounds, from our internal assessments, with the intention of providing LRRK2 researchers with additional data to better enable selecting of the best compound(s) for their studies.

The LRRK2 inhibitors described below have been characterized in a number of in vitro and in vivo models. Activity against LRRK2 wild-type and G2019S mutant was assayed using truncated enzyme from Invitrogen in a LanthaScreen format at 50 μM and 1 mM ATP concentrations [58]. The whole cell potency is measured in HEK293 cells transfected with WT LRRK2, using the antibody against pS935 [64]. The RRCK (Ralph Russ Canine Kidney) assay is a measure of passive permeability, using a canine kidney cell line which expresses low levels of efflux transporters [161]. MDR1 is a measure of efflux by human P-gp transporter and is used to assess the likelihood of brain impairment [162]. HLM and RLM are an assessment of metabolism in human and rat liver microsomes, respectively. Several safety assays are performed; "dof" is a measure of the ability of compounds to displace [^{3}H]-labeled dofetilide from the hERG K^+-channel, important for cardiac safety in clinical candidates [163]. General cytotoxicity is measured using the THLE assay [164]. Effects on cell health are followed up with a panel of assays performed in HepG2 cells, measuring mitochondrial function [165]. Kinome selectivity is reported from a Dundee Panel [166] using recombinant enzymes at the ATP K_M or ActivX data [167], which used lysed PBMC cells to assay selectivity in a more native system.

Table 3 Pharmacological profile of key LRRK2 kinase inhibitors

	Assay	36	37	42	40	45
LRRK2 (in vitro)	1 mM ATP LRRK2 IC_{50} (nM)	68	22	150	37	8
	1 mM ATP LRRK2 (G2019S) IC_{50} (nM)	25	13	247	23	34
	pS935 WCA LRRK2 IC_{50} (nM)	233	4.95	992	194	53
	ActivX huPBMC LRRK2 IC_{50} (nM)	> 300 [142]	15	400	nt	15
Physico-chem	LE	0.23	0.31	0.31	0.33	0.48
	LipE	5	4.1	2.5	3.5	5.5
	CNS MPO	4.52	2.12	3.82	4.6	5.83
ADME	RRCK AB (x 10^{-6} cm/sec)	3.88	14.7	2.87	12.7	27.2
	MDR1 ER	22	3.67	2.24	1.07	1.02
	HLM $CL_{IA,S}$ (mL/min/kg)	22	17	< 8	< 8	36
	RLM $CL_{IA,S}$ (mL/min/kg)	39	101	nt	nt	89
Safety	Dof IC_{50} (μM)	32	16	> 100	50	44
	HepG2 (24h) Gal IC_{50} (μM)	90	> 300	> 300	103	> 300
	HepG2 (24h) Glu IC_{50} (μM)	103	> 300	> 300	159	> 300
	HepG2 (72h) Glu IC_{50} (μM)	26	112	> 300	42	> 300
	THLE Cv IC_{50} (μM)	18	17	> 300	75	> 223
Rat BA	C_{bu}/C_{pu} (AUC)	< 0.1	nt	nt	0.5	1
KSS	Dundee Panel 1 μM (hits\total)	2/121	15/131	2/131	20/140	7/159[a]
LRRK2 (*in vivo*)	Mouse brain free drug pS935 IC_{50} (nM)	7,798[b]	nt	nt	75	<20

Colors represent a stop-light analysis of the data based on Pfizer cut-offs (green low; yellow moderate; red high)
[a] ActivX data at 1 μM
[b] Kidney data. *nt*, not tested. See text for description of assays

5 Summary, Conclusions, and Outlook

The answers to some key questions are important for a more thorough understanding of LRRK2 structure, function, and the development of inhibitors, including how the Roc domain regulates the kinase domain, what the role of the COR domain is, and how the pathogenic mutations alter the function of the LRRK2 protein. X-ray crystal structures of the LRRK2 enzymatic domains, including both WT and the pathogenic mutations, could contribute to an understanding of these questions and would be a key accomplishment to help drive inhibitor design.

The strength of research in the field of LRRK2, both academic and industrial, has generated a wealth of chemical tools for the study of LRRK2. Where the initially reported compounds had activity across multiple kinase targets, new inhibitors have a high degree of kinome selectivity along with improved physicochemical properties, providing opportunities to study inhibition of LRRK2 kinase activity

from the isolated enzyme through to in vivo assays. Ultimately, this collection of inhibitors should allow researchers the flexibility to design experiments to probe LRRK2 function, knowing that they have the right tools for the job.

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