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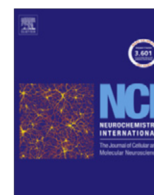

Vitamins K interact with N-terminus α-synuclein and modulate the protein fibrillization *in vitro*. Exploring the interaction between quinones and α-synuclein

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ABSTRACT

In the last decades, a series of compounds, including quinones and polyphenols, has been described as having anti-fibrillogenic action on α-synuclein (α-syn) whose aggregation is associated to the pathogenesis of Parkinson's disease (PD). Most of these molecules act as promiscuous anti-amyloidogenic agents, interacting with the diverse amyloidogenic proteins (mostly unfolded) through non-specific hydrophobic interactions. Herein we investigated the effect of the vitamins K (phylloquinone, menaquinone and menadione), which are 1,4-naphthoquinone (1,4-NQ) derivatives, on α-syn aggregation, comparing them with other anti-fibrillogenic molecules such as quinones, polyphenols and lipophilic vitamins. Vitamins K delayed α-syn fibrillization in substoichiometric concentrations, leading to the formation of short, sheared fibrils and amorphous aggregates, which are less prone to produce leakage of synthetic vesicles. In seeding conditions, menadione and 1,4-NQ significantly inhibited fibrils elongation, which could be explained by their ability to destabilize preformed fibrils of α-syn. Bidimensional NMR experiments indicate that a specific site at the N-terminal α-syn (Gly31/Lys32) is involved in the interaction with vitamins K, which is corroborated by previous studies suggesting that Lys is a key residue in the interaction with quinones. Together, our data suggest that 1,4-NQ, recently showed up by our group as a potential scaffold for designing new monoamine oxidase inhibitors, is also capable to modulate α-syn fibrillization *in vitro*.
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1. Introduction

Parkinson's disease (PD) is the second most common age-related neurodegenerative disease and is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta and dopamine deficiency in the striatum (Hoehn and Yahr, 1967; Lees et al., 2009; Hughes et al., 1992; Burns et al., 1983). Although the etiology of PD is unknown, the neurodegeneration has been suggested to be result of a combination of factors including damages associated with oxidative stress (Jenner, 2003) and the formation of cytotoxic inclusion composed by the protein α-synuclein (α-syn) (Polymeropoulos et al., 1997).

Physiologically, α-syn is believed to exist as a disordered monomer (Fauvet et al., 2012), which aggregates into fibrillar structures that can accumulate as intracellular inclusions and abnormal neuritis (Lewy bodies and Lewy neuritis). However, some studies indicate that α-syn might behave as a helicoidal tetramer that likely undergoes destabilization before protein aggregates into fibrillar assemblies (Bartels et al., 2011). Besides PD, α-syn is involved in some variants of Alzheimer's disease (AD), dementia with lewy bodies and multiple system atrophy (Goedert, 2001; Spillantini et al., 1997; Baba et al., 1998; Wakabayashi et al., 1998). *In vitro* studies show that α-syn mutation A30P, linked to early onset cases of PD (Conway et al., 2000), increases the formation of nonfibrillar oligomers (protofibrils). Interestingly, α-syn protofibrils but not either fibrils or the monomer, cause permeabilization of synthetic vesicles similarly to found with some pore-forming protein toxins (Volles et al., 2001; Lashuel et al., 2002).

In vitro assays have demonstrated that several compounds, including catecholamines, anti-parkinsonian drugs and certain natural products inhibit the fibrillization of α-syn, leading to the formation of non-toxic aggregates (Ono and Yamada, 2006; Ono et al., 2007; Follmer et al., 2007; Braga et al., 2011). However, many of these molecules, notably those highly hydrophobic, behave as promiscuous anti-fibrillogenic agents interacting with both α-syn and amyloid-β peptide (involved in AD) by non-specific hydrophobic interactions. In light of these findings we are looking for

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potential pharmacophoric groups that specifically bind to α-syn monomer leading to the formation of aggregates incapable to promote vesicle permeabilization.

The 1,4-naphthoquinones (1,4-NQ) have been described to exhibit a broad spectrum of activities on important targets associated to neurodegenerative disorders including inhibition of the aggregation of amyloid-β peptide (Bermejo-Bescós et al., 2010), inhibition of Hsp90 (Hadden et al., 2009) and inhibition of monoamine oxidase (MAO) activity (Coelho-Cerqueira et al., 2011). For instance, the 2,3,6-trimethyl-1,4-naphthoquinone, a component of flue-cured tobacco leaves and smoke, is a competitive inhibitor of both MAO-A and MAO-B that exhibits protective properties against MPTP toxicity in mice (Castagnoli et al., 2003). The 1,4-NQ scaffold is usually found in bioactive molecules such as vitamins K, which are characterized by a methylated naphthoquinone ring with an aliphatic chain substituent at the 3-position. Interestingly, menadione (2-methyl-1,4-naphthoquinone; vitamin K3), which acts as a precursor of vitamin K2, has been recently pointed out as a competitive and reversible inhibitor of human MAO as selective for MAO-B as rasagiline (Coelho-Cerqueira et al., 2011). Inhibition of monoamine oxidase activity is one of the most important pharmacological targets in the therapy for PD.

Herein, we investigated the effects of multiple forms of vitamin K [phylloquinone (K1), menaquinone (MK-4) (K2) and menadione (K3)] and 1,4-NQ itself on α-syn fibrillization. Our results suggest that these molecules prevent α-syn fibrillization by interacting with specific residues of α-syn monomer located at N-terminal repeat domain (Gly31/Lys32) as indicated by ^{1}H-^{15}N HSQC NMR data. This interaction results in protein assemblies (amorphous aggregates and small fibrils) with a reduced capacity to produce vesicle leakage compared with the control. Taken together, our data suggest that 1,4-NQ, recently reported by for our group as a potential scaffold for designing new MAO inhibitors, is also an important scaffold to develop drugs targeting α-syn fibrillization.

