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Innovative treatment targeting gangliosides aimed at blocking the formation of neurotoxic α -synuclein oligomers in Parkinson's disease.

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Abstract

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Parkinson's disease (PD) is a major neurodegenerative disorder which exhibits many of the characteristics of a pandemic. Current therapeutic strategies are centered on the dopaminergic system, with limited efficacy, so that a treatment that has a direct impact on the underlying disease pathogenesis is urgently needed. Although α -synuclein is a privileged target for such therapies, this protein has been in the past wrongly considered as exclusively intracellular, so that the impact of paracrine neurotoxicity mechanisms in PD have been largely ignored. In this article we review the data showing that lipid rafts act as plasma membrane machineries for the formation of α -synuclein pore-like oligomers which trigger an increase of intracellular Ca^{2+} . This Ca^{2+} influx is responsible for a self-sustained cascade of neurotoxic events, including mitochondrial oxidative stress, tau phosphorylation, Ca^{2+} release from the endoplasmic reticulum, Lewy body formation, and extracellular release of α -synuclein in exosomes. The first step of this cascade is the binding of α -synuclein to lipid raft gangliosides, suggesting that PD should be considered as a ganglioside membrane disorder lipidopathy. Accordingly, blocking α -synuclein-ganglioside interactions should annihilate the whole neurotoxic cascade and stop disease progression. A pipeline of anti-oligomer molecules is under development, among which an in-silico designed synthetic peptide AmyP53 which is the first drug targeting gangliosides and thus able to prevent the formation of α -synuclein oligomers and all downstream neurotoxicity. These new therapeutic avenues challenge the current symptomatic approaches by finally targeting the root cause of PD through a long-awaited paradigm shift.

Parkinson's disease: a pandemic

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Neurological disorders are currently the leading source of disability around the world, and the fastest growing of these neurodegenerations is Parkinson's disease (PD), which, although noninfectious, exhibits many of the characteristics of a pandemic [1]. The risk of developing PD increases with age: the disease affects approximately 0.5% to 1% of people aged between 65 to 69, rising to 1% to 3% among persons 80 years of age and older, and it is about 1.4 times more frequent in men than women [2].

PD is characterized by a broad range of symptoms including postural instability, rigidity, tremor, gait and motor impairment, cognitive deficits and depression [3]. Accordingly, the disease has a severely negative impact on the quality of life of patients and their caregivers. The global burden of PD has been evaluated by the disability-adjusted life year (DALY) index, which is an aggregate measure of years of life lost due to premature death, with 1 DALY equating to 1 healthy year of life lost [1].

The current therapeutic proposal for PD patients is centered on the dopaminergic system, which is quite logical given the typical loss of dopaminergic neurons in this disease [3]. The shortcomings of such pharmacotherapies (dopamine agonists and Levodopa) include : i) their diminished effectiveness over time, ii) the development of medication-related complications such as motor fluctuations and dyskinesia, iii) well-known side effects such as impulse control disorders, sleepiness or sudden-onset sleep, and dopamine dysregulation syndrome (addiction problems), iv) the absence of impact on stopping or even slowing down disease progression [3]. Hence, targeting the dopaminergic system does no longer seem to be a suitable approach. Clearly there is an urgent need for a treatment that has a direct impact on the underlying disease pathogenesis. The development of such treatment requires a good comprehension of the molecular mechanisms that trigger PD.

PD is a membrane disorder involving gangliosides

PD is characterized by a dysfunction of α -synuclein (α -syn), a membrane-interacting protein [4] involved in several physiological functions including synaptic vesicle recycling, dopamine release, exocytosis, endocytosis, vesicular trafficking and lipid metabolism and transport [5-8]. Consistently, the abnormal behavior of α -syn is the common cause of several forms of PD and related disorders such as dementia with Lewy bodies (DLB) and multiple systems atrophy (MSA) [9]. In all these diseases, α -syn forms various oligomeric protein aggregates that induce important perturbations of brain cell functions and eventually cell death [10]. Consistently, α -syn oligomers are detected in postmortem brains of patients with PD [11] and in blood plasma [12] and cerebrospinal fluid (CSF) of PD patients [13]. The presence of α -syn oligomers in extracellular body fluids is consistent with the extracellular propagation of these assemblies in brain tissues.