2. Methods

2.1. Expression and purification of α-syn

The pT7-7-wt plasmid (kindly provided by H.A. Lashuel and D. Foguel) containing wild-type (WT) human α-syn was transfected into BL21(DE3)pLysS *E. coli* and the expression induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG). The expression and purification procedures were carried out as described by Conway et al. (1998) with modifications. Briefly, the expression was induced at absorbance (600 nm) 0.5 with 0.5 mM IPTG for 4 h and then cells were harvested by centrifugation (8000*g*) for 30 min. The pellet was ressuspended in 20 mM Tris–HCl, 5 mM EDTA, 1 mM PMSF pH 8.0 and then cells were disrupted by sonication. The supernatant was dialyzed overnight against 20 mM Tris–HCl, pH 8.0, 1 mM EDTA and then loaded onto a Q-Sepharose® Fast Flow column (GE Biosciences). The fraction eluted with 0.2–0.4 M NaCl was loaded onto a Sephacryl S-200 (GE Biosciences) equilibrated with 20 mM Tris–HCl, pH 7.5, 150 mM NaCl and the fractions containing α-syn were combined and dialyzed against MilliQ water and further lyophilized. Purified α-syn showed a single 18 kDa band in 15% SDS–PAGE.

2.2. Measurement of α-syn fibrillization and fibril-destabilizing activity

Purified α-syn (100 μM) in 20 mM Tris, pH 7.4, 0.02% sodium azide, 150 mM NaCl plus 5 μM Thioflavin-T (Thio-T) was incubated in the presence of 5 to 100 μM of 1,4-NQ, menadione, menaquinone or phylloquinone. DMSO was used as control since all compounds was dissolved in this solvent. The fibrillization was monitored *in situ* in a 96-well plate (Greiner 96-well white plate) at 37 °C in Eppendorf Thermomixer with continuous shaking (350 rpm). Thio-T binding was evaluated by fluorescence intensity by exciting at 446 nm and collecting the emission at 485 nm (slits: 5 nm) in a Cary Eclypse Fluorimeter (Agilent Inc.). The effective concentrations (EC_{50}) were defined as the concentration of drug that inhibits the formation of α-syn fibrils to 50% of the control value (α-syn plus DMSO). Fibril destabilization activity was measured by incubating 20 μM mature α-syn fibrils (in terms of monomer concentration) in 20 mM Tris, pH 7.4, 0.02% azide, 150 mM NaCl and 5 μM Thio-T in a 96-well plate at 37 °C under agitation (350 rpm) in the presence of 5–100 μM 1,4-NQ, menadione, menaquinone or phylloquinone. Fibril disassembling was monitored by Thio-T fluorescence as described above.

2.3. Nucleation-dependent fibrillization of α-syn

Seeds of α-syn were prepared by incubation of 100 μM α-syn in 20 mM Tris, pH 7.4, 0.02% azide, 150 mM NaCl for 8–10 days at 37 °C under agitation (350 rpm) followed by sonication by ultrasound for 60 min. For the fibrillization in seeding conditions, α-syn monomer (40 μm) in PBS buffer plus 5 μM Thio-T were incubated in presence of seeds (5%, w/w) at 37 °C, without shaking. To evaluate the effect of vitamins K and 1,4-NQ, α-syn was preincubated for 30 min with equimolar concentration of these compounds prior the addition of seeds. Equivalent amounts of DMSO were used as control.

2.4. Atomic force microscopy (AFM)

An aliquot of 5 μl, withdrawn of the α-syn solutions (100 μM monomer) under different aggregation conditions, was deposited on freshly cleaved mica. After 5 min incubation at room temperature, it was rinsed with MilliQ water, gently dried under nitrogen flow and used for AFM imaging, as described below. The acquisition of AFM images was done by using a Nanowizard JPK AFM (Berlin, Germany), operating in dynamic mode under ambient conditions, with 46% relative humidity, using AC240 cantilevers (Veeco, USA) with a spring constant of 2 N/m and a resonance frequency of ∼70 kHz. The nominal tip radius was <10.0 nm. AFM image analysis was performed using the JPK Image Processing software.

2.5. NMR experiments

The NMR measurements were carried out at 12 °C on a Bruker Avance III (Bruker Biospin GmbH, Reinstetten, Germany) operating at ^{1}H frequency of 800 MHz. The sample contained 100 μM ^{15}N-α-syn in PBS pH 7.0 [10% D_2O (v/v)] in the presence or absence of equimolar concentration of phylloquinone, menaquinone, menadione or 1,4-NQ. Equivalent amounts of DMSO were used as control. The ^{1}H-^{15}N Heteronuclear Single Quantum Coherence (HSQC) experiments were done with WATERGATE water suppression (Piotto et al., 1992). The data were processed with TOPSPIN 3.1 (Bruker Biospin Corporation, USA) and the assignment was introduced using CCPNmr Analysis (Vranken et al., 2005). The ^{1}H-^{15}H HSQC spectra of α-syn in the absence or in the presence of some compounds were assigned according to Rao et al. (2009).

2.6. Vesicle leakage experiments

Large unilamellar vesicles (LUVs) were prepared by the extrusion method (Hope et al., 1985). All preparations were made in

25 mM Tris, pH 7.5. Unspecific charge interactions were reduced by carrying out the experiments in presence of 100 mM NaCl. A two-syringe extruder was used, equipped with a polycarbonate filter (Nuclepore®, Whatman) with 80-mm pore size. Twenty passages were performed each time. L-α-phosphatidyl-DL-glycerol (PG) (Avant Polar Lipids, USA) (10 mg) were solubilized in 500 μL of chloroform and the solvent completely evaporated in a nitrogen atmosphere until the formation of a uniform lipid film on the inner surface of the glass tube. A self-quenching solution of 100 mM carboxyfluorescein [2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; Molecular Probes], in 25 mM Tris pH 7.5, was entrapped in the LUVs by mixing 500 μL of carboxyfluorescein solution with the lipid film previously prepared. The free labels were separated from the labeled vesicles by gel filtration in Sephacryl® S-100 column (GE Bioscience). The leakage promoted by α-syn aggregates (final concentration of 10 μM in 25 mM Tris, pH 7.4, 100 mM NaCl), generated after 3 days of incubation at 37 °C under agitation, was evaluated by the carboxyfluorescein release assay (Kendall and MacDonald, 1982). The concentration of LUVs in the experiment was estimated based on the absorbance of the fluorescent probe (490 nm). In all experiments we have considered a final concentration of LUV corresponding to value of absorbance of 0.1 at 490 nm. In the leakage assays, fluorescence intensity (excitation at 490 nm and emission 518 nm) of the reactional mixture was recorded as a function of time. All measurements were carried out in a Cary Eclipse fluorescence spectrophotometer (Agilent Inc). The absence of leakage (0%) corresponds to the fluorescence of the vesicles at time zero; 100% leakage was taken as the value of fluorescence intensity obtained after addition of 1% (v/v) Triton X-100. The degree of permeabilization was then inferred from the relation: $\% \text{ leakage} = [(F - F_0)/(F_t - F_0)] \times 100$, where F is the fluorescence intensity after the addition of α-syn aggregates; F_0 is the initial fluorescence of the intact LUV suspension and F_t is the fluorescence after the addition of Triton X-100.