As a matter of fact, α -syn has long been considered as an intracellular protein because it lacks a signal peptide sequence [14]. However, this notion has been challenged by a number of in vitro and in vivo studies that converged to incriminate a paracrine effect of α -syn in PD [15, 16]. Using a highly sensitive ELISA in conjugation with in vivo microdialysis, Emmanouilidou et al. [17] detected α -syn in the interstitial fluid of human brain patients. Danzer et al. [18] have demonstrated the presence and uptake of secreted α -syn oligomers by neurons and their detrimental effect on neuronal survival. It has also been shown that α -syn is transported in the brain through the blood-brain-barrier as a free protein [19]. Moreover, extravasation of red blood cells might also contribute to disseminate α -syn in the striatum of PD patients [20]. Finally, soluble α -syn and α -syn oligomers may also be released by damaged neurons, which may intoxicate neighbor cells [21]. Taken together, these data

1 suggest that α -syn is indeed physiologically secreted and pathologically released in vivo by
2 brain neurons and that the interstitial pool of the protein plays a key role in PD pathology
3 [22]. Thus, it is time to consider extracellular α -syn as a privileged target for PD treatment,
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5 providing that the molecular mechanisms linking extracellular α -syn and PD are unraveled.
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7 In this article, we review the literature incriminating plasma membrane gangliosides as
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9 critical partners that control the neurotoxicity of α -syn oligomers in PD and related
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11 synucleinopathies.
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16 α -syn oligomers affect a broad range of cellular processes, including alterations of
17 calcium signaling pathways due to plasma membrane permeabilization [23]. The
18 aggregation of α -syn has also been reported to induce major dysfunction of the ubiquitin-
19 proteasome system (UPS) and autophagy-lysosomal pathway (ALP), leading to impaired
20 protein clearance resulting in its intracellular accumulation [24]. This self-sustained
21 mechanism is triggered by changes in ganglioside and cholesterol content of lipid rafts
22 which catalyze the formation of Ca^{2+} -permeable neurotoxic oligomers in the plasma
23 membrane of brain cells [25-27] (**Figure 1**). Thus, PD and other synucleinopathies can be
24 defined as both proteinopathies and lipidopathies, with a main protein culprit, α -syn and
25 membrane lipid partners, especially lipid rafts that can be considered as the actual triggers
26 [28-31]. Most importantly, the composition of lipid rafts, α -syn levels and thus the lipid/ α -
27 syn ratio are key parameters that control the balance between the physiological and
28 pathological functions of α -syn [32] (**Figure 1**). In line with this notion, it has been shown
29 that PD grey matter exhibited distinct patterns of raft lipids compared to age-matched
30 control subjects [33]. From a mechanistic point of view, lipid rafts may be considered as
31 integrated molecular machines that concentrate α -syn proteins and promote their
32 oligomerization in Ca^{2+} permeable amyloid pores [27, 34-39]. The increase in intracellular
33 Ca^{2+} triggered by α -syn oligomers induces a cascade of neurotoxic events [40-42] including
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1 (according to the type of synucleopathy) mitochondrial oxidative stress [43, 44], tau
2 phosphorylation and dysfunction [45, 46], Lewy body formation [42], and secretion of
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(according to the type of synucleopathy) mitochondrial oxidative stress [43, 44], tau phosphorylation and dysfunction [45, 46], Lewy body formation [42], and secretion of exosome-associated α -syn [47, 48] which ensures the extracellular propagation of α -syn in the brain via a prion-like mechanism [49, 50]. The changes in Ca^{2+} homeostasis [51] and the mitochondrial oxidative stress [44] are further amplified by intracellular vesicular α -syn oligomers localized at the endoplasmic reticulum-mitochondrial interface [52] and by the perforation of the mitochondrial membrane by oligomeric pores of α -syn [53]. Moreover, intracellular Ca^{2+} contributes to maintain this chain reaction by stimulating α -syn oligomerization and aggregation [30, 54]. All these events induce neural dysfunction, neuroinflammation, neurodegeneration and eventually cell death [24]. The interaction of α -syn oligomers with the plasma membrane of brain cells is influenced by the lipid composition which is a key modulator of the downstream cellular toxicity [32, 55-57].

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The interaction of α -syn with lipid rafts is facilitated by the strong electropositive surface potential of the N-terminal region of the protein [58] which is attracted by the electronegative charges of gangliosides (**Figure 2**), which account for 80% of all brain glycans [59]. Lipid rafts of adult human brain neurons display 4 principal types of gangliosides: GM1, GD1a, GD1b, and GT1b [57, 60]. Astrocytes, which are also involved in PD [61, 62], express chiefly GM3 [63]. The link between PD and ganglioside levels [64] is supported by quantitative analysis of ganglioside content in the substantia nigra of PD patients compared to healthy subjects: decrease of GM1, GD1a, GD1b and GT1b [65, 66] and increase of GM3 in the cerebrospinal fluid (CSF) [66]. These data are in line with a similar shift from complex to simple gangliosides in aging brains [67]. Interestingly, physicochemical studies revealed that α -syn can bind to several brain glycosphingolipids in the following order: GM3 > Gb3 > GalCer > GM1 > sulfatide > LacCer > GM4 > GM2 >