3. Results and discussion

3.1. Vitamin K inhibit α-syn fibrillization

Recently, the 1,4-NQ scaffold has been reported to exhibit important activities on biochemical pathways associated to neurodegenerative disorders such as inhibition of Hsp90, inhibition of β-secretase (involved in amyloid-β proteolysis), inhibition of MAO-A and MAO-B isoforms (involves in both PD and AD) and inhibition of amyloid-β fibrillization (Hadden et al., 2009; Bermejo-Bescós et al., 2010; Coelho-Cerqueira et al., 2011).

Herein, the *in vitro* aggregation properties of α-syn were investigated in the presence of vitamins K by monitoring the formation of fibrillar structure through the Thio-T fluorescence as well as by AFM imaging. The chemical structures of these molecules and other anti-fibrillogenic compounds discussed in this article are shown in the Fig. 1. The compounds were assayed in a micromolar range to keep the molar ratio protein/compound at 1:2 to 10:1, although physiologically vitamins K levels do not reach micromolar concentrations. We have also evaluated the effect of 1,4-NQ since all members of vitamins K displays this scaffold. Fig. 2 shows the characteristic sigmoidal curves for the fibrillization of α-syn at 37 °C and under agitation (350 rpm), given by the fluorescence of Thio-T in a specific time (F) divided by the initial value (F_0). Since the compounds tested were solubilized in 100% DMSO, equivalent amounts of the solvent were used as control [1–5% (v/v)]. All members of vitamin K as well as 1,4-NQ produced a dose-dependent decreasing of α-syn fibrillization (Fig. 2A–D), whereas no effect was observed for DMSO alone. In the kinetic of fibrillization, these compounds produced a slight prolongation of lag phase accompanied by a reduction of the content of fibrils formed at the steady state. This effect is distinct from other compounds such as selegiline, an anti-parkinsonian drug, which delays the nucleation step in the aggregation process but it does not change the final content of fibrils (Braga et al., 2011). The EC_{50} values were estimated by plotting the percentage of fluorescence at the steady state, obtained from the sigmoidal curve fitting of the data presented in Fig. 2, versus the logarithm of inhibitor concentration in micromolar (Fig. 2A–D, insets). For all compounds assayed, the inhibition was verified at substoichiometric concentrations. All molecules display similar ability to inhibit α-syn fibrillization with EC_{50} values in range of 15–30 μM. Interestingly, menadione (EC_{50} value of 18 μM for α-syn fibrillization) did not exhibit inhibitory activity on amyloid-β peptide (Bermejo-Bescós et al., 2010).

The EC_{50} values determined by using Thio-T as a fibril-probe must be carefully evaluated, presumably because Thio-T assay might be biased by the presence of certain exogenous compounds, notably polyphenols, resulting in a reduction of EC_{50} values and overestimation of the anti-fibrillogenic activities. Polyphenols are usually chromophoric and even intrinsically fluorescent since their phenolic aromatic rings are often connected by conjugated systems, resulting in strong $\pi \rightarrow \pi*$ electronic transitions. For instance, by using real-time Thio-T assay, quercetin and curcumin (referred as potent anti-fibrillogenic molecules) cause significant inner filter effects leading to effective quenching of the Thio-T fluorescence (Hudson et al., 2009). Herein, we evaluated the effect of vitamins K in both *in situ* real-time Thio-T assays and by single time-point dilution Thio-T-type assay and no significant differences were observed. Additionally, vitamins K did not display any effect on Thio-T fluorescence emission spectra over short period of time when these compounds were added to samples containing fibrils plus Thio-T (data not shown).

Fig. 2E shows the AFM images of α-syn aggregates at end of the aggregation process in the presence of vitamins K or DMSO (control). We can note the presence of large fibrillar structures (radius: 101 ± 49 nm and height: 8.9 ± 6.1 nm) in the sample containing 1% (v/v) DMSO instead of the compounds tested. Similar morphology was verified for α-syn incubated in the presence of buffer (data not shown). On the other hand, α-syn incubated with phylloquinone or menaquinone at a molar ratio of 2:1 protein/compound displays amorphous aggregates besides short fibrillar structures. α-syn fibrils exhibited average radii of 57 ± 26 nm (height of 5.7 ± 1.5 nm) and 46 ± 18 nm (height of 4.6 ± 1.2 nm) when incubated in the presence of phylloquinone and menaquinone, respectively. The radius of these fibrils was statistically smaller than those observed for fibrils in the presence of buffer or DMSO (*p* values for DMSO versus phylloquinone and menaquinone were <0.05 and <0.01, respectively). Similar results were observed for menadione. For 1,4-NQ, only few small fibrils were noted besides amorphous aggregates. It is important to note that in all cases, fibrils coexist with amorphous aggregates. Importantly, in the presence of the vitamins K, we did not observe a significant population of oligomers at the steady state, at least by using AFM. Note that α-synuclein oligomers (2–7 nm), including those acting as pore-forming toxins (Volles et al., 2001; Lashuel et al., 2002), are easily observed by AFM (Apetri et al., 2006). Several anti-fibrillogenic molecules inhibit the fibrillation process resulting in the formation oligomeric species that, as transient intermediates of the aggregation process, display diverse morphologies and biological properties (Caughey and Lansbury, 2003).

Besides vitamins K, multiforms of vitamin A have been reported as inhibitors of α-syn fibrillization (Ono and Yamada, 2007). Unfortunately, biological properties of α-syn species formed in the presence of retinols have not been evaluated. The low EC_{50} values showed by retinol, retinal and retinoic acid (0.19–3 μM) might be associated to the high hydrophobic character of these molecules.

Fig. 1. Chemical structures of the anti-fibrillogenic molecules: vitamins K, vitamin A, CoQ10, baicalein, PQQ, dopaminechrome and selegiline.