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asialo-GM1 > GD3, indicating a marked preference for GSLs with one, three, or five sugar units, such as GM3 and GM1 [68]. Several reports confirmed the capacity of wild-type and mutant α -syn to interact with lipid rafts and gangliosides [68-71]. A ganglioside binding domain (GBD) was identified in region 34-45 of α -syn by peptide mapping analysis [68]. The amino acid sequence of this motif (KEGVLYVGSKTK) fulfills the consensus criteria of classical GBD found in other ganglioside binding proteins: (K/R)-X₁₋₅-(Y/F/W)-X₁₋₅-(K/R) [68, 72, 73]. The organization of amino acid residues in α -syn₃₄₋₄₅ is particularly interesting. The electric net charge of the GBD at pH7 is +3, and the surface potential is highly positive all over the domain, except for a membrane-repulsive electronegative spot corresponding to the acidic E35 residue (**Figure 2A**). The combination of a strong electropositive surface with an electronegative spot determines a functional orientation of the GBD with respect to the membrane surface. Consequently, the 34-45 region of α -syn is efficiently attracted by the electronegative field of brain lipid rafts. This region is a turn which links two α -helix domains when α -syn interacts with membrane lipids [55]. The C-terminal part of α -syn is a highly acidic disordered domain with a strong electronegative surface. This domain has been characterized as a Ca²⁺ sensor [74].

As discussed above, the surface potential of α -syn is important for determining which face of the protein interacts with lipid raft gangliosides. On this criterion, a typical orientation of α -syn bound to lipid rafts is shown in **Figure 2**. The electropositive GBD (amino acids 34-45) looks like the upper jaw of a snake with the hooks biting the cell surface (**Figure 2A**). The peculiarity of the α -syn-ganglioside interaction is that the GBD finds its room between the extracellular glycone parts of two vicinal gangliosides in a typical chalice-like organization [73]. Once bound to its ganglioside receptors, the central tyrosine residue Y39 makes its way through the outer leaflet of the plasma membrane, separating the ganglioside dimer in two monomers (**Figure 2B**). As a consequence, the 34-

1 45 loop is inserted deeply in the plasma membrane, until it emerges in the cytosol [72]
2 (Figure 2C). At this stage, the 69-78 region of α -syn, which has been characterized as a
3 functional cholesterol binding domain (CBD) is available for an interaction with cholesterol
4 in the outer leaflet [75]. The α -syn oligomerization process leading to the formation of a
5 Ca^{2+} permeable oligomeric channel (amyloid pore) is controlled by membrane cholesterol
6 [25, 76, 77] which forms an annular interface between the pore and surrounding membrane
7 lipids (Figure 2C).
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10 In this process, gangliosides play a critical role in the initial binding of α -syn (receptor
11 function) but also in the structuration of membrane-bound α -syn (chaperone function). Thus,
12 blocking this critical step by targeting those gangliosides involved in oligomer formation
13 will prevent all downstream neurotoxic events caused by intracellular Ca^{2+} influx. The
14 difficulty of this strategy is to find molecules able to target the exact pool of gangliosides
15 recognized by α -syn without interfering with gangliosides associated with physiologically
16 important membrane proteins [57]. The difference between these two categories of
17 gangliosides is essentially topologic. Gangliosides that control receptor function are masked
18 by the protein with which they interact and are thus inaccessible to extracellular proteins
19 such as α -syn [57]. The pool of gangliosides recognized by α -syn not only has to be
20 accessible but should also have the capability to form functional chalice-like dimers [73,
21 78]. We anticipate that these gangliosides are necessarily located at the periphery of lipid
22 rafts, where their glycone part has a maximal degree of freedom allowing the recruitment of
23 two adjacent gangliosides by extracellular α -syn.
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51 **Ganglioside-centered therapies for PD**

52 Besides α -syn, other amyloid proteins associated with neurodegenerative diseases use
53 plasma membrane gangliosides as primary binding sites during the course of oligomer
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1 formation [79]. Indeed, α -syn and Alzheimer's β -amyloid peptide ($A\beta$) share a common
2 structural GBD with a central tyrosine residue flanked by two basic amino acids [73]. This
3 unique situation allowed us to decipher the biological code controlling the interaction of
4 these amyloid proteins with cell surface gangliosides [73]. A molecular modeling strategy
5 based on this study was then developed to design a synthetic peptide (now referred to as
6 AmyP53) with broad ganglioside recognition properties (**Figure 3**). At nanomolar
7 concentrations, AmyP53 efficiently prevents the formation of α -syn and $A\beta$ neurotoxic
8 oligomers by competition for ganglioside binding, including GM1 (**Figure 3A**) and GM3
9 (**Figure 3B**) [25, 26]. Most importantly, AmyP53 is able to prevent the formation of α -syn
10 pores formed by oligomers of wild-type and mutant forms including A30P, E46K and A53T
11 that are associated with inherited forms of PD [26]. This activity of AmyP53 is particularly
12 promising since α -syn mutations A30P and A53T were shown to promote an increased
13 formation of neurotoxic oligomers [80], which was suggested to be the pathophysiological
14 mechanism of early onset PD. Hence, AmyP53 is a potential therapy for any case of
15 synucleinopathies including the most aggressive sporadic and genetic forms of PD, DLB and
16 MSA. Moreover, there is now substantial evidence of α -syn pathology, including LBD and
17 Lewy neurites (LN) in about 50% of AD brains [81-84]. Overall, it is now known that pure
18 neurodegenerative pathologies represent a minority of cases, the rule being mixed
19 proteinopathies shared by several neurodegenerative diseases [85]. From a therapeutic
20 perspective, this situation underscores the difficulty to treat patients with neurodegenerative
21 disorders with a single drug [86]. In this respect, the dual activity of AmyP53 against the
22 formation of both α -syn and $A\beta$ oligomers raises the remarkable possibility of a combination
23 therapy - with one molecule - for two distinct neurodegenerative diseases (PD and AD).
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Another therapeutic approach also based on gangliosides is to treat PD patients with intravenous injections of GM1 sodium salt solutions [87]. The rationale is that GM1 has general neurotrophic and neuroprotective effects [88], potentially through modulation of lipid raft/structure function [87]. Indeed, several PD-associated proteins such as LRRK2, parkin and PINK1 co-localize with GM1 in lipid rafts [89, 90]. GM1 has also been shown to induce a functional improvement of dopaminergic neural functions [91]. Finally, a deficiency of ganglioside GM1 has been shown to correlate with PD [92]. Extracellular GM1 may also bind to α -syn exosomes and inhibit both the prion-like propagation of α -syn and the formation of Ca^{2+} permeable oligomeric pores in brain cells. In this case, GM1 might be considered as a disease-modifying therapy for PD able to prevent α -syn aggregation and promote its clearance [87, 91, 93].

The oligosaccharide derived from the glycone part of GM1 (oligo-GM1) is also being considered as a surrogate for ganglioside-based therapy of PD [94]. In this case, the rationale is to compensate the loss of neuronal regulating functions associated with decreased GM1 levels by providing the portion of the ganglioside that is neuroprotective instead of the whole molecule. Interestingly, oligo-GM1 has also been shown to regulate mitochondrial functions [95] and to cross the blood brain barrier more efficiently than GM1 [96].

α -syn Oligomer-centered therapies for PD

Alternatively, one could try to block the formation or the Ca^{2+} dependent neurotoxic activity of oligomers in PD by targeting these oligomers with small molecules [97-99] or antibodies [100]. Trodusquemine (**Figure 4**) is an aminosterol derived from squalamine that has been shown to displace various neurotoxic oligomers (including α -syn) from the plasma

1 membrane of neural cells [99]. NPT-200-11 (**Figure 4**) was designed from structure-based
2 molecular modeling studies aimed at identifying the regions of α -syn that are critical for
3 oligomer formation [101]. Anle138b (**Figure 4**) is an anti-aggregation drug with a broad
4 neutralization effect of amyloid pores formed by various amyloid proteins, including A β
5 [101]. Both drugs showed interesting anti-PD effects in animal models of PD, yet at
6 relatively high dosage (5mg/kg body weight daily for NPT-200-11, 5mg per mice two times
7 per day for Anle138b) [97, 98]. However, these results should be taken with caution, since
8 in the past, promising drugs based on work in rodent models failed in clinical trials [102-
9 104]. While many animal models of PD and AD have been created, no single model, either
10 based on pesticides or genetically induced, has been able to recreate all the key features of
11 these neurodegenerative diseases [102, 105, 106]. Several α -syn transgenic mice have been
12 developed, but they fail to reproduce the combination of progressive and specific dopamine
13 cell loss, and the main neurological manifestations of PD, including movement disorders
14 and cognitive decline [106-110]. As a matter of fact, the classic scheme [in silico => in vitro
15 => in vivo => clinical trials] has not been successfully applied for the neurodegenerative
16 domain, emphasizing the difficulty of translational neurosciences in this case. The problem
17 is that PD is a human specific disease which has not been observed in any other animal
18 species [111].

19 It should be noted that some rodent models of PD are based on an impairment of
20 ganglioside expression. For instance, the B4GALNT1 mouse model [112] partially
21 (heterozygous) or totally (homozygous) lacks all complex gangliosides (including GM1) due
22 to deficiency of β -1,4 N-acetylgalactosaminyltransferase 1, the enzyme that catalyzes the
23 addition of N-acetylgalactosamine to GM3 in the ganglioside biosynthetic pathway [113]. In
24 this case, the formation of neurotoxic oligomeric pores might still be possible through α -syn
25 binding to GM3, which has a higher affinity for the protein than GM1 [68]. In this respect,
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1 the accumulation of α -syn aggregates and the PD-like symptoms in the B4GALNT1 model
2 is not in contradiction with the crucial role of gangliosides for the neurotoxic insertion of α -
3 syn into the plasma membrane. Then the protection conferred by oligo-GM1 in this model
4 [94] may be due to the broad neuroprotective effect of this oligosaccharide, i.e. a
5 symptomatic instead of a disease-modifying effect.
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11 In a non-genetic model (mice treated with MPTP), an intraventricular administration of
12 sialidase resulted in a local increase of GM1 in the plasma membrane (enzymatic conversion
13 of polysialogangliosides into GM1), which appeared to be partially protective against PD-
14 like symptoms [114]. Again this effect is probably not directly linked to α -syn, as according
15 to the authors of this study, increasing GM1 levels in the brain of PD patients may result in a
16 neuroprotective effect on the damaged nigrostriatal dopamine system, as also suggested by
17 other studies [88]. Finally, Brekk et al. [115] reported that the upregulation of β -
18 hexosaminidase in the striatum of transgenic rats led to a modest local increase in GM3
19 levels that appeared to be protective in this human α -syn expressing rodent model. Given the
20 complexity of this virus-induced animal model, it is difficult to determine whether GM3 is
21 fully responsible for the observed effects.
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39 Nevertheless, the pipeline of anti-oligomer therapeutic compounds (**Figure 4**) is now
40 under development with several molecules that target either gangliosides (AmyP53) or
41 neurotoxic oligomers [25, 26, 97-99, 101].
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48 **Other therapies**