Although vitamins K, notably phylloquinone and menaquinone, are also nonpolar molecules, they contain the reactive quinone group, which is capable to react with amines to form a Schiff base. On the other hand, retinol derivatives are largely hydrophobic and contain a single polar group [carboxylic acid (retinoic acid), hydroxyl (retinol) or aldehyde group (retinal)] on the extremity of a long aliphatic chain (Fig. 1). Therefore, the anti-fibrillogenic activity of vitamins A is presumably associated to their ability to interact with the highly hydrophobic NAC domain of α-syn rather than any specific site. Furthermore, these molecules efficiently inhibit the aggregation of both α-syn and amyloid-β peptide (Ono and Yamada, 2007) that are unfolded proteins without any sequence similarity and with a large amount of hydrophobic residues exposed to water. Therefore, we can speculate that retinol derivatives behave as promiscuous anti-fibrillogenic agents. In case of phylloquinone and menaquinone, the long aliphatic substituent in the 3-position of 1,4-NQ ring does not improve their ability to inhibit α-syn aggregation as was expected for a hydrophobic interaction. Conversely, phylloquinone and menaquinone have higher EC_{50} values than either menaquinone or and 1,4-NQ (29–30 μM against 18–15 μM). Interestingly, ubiquinol (CoQ10), with a 1,4-benzoquinone scaffold and a long aliphatic substituent at the position 3 in the ring, has similar structure to vitamins K (Fig. 1) and displays a EC_{50} value of 10 μM, which is in the same range of vitamins K (Ono and Yamada, 2007).

3.2. Vitamins K affect the nucleation step of α-syn fibrillization

α-syn fibrillization is a nucleation-dependent process involving two main pathways: a nucleation step (lag phase), which results in protofibrils formation, followed by elongation step (exponential phase) in which the fibrils grow. Anti-fibrillogenic compounds might interfere in one of these steps or in both and thus generate in a wide spectrum of species (small oligomers, protofibrils, fibrils or amorphous aggregates).

To verify if the observed effect of vitamins K is restricted to the nucleation step, α-syn was incubated under aggregating conditions and then 50 μM of vitamins K or 1,4-NQ (or 2% DMSO) were added to the samples when the lag phase was already concluded. Fig. 3A shows the Thio-T fluorescence at the steady state in the system in which the compounds were added to α-syn monomer at either the initial time or after 3 days under aggregation conditions. We can observe that the effect of phylloquinone and menaquinone was prevented when lag phase was completed, suggesting that these compounds might be interfering in the formation of the nucleus. For menadione and 1,4-NQ, the inhibitory effect remained even these compounds were added after the lag phase, which can be ascribed to either a destabilization of preexisting nucleus/protofibrils or an inhibition of the fibril elongation process. To investigate these two hypotheses, α-syn aggregation was carried out in seeding conditions.

It has been established that preformed fibrils submitted to sonication by ultrasound act as "seeds" when they are added to α-syn monomer, resulting in acceleration of the fibrillization process by shortening the nucleation phase. These seeds are fragments of fibrils acting as a template for addition of α-syn monomer to fibril but they are structurally different than the nucleus specie. Fig. 3B shows that the addition of seeds to α-syn monomer significantly abolished the lag phase and accelerated the formation of fibrils. In the absence of agitation, no aggregation was observed in unseeding condition (open symbols). When α-syn monomer plus seeds is incubated in the presence of equimolar concentration of phylloquinone or menaquinone, the inhibitory effect of these compounds was abolished, suggesting these compounds did not interfere in the fibril elongation process. On the other hand, seeds did not abolish the effect of menadione or 1,4-NQ on the fibrillization process.

The inhibitory effect of menadione and 1,4-NQ on α-syn fibrillization in the presence of seeds could be ascribed to either an inhibition of the fibril elongation process or a destabilization of preexisting seeds. If the seeds have their structure destabilized