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50 Several disease-modifying strategies have recently been tested in clinical trials, but with
51 unsuccessful results due to the extent and severity of PD symptoms. Such protocols include
52 anti-oxidant agents, dopamine agonists, monoamine oxidase type B (MAOB) inhibitors,
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1 trophic factors, anti-inflammatory, and gene therapy based approaches aimed at restoring
2 striatal dopamine production [3].
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7 Deep brain stimulation (DBS) may transiently improve some motor symptoms, but has
8 a number of drawbacks and undesirable side effects such as dyskinesias, hypophonia, eyelid
9 apraxia and decreased memory, in addition with the adverse events related to general
10 neurological and surgical complications (cognitive decline, depression, peri-operative
11 confusion, hemorrhage and CSF leak) [116, 117]. Moreover, DBS is highly invasive without
12 a real appreciation of detrimental consequences on brain functional integrity, it cannot be
13 generalized to all patients, it remains not readily accessible for financial reasons [118].
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24 Monoclonal antibodies against aggregated forms of α -syn are currently under trial
25 [100]. However, brain immunotherapies are associated with numerous side effects, including
26 severe brain inflammatory processes (Amyloid-Related Imaging Abnormalities, ARIA
27 syndrome) [119]. Besides toxicity, there is a specificity issue for such monoclonal
28 antibodies which may bind to monomeric, oligomeric and aggregated forms of α -syn [120].
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39 **Conclusion**

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41 A growing line of evidence suggest that an essential cause of PD is an overdose of Ca^{2+} that
42 enters brain cells through membrane-inserted α -syn oligomers [121]. Intracellular Ca^{2+} then
43 triggers a self-sustained cascade of neurotoxic events, resulting in definitive loss of neurons.
44 Preventing this membrane damage process is of primary importance to develop an efficient
45 therapy for PD [31, 32]. The responsibility of gangliosides in this process places AmyP53 as the
46 first molecule ever able to prevent the formation of any calcium-permeable pores and thus all
47 disease-associated downstream neurotoxicity [121]. By its innovative design as a chimeric α -
48 syn/A β peptide, it is also the first therapeutic candidate that considers neurodegenerative diseases
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1 such as PD and AD as concomitant pathologies involving distinct amyloid proteins in brain
2 patients. By targeting for the first time lipid raft gangliosides, i.e. the machinery of oligomer
3 formation, it acts at an early step of the disease by preventing the oligomerization process. The
4 interplay between gangliosides, neurodegenerative diseases and therapeutic options relies on an
5 up growing scientific literature [31, 122]. Several other strategies targeting α -syn oligomers are
6 now emerging from the research pipeline, which indicates that the paradigm is changing, giving
7 a new hope for curing PD and related synucleinopathies .
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18 **Declaration of interests.**

19 N. Y. and J. F. are co-inventors of the AmyP53 peptide (patent Application EP15709163.8A), currently
20 under development by AmyPore (France). H. C. is President of the Ethics and Scientific Committee of
21 AmyPore. C. D. is member of the Ethics and Scientific Committee of AmyPore.
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Figure legends.

Figure 1. Overview of the role of α -syn oligomers in PD.

Lipid raft composition (gangliosides and cholesterol) and lipid/ α -syn ratio are key parameters whose weight controls the balance between the physiological and pathological functions of α -syn and may promote PD when that balance is upset. Key steps linking α -syn oligomerization, intracellular Ca^{2+} and neural dysfunction are indicated.

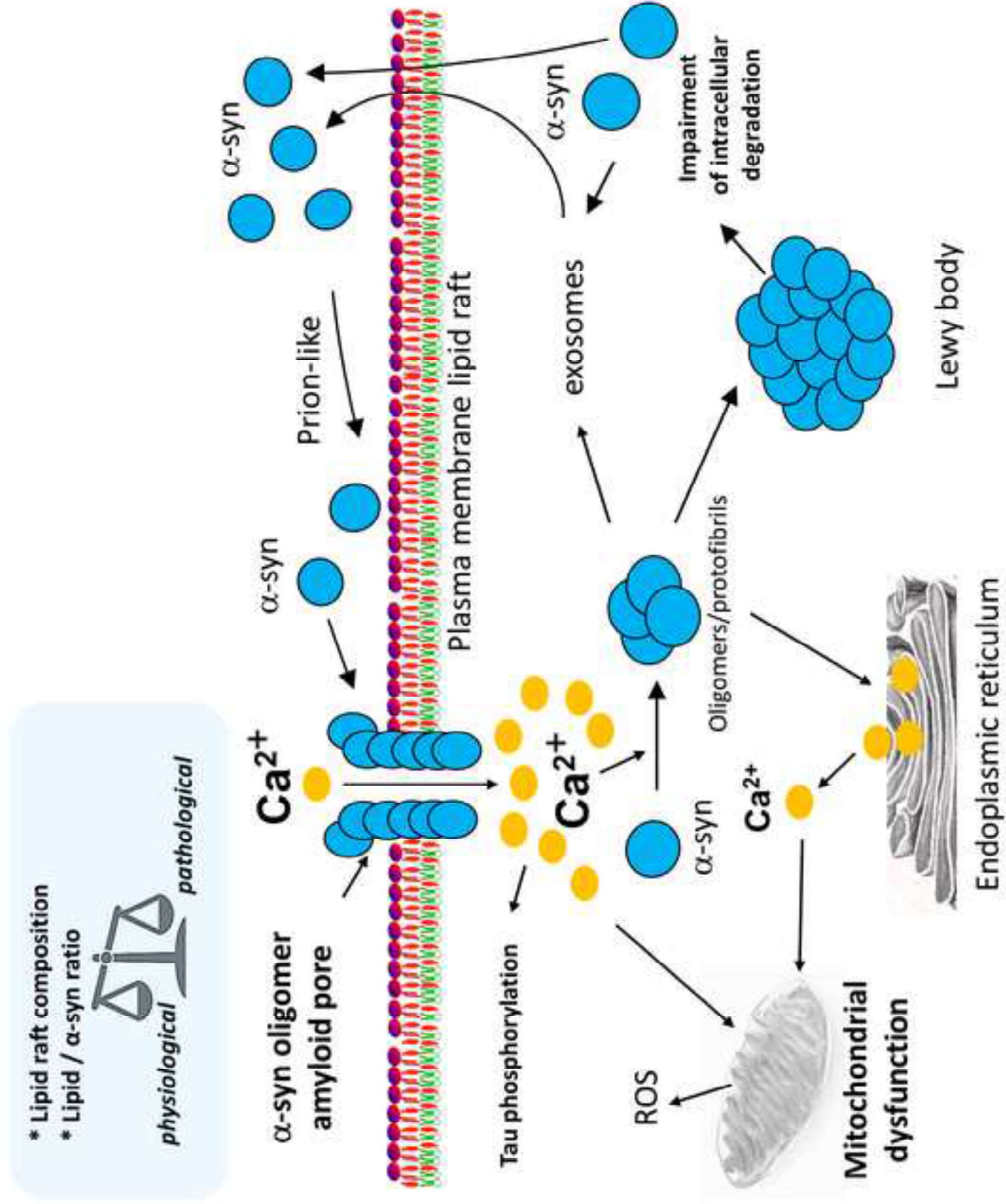
Figure 2. Lipid rafts as a machinery for α -syn oligomer formation.