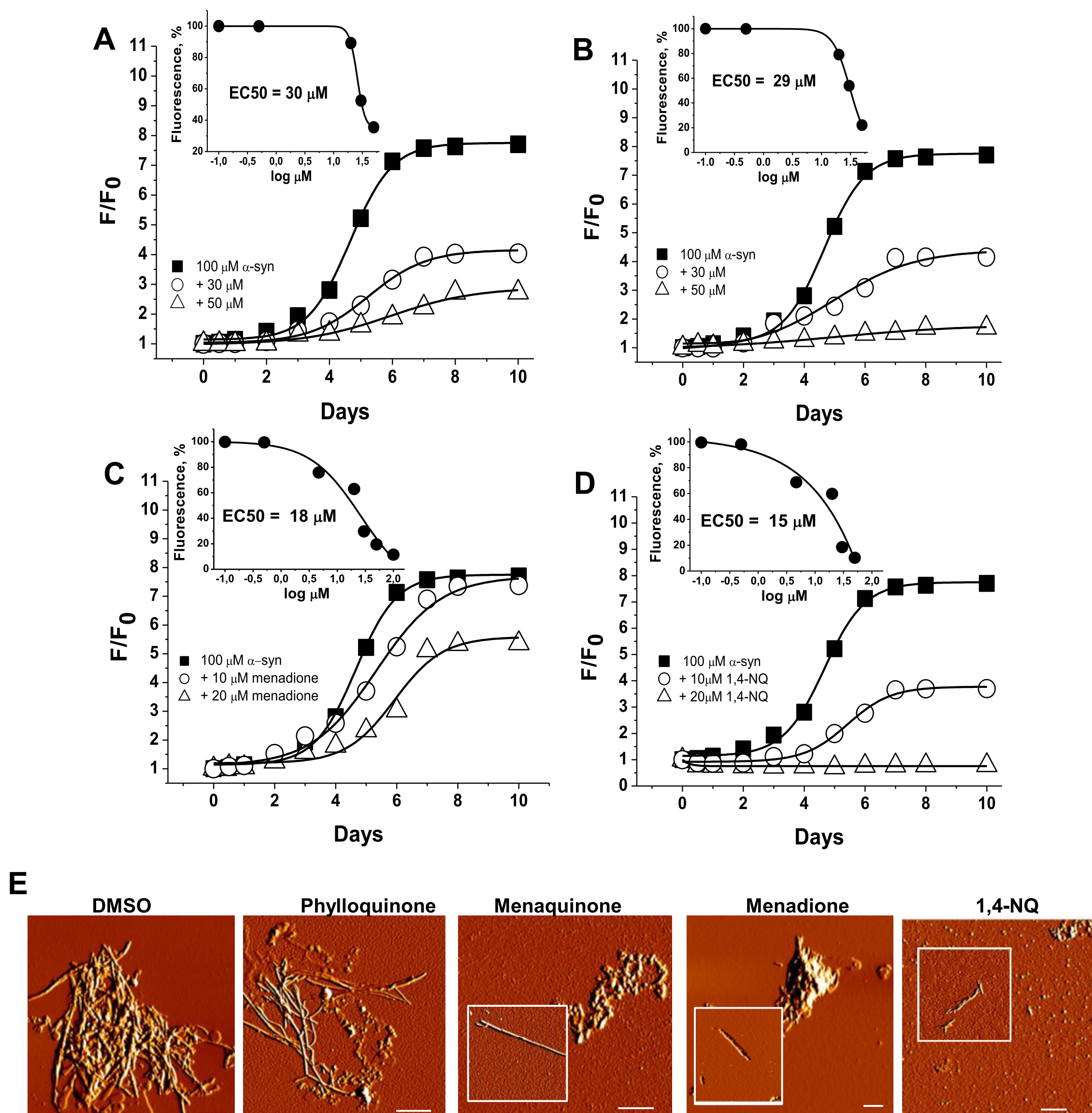

Fig. 2. Effect of vitamins K and 1,4-NQ on α-syn fibrillization. Kinetic of fibrillization of 100 μM de human WT-α-syn incubated at 37 °C (350 rmp) in the presence of several concentrations of vitamins K: phylloquinone (A), menaquinone (B), menadione (C) or 1,4-NQ (D). The content of fibrils was monitored *in situ* by measurement of Thio-T fluorescence. The *insets* show the determination of EC_{50} by plotting of the percentage of fluorescence at the steady state obtained from the sigmoidal curve fitting versus the logarithm of inhibitor concentration in μM. (E) AFM images of α-syn aggregates generated when 100 μM de α-syn monomer was incubated at 37 °C (350 rmp) with 50 μM of phylloquinone, menaquinone, menadione or 1,4-NQ for ten days. Bars: 500 nm. Fibrillar structures were highlighted. DMSO was used as control.

by menadione or 1,4-NQ, they could not act properly as a template for fibrils growth. Thus the effect of 1,4-NQ and vitamins K on the disaggregation of preformed fibrils of α-syn was investigated. As shown in Fig. 3C, the Thio-T fluorescence remains unaltered during the incubation of α-syn fibrils at 37 °C under agitation, in the presence of 1% DMSO. No differences were observed between DMSO and buffer (data not shown). However, the Thio-T fluorescence dose-dependently decrease immediately after the addition of 1,4-NQ (Fig. 3C). AFM images (insets) show that only amorphous aggregates were observed after 7 days of incubation in the presence of 50 μM 1,4-NQ. Although in lower extension than 1,4-NQ, menadione is also capable to destabilize preformed fibrils of α-syn (Fig. 3D). In this case, the concomitant presence of fibrils and amorphous aggregates were observed by AFM after 7 days of incubation. Phyloquinone and menaquinone did not display any effect on the stability of fibrils (data not shown), which could be associated to their higher molecular volumes in comparison with menadione or 1,4-NQ, that reduce their diffusion into fibril structure and hence their interaction with protein sites involved in the fibril destabilization process. These data suggest that the ability to dissociate preformed fibrils exhibited by vitamins K was not necessarily associated with their capacity to inhibit α-syn fibrillization.

Certain anti-parkinsonian drugs are capable to inhibit or retard α-syn aggregation (Ono et al., 2007). One of these compounds is selegiline, which binds to α-syn and delay the formation of protein fibrils (Braga et al., 2011). Selegiline inhibits α-syn fibrillation precisely by delaying the nucleation step but not interfering in the elongation process, similarly to vitamins K. However, selegiline was effective only in large micromolar concentrations whereas vitamins K exhibit inhibitory activity at substoichiometric concentrations. Selegiline, with a tertiary amine and an acetylene group, is not structurally related with vitamins K or other anti-amyloidogenic molecules (Fig. 1).

3.3. Effect of vitamins K on vesicle leakage induced by α-syn

In the early stage of fibrillization process, the system is populated by soluble oligomers and protofibrils with diverse

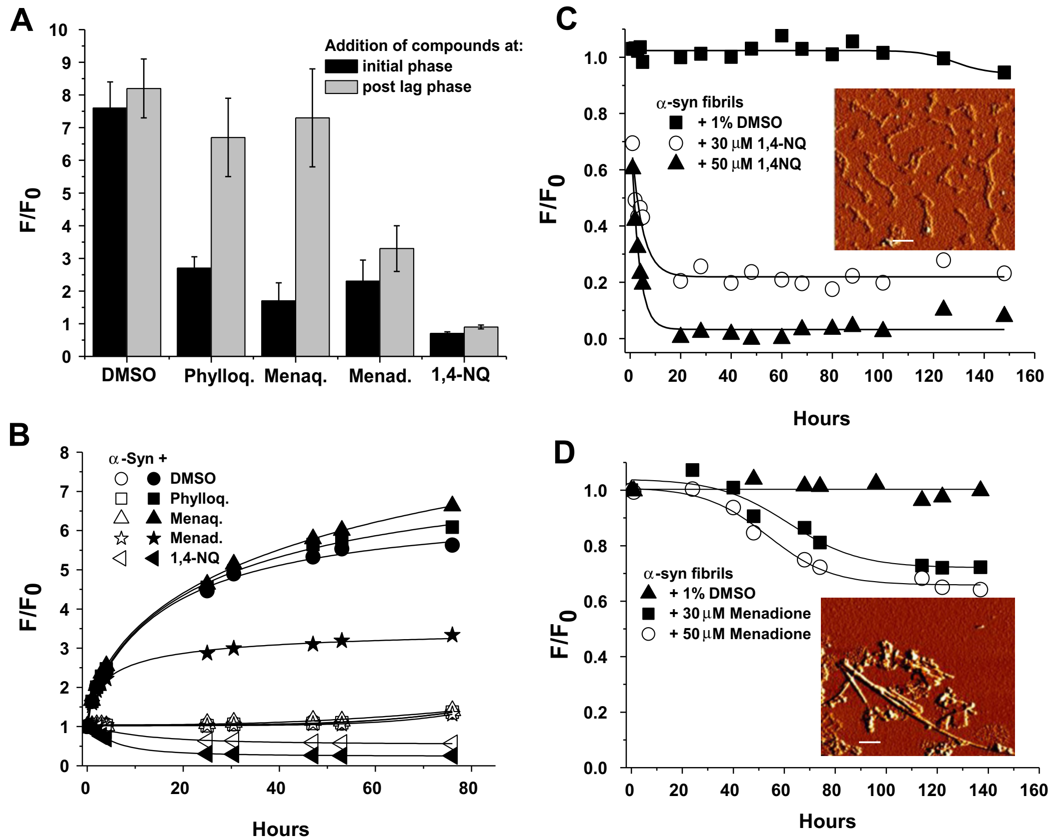

Fig. 3. Vitamins K and 1,4-NQ affecting different steps of α-syn fibrillization. (A) Thio-T fluorescence at the steady state in the system in which the compounds (30 μM) were added to α-syn monomer at either the initial time or after 3 days of aggregation at 37 °C under agitation. (B) Fibrillization of α-syn in seeding conditions. α-syn monomer, in the absence (open symbols) or the presence of 5% (w/w) seeds (black symbols), was incubated at 37 °C, without shaking, in the presence of 50 μM phylloquinone, menaquinone, menadione or 1,4-NQ or 1% DMSO. Disaggregation of α-syn fibrils induced by menadione or 1,4-NQ was measured by incubating mature α-syn fibrils at 37 °C (350 rpm) in presence of 1,4-NQ (C) or menadione (D). Fibril disassembling was monitored by Thio-T fluorescence as previously described. Insets show the AFM image in presence of the respective compounds (bars: 500 nm).

morphologies including annular, spherical and tubular protofibrils (Caughey and Lansbury, 2003). α-syn protofibrils can bind to synthetic vesicles causing transient permeabilization that could trigger off toxic events to the cell (Volles et al., 2001; Lashuel et al., 2002). This finding could explain the connection between α-syn mutations, which slightly accelerate *in vitro* oligomerization of α-syn, and PD. The α-syn monomer or mature fibrils do not display vesicle permeabilization property. Herein, we investigate the effect of α-syn aggregates, generated in the presence of vitamins K, on vesicle integrity using LUV composed by PG (acidic vesicles). Vesicles composed by PG have been previously used in studies of vesicle permeabilizaiton by protofibrillar α-syn (Volles et al., 2001). The vesicles (approximate diameter of 80 nm) were loaded with carboxyfluorescein and the enhancement of fluorescence signal due to the fluorescent dye release, which occurs during vesicle leakage, was monitored. A leakage of 100% was taken as that produced by addition of 0.1% Triton X-100. α-syn monomer was incubated with vitamins K or DMSO under aggregation conditions and the aggregates formed at the end of lag phase, i.e. prior the formation of large fibrils, were added to the extravesicular space. Alpha-syn aggregates (monomer concentration of 20 μM) incubated for three days with 1% DMSO produced 100% of leakage whereas those formed in the presence of 100 μM of phylloquinone, menaquinone or menadione have a reduced leakage activity (10–40%) (Fig. 4). Similar results were verified for aggregates incubated with 1,4-NQ. Either compounds alone or DMSO did not produce fluorescence enhancement. Additionally, no effect was observed for α-syn fibrils in concordance with previous studies. Our data indicate that the incubation of α-syn monomer for short period of time leads to formation of aggregates capable to promote vesicle leakage, whereas the incubation in the presence of either vitamins K or 1,4-NQ produced, aggregates with a reduced ability to leak vesicles. These data suggest that vitamins K could preclude the formation of protofibrils involved in vesicle permeabilization.

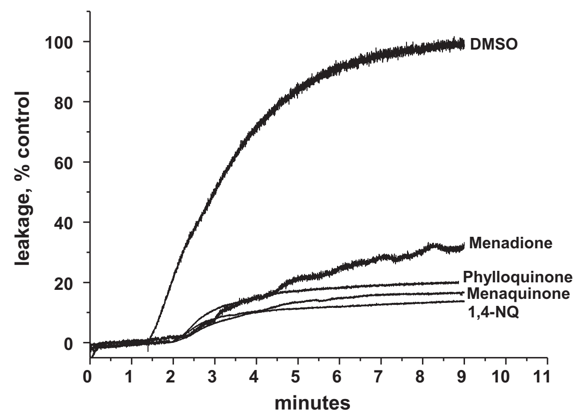

Fig. 4. Vesicle leakage induced by α-syn protofibrils. α-syn protofibrils (20 μM in terms of monomer concentration) formed in the presence of equimolar concentrations of vitamins K or 1,4-NQ (or 1% DMSO) were added to the LUV composed by PG. The vesicles of PG were loaded with carboxyfluorescein and the enhancement of fluorescence signal due to the fluorescent dye release was monitored. A leakage of 100% was taken as that produced by addition of 0.1% Triton X-100.

3.4. Structural features of the interaction between α-syn and vitamins K

To shed light on the mechanism involved in the interaction between vitamins K and α-syn, we carried out ^{1}H-^{15}N HSQC NMR analysis of ^{15}N-α-syn in the presence of the compounds. With this approach, we sought to determine the residues that could be taking part on this interaction through the ^{15}N and ^{1}H chemical shift perturbations. Fig. 5A shows the HSQC spectrum of α-syn (blue) superposed with the spectrum of α-syn plus phylloquinone (red). This profile was quite similar for α-syn in the presence of menaquinone, menadione or 1,4-NQ (data not shown). From the comparison of the spectra, we have determined the variation of chemical shifts ($\Delta\delta$ ^{1}H,^{15}N) of each α-syn residue caused by the presence of the compounds (Fig. 5B–E). The greatest values for $\Delta\delta$ (^{1}H,^{15}N) are presumably associated to α-syn residues involved to the interaction with the compounds. The changes in the His50 were neglected since this residue is very sensitive to slight pH changes and then its involvement in the interaction could be overestimated. Analyzing the similarities among vitamins K and 1,4-NQ, we can observe that all these compounds have perturbed the Gly31/Lys32 residues suggesting that these residues might play an important role on the interaction with 1,4-NQ scaffold. The Val3 was slightly perturbed by menaquinone, menadione and 1,4-NQ but not by phylloquinone,

Three distinctive domains can be noted in the α-syn sequence (Fig. 5F). The first one is the highly conversed N-terminal domain (residues 1–61) involved in the binding to phospholipid vesicles (Eliezer et al., 2001). This domain contains four imperfect KTKEGV repeats and shows an amphipathic character with propensity to acquire α-helical structure (Chandra et al., 2003). The second domain is a variable internal hydrophobic NAC (non-amyloid component) domain (residues 61–95), which is essential for the protein aggregation. The third one is the C-terminal acid tail (residues 95–140), which is composed primary by negatively charged aspartate and glutamate residues. The interacting environment of the N-terminal permits to α-syn exhibit different conformations from random coil to α-helices or β-conformation. According to the chemical shift changes observed in the HSQC spectrum of α-syn in the presence of vitamins K, the second repeat domain in the N-terminal (containing the Lys32) is involved in the binding to these molecules. This interaction is likely mediated by the 1,4-NQ scaffold since the chemical shift changes at Gly31/Lys32 are not greatly affect by the substituent in the 3-position of the ring.