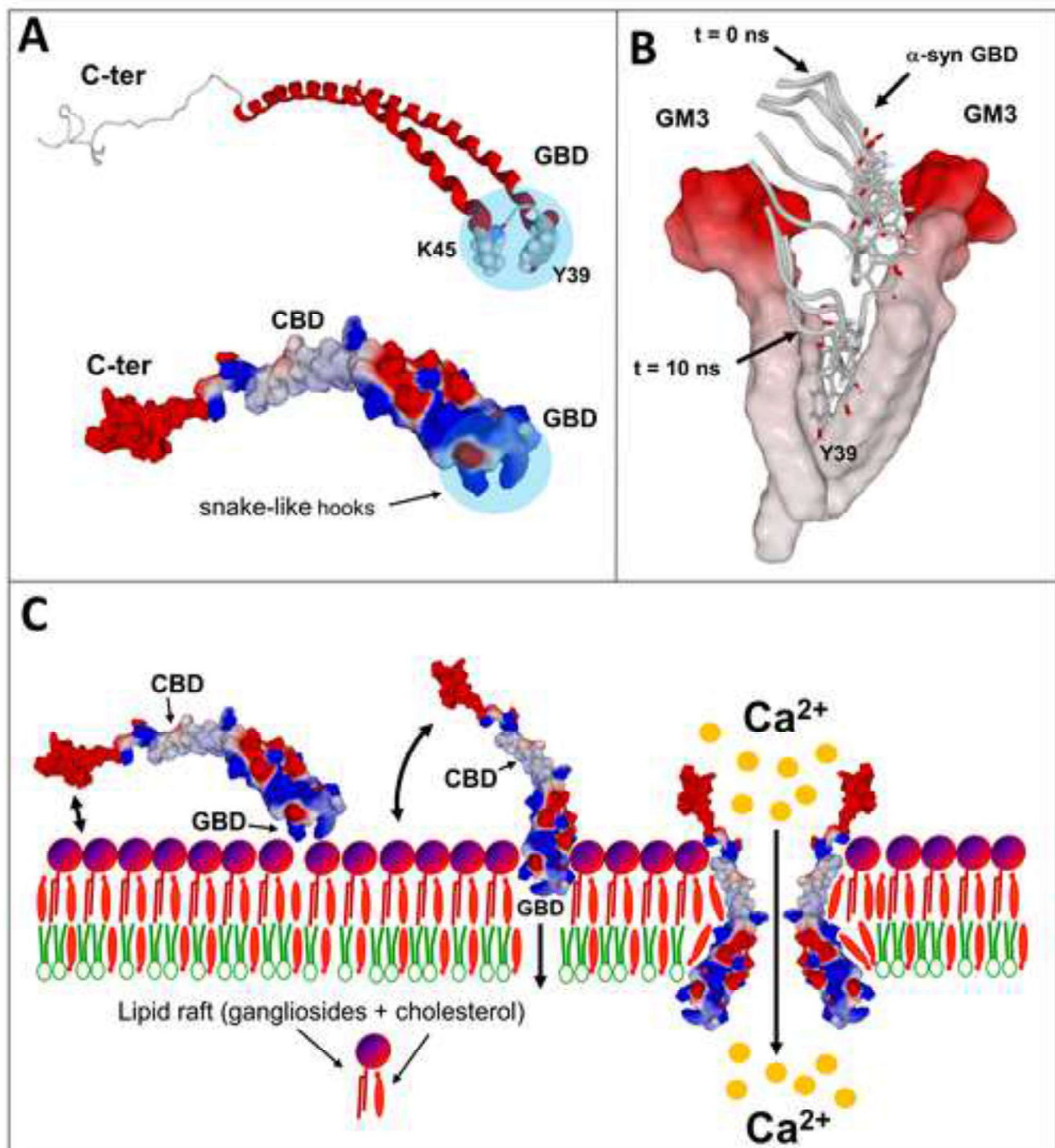
A. Typical helical structure of α -syn bound to the membrane surface (in secondary structure and surface potential representation). The snake-like hooks (residues Y39 and K45) in the ganglioside-binding domain (GBD, blue disk) are highlighted. The electronegative spot due to the negative charge of E35, the cholesterol-binding domain (CBD) and the C-ter domain of α -syn are also indicated. **B.** Molecular dynamics of α -syn GBD insertion driven by the OH group of the aromatic side chain of Y39. **C.** Overview of the molecular mechanism of lipid raft controlled oligomerization of α -syn in Ca^{2+} permeable amyloid pores.

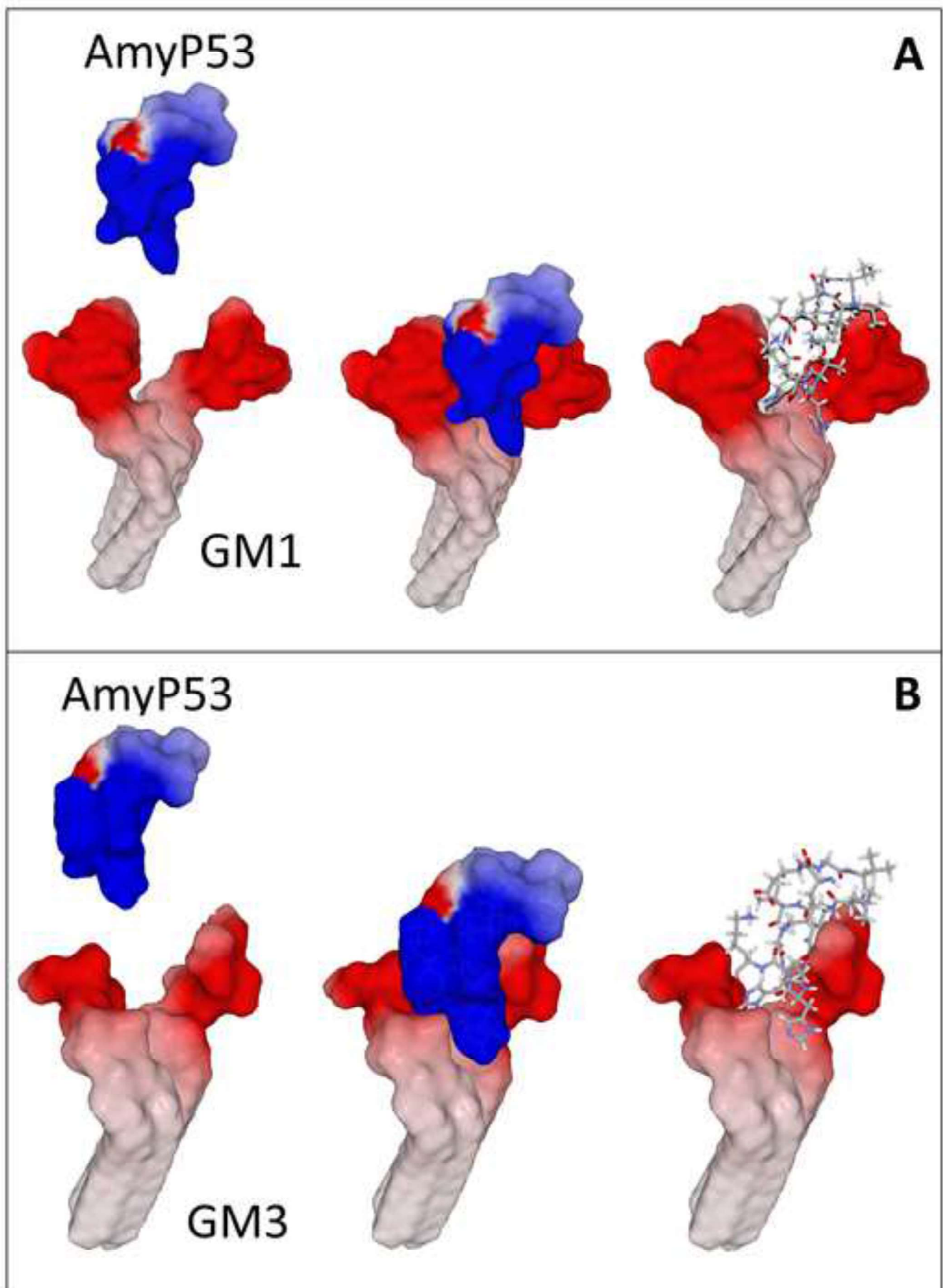
Figure 3. Ganglioside binding properties of AmyP53.