Analyzing the hydrophobic character of the α-syn residues (Fig. 5F) we can verify that either vitamins K or 1,4-NQ did not produce any perturbation in the residues located in the most hydrophobic portion (NAC) of the protein. Furthermore, the residues Gly31/Lys32 belong to a low hydrophobic region (indicated by an arrow in Fig. 5F) reinforcing the idea of the effect observed for

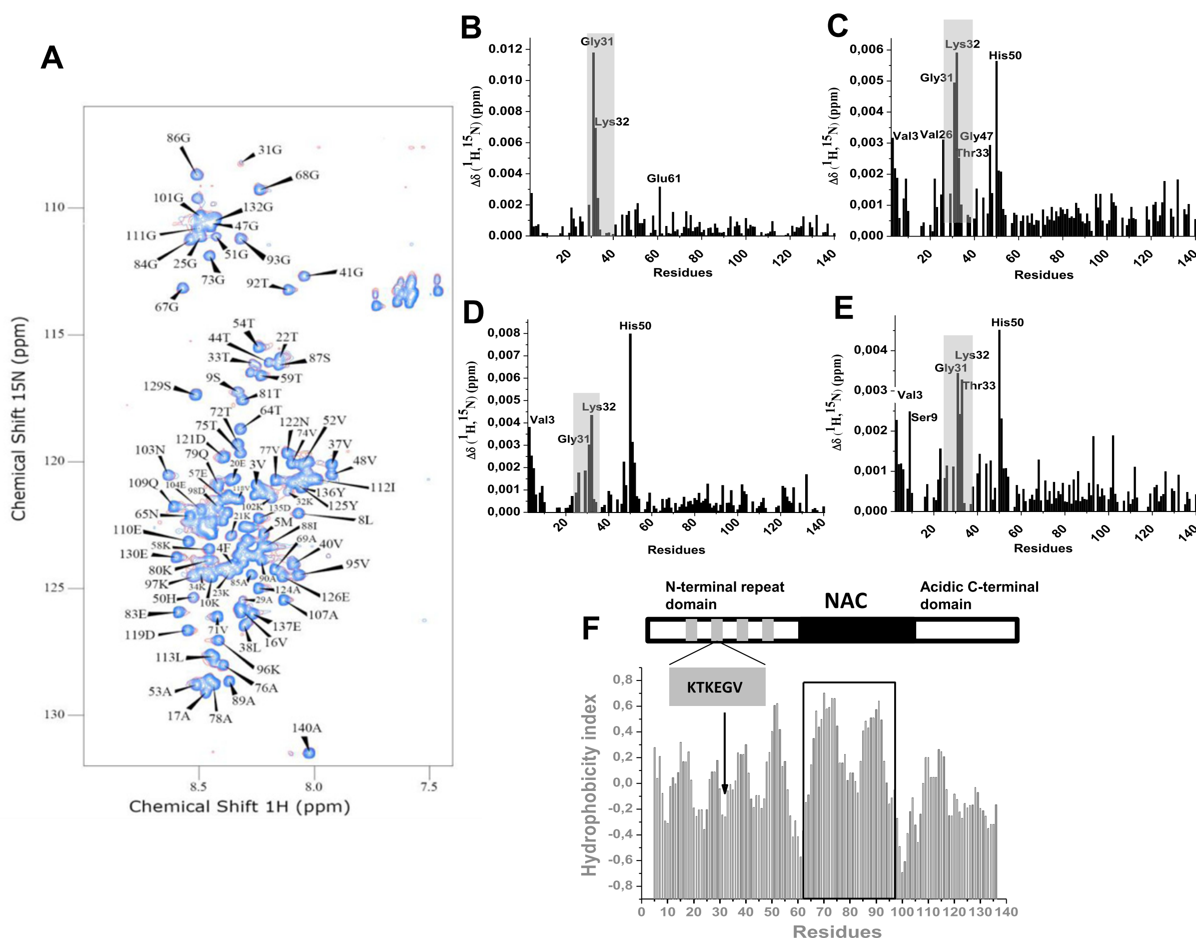

Fig. 5. Binding of α-syn monomer to vitamins K or 1,4-NQ. (A) ^{1}H-^{15}N HSQC NMR spectrum of 100 μM α-syn alone (blue) or in presence of equimolar concentration of Phylloquinone (red). The panels (B) to (E) show the changes in chemical shifts of α-syn in presence of phylloquinone, menaquinone, menadione and 1,4-NQ, respectively. A significant chemical shift changes were defined based on values above the average of all chemical shifts. (F) Hydrophobicity of the α-syn domains. The N-terminal domain (residues 1–61), containing four imperfect KTKEGV hydrophobic, is suggested to be involved in the binding to vitamins K or 1,4-NQ. These molecules do not produce any perturbation in the residues located in the more hydrophobic portion (NAC) (residues 61–95). The arrow indicates the location of Gly31/Lys32 residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vitamins K is not merely resulted of a promiscuous hydrophobic interaction. The N-terminal α-syn region contains the three missense mutations linked to familial PD (A30P, E46K, and A53T), which have been associated to conformational changes of the protein, notably in secondary structure (A30P or A53T) or a net charge (E46K). Therefore, the binding of vitamins K to this domain might also alter the structure propensity of α-syn.

The repeat domain in α-syn plays a critical role in both kinetic of fibrillation and in the morphology of the fibrils formed (Harada et al., 2009). For instance, the mutation E46K occurs in the repeat sequences and accelerates the fibrillization *in vitro* and in cultured cells (Greenbaum et al., 2005; Pandey et al., 2006). In addition, the E35K, E61K and E83K α-syn mutations perturb the cross β-structures in N-terminal region, resulting in the formation of short fibrils rather than the mature fibrils (Harada et al., 2009). In our studies, vitamins K as well as 1,4-NQ are suggested to interact with the N-terminal α-syn and promote the formation of short and sheared fibrils similarly to reported for some mutations in the repeat sequences.