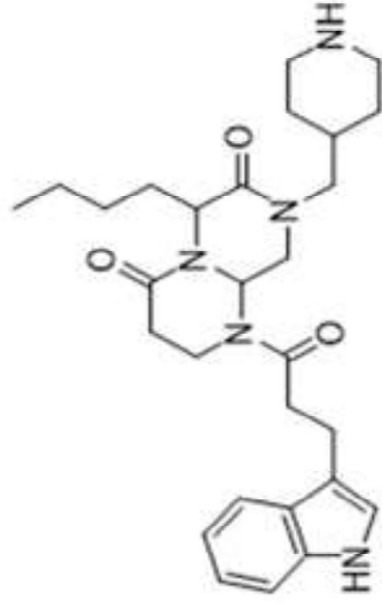
A. Interaction of AmyP53 with GM1. **B.** Interaction of AmyP53 with GM3. In both cases, the models represent the approach of the electropositive surface of AmyP53 to the electronegative glycone part of the ganglioside (left panels), and the formation of the complex in electronegative surface potential rendition (middle panels) or with AmyP53 amino acid residues represented (right panels).

Figure 4. Pipeline of anti-oligomer molecules for PD treatment. For clarity, AmyP53 is represented in a linear conformation. The other molecules are represented as chemical structures.

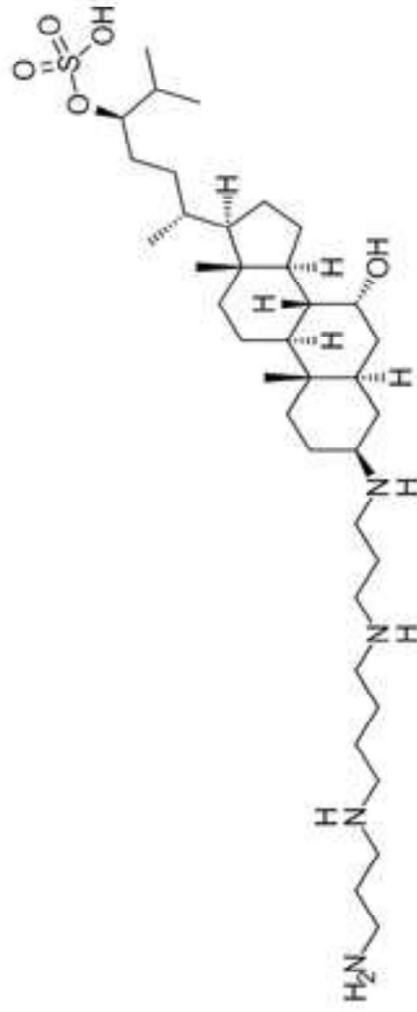




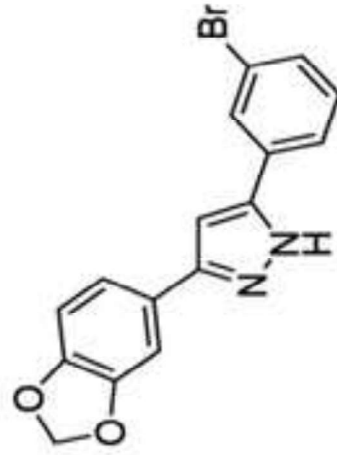




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AmyP53