In contrast with our results, the catechol derivatives entacapone, tolcapone and quercetin, which inhibit α-syn fibrillization, do not bind to α-syn monomer as verified by NMR spectroscopy even in a molar radios up to 1:10 α-syn:compound (Di Giovanni et al., 2010). These compounds do not produce any significant chemical shift alterations in the HSQC spectrum of α-syn monomer, with exception of very minor chemical shift changes for His50 and some N-terminal residues. These chemical shift changes in His50 and the two to three N-terminal residues are also observed for vitamins K and were ascribed to slight changes in pH. Similar results were reported for α-syn in the presence of selegiline (Braga et al., 2011). Selegiline did not produce any perturbation in the HSQC spectrum of α-syn monomer (Braga et al., 2011), suggesting the mechanism lying behind the anti-fibrillogenic activity of these molecules is different than that observed for vitamins K.

3.5. Binding of α-syn to quinones and polyphenols

The molecular basis of vitamins K acting as anti-fibrillogenic agent might be clarified comparing these molecules to others quinones. Imino bonds might be formed by nucleophilic attack of amino groups of α-syn residues on quinones. Similar mechanisms might lie behind the anti-fibrillogenic activity of polyphenols. Polyphenols can be spontaneously oxidized in the presence of oxygen into quinones. Epigallocatechin gallate, a polyphenol from green tea, inhibits α-syn fibrillization with a EC_{50} value of 20 μM, which is in the same range of those observed for vitamins K or 1,4-NQ (Li et al., 2005).

It has been reported that the reaction of α-syn with quinones presumably involves the side chains of Tyr, Lys or Met residues (Li et al., 2005). Studies with α-syn mutants suggested that Tyr residues do not participate of the interaction with quinones (Norris et al., 2005), whereas Met residues are more prone to be oxidized by reactive oxygen species generated during the oxidation of the polyphenols, catechols or hydroquinones into quinones that might occurs during the incubation process (Li et al., 2005). On the other hand, chemical shift changes in ^{1}H-^{15}N HSQC NMR spectrum of α-syn in the presence of quinone or hydroquinones provide evidences for the reaction between quinone and ε-amino groups of Lys residues or the α-amino group in the protein N-terminus. Interestingly, the nonamyloidogenic variant of α-syn (α-syn1–60) is also able to react with quinone, suggesting that Lys at N-terminal domain might be involved in this interaction (Li et al., 2005). The Lys residues are majority found in the N-terminal region of α-syn and the KTKEGV repeat sequences might represent probable sites for the interaction with quinones. The Lys32 is found in one of these KTKEGV repeats and it is candidate to interact with 1,4-NQ scaffold of vitamins K.

The flavonoid baicalein (Fig. 1) exhibits anti-fibrilligenic action on α-syn that promotes the formation of oligomers rather than fibrils (Zhu et al., 2004). Although the inhibition of α-syn fibrillization by baicalein occurs in the micromolar range, similarly to vitamins K, the product of baicalein action on α-syn is soluble oligomers, whereas vitamins K action results in short fibrils and amorphous aggregates. Like vitamins K, baicalein affects α-syn nucleation but not the fibril elongation, and is capable to disaggregate mature fibrils similarly to menadione and 1,4-NQ. Baicalein covalently bind to the protein molecule through a binding between the quinone moiety and the Lys side chain resulting in a Schiff base. Taken together, these data reinforce the idea of vitamins K interact with α-syn through the N-terminal domain and, more important, Lys residues participate in the binding process.

The naturally occurring pyrroloquinoline quinone (PQQ) (Fig. 1) is capable to inhibit the fibrillization of α-syn, even though it is a less effective anti-fibrillogenic agent than baicalein (Kobayashi et al., 2006). Regarding its broad spectrum of activities and positive effect on cellular growth, PQQ has been suggested as an essential nutrient and even considered as a new form of vitamin B (Killgore et al., 1989; Kasahara and Kato, 2003). Besides α-syn, PQQ also prevents the *in vitro* fibrillization of both amyloid-β (1–42) and mouse prion protein (Kim et al., 2010a). Additionally, PQQ is able to inhibit the fibrillization of C-terminal truncated α-syn119 and 133 resulting in the formation of species with reduced cytotoxicity (Kim et al., 2010b). Interestingly, C-terminal truncation of α-syn accelerates the fibrillization and, more importantly, these forms have been found in both the normal and the pathogenic brain (Muntané et al., 2012).

In contrast with vitamin A, the anti-fibrillogenic activity of PQQ on α-syn seems not to be due to nonspecific hydrophobic interactions. It has been demonstrated that PQQ interact with α-syn via formation of Schiff base with Lys residues, resulting in a PQQ-conjugated α-syn (Kobayashi et al., 2006, 2009). In this context, the inhibitory property of PQQ is directly associated to number and location of Lys that are modified, notably those located in N-terminus region (Kim et al., 2010a). The formation of Schiff base with Lys residues was also proposed to occur between α-syn and other quinones such as baicalein, dopaminechrome and vitamins K. It must be kept in mind that 10 from 15 Lys residues are located in the imperfect repeat sequences in N-terminal α-syn.

Additional valuable data on the anti-fibrillogenic action of quinones might be gained from the investigation of the effect of dopamine quinones such as dopaminechrome (DAQ), on α-syn aggregation. Oxidative stress, dopamine metabolism and α-syn misfolding are tightly interconnected events in PD neuropathogenesis. Lansbury and co-workers demonstrated for the first time that DAQ and others catecholamines are capable of inhibiting α-syn fibrillization by formation of a covalent adduct (Conway et al., 2001). DAQ is also capable to promote degradation of pre-existing fibrils and stabilize cytotoxic protofibrils of α-syn variants, notably from A30P mutant (Li et al., 2004; Follmer et al., 2007). Structurally, DAQ displays a 1,2-benzoquinone scaffold in contrast with 1,4-NQ of vitamins K (Fig. 1).

The molecular basis of the interaction between DAQ and α-syn has been widely investigated but the results are often contradictory (To review see: Leong et al., 2009a). While some investigations have indicated that the generation of toxic protofibrils occurs through the formation of covalent adducts between of α-syn and DAQ (Conway et al., 2001; Li et al., 2005; Cappai et al., 2005), a number of other studies suggest for a non-covalent nature of this interaction (Norris et al., 2005; Bisaglia et al., 2010). Apparently, both mechanisms seem to coexist: while a small fraction of α-syn forms a covalent adduct with DAQ, the majority of the protein

interacts by non-covalent interaction (Bisaglia et al., 2010). In the covalent adducts hypothesis, DAQ was covalently incorporated into α-synuclein by formation of a Schiff base by nucleophilic attack of Lys on quinone (Li et al., 2005). A covalent bond between quinone and Lys is also suggested to occur in the interaction of baicalein with α-syn (Zhu et al., 2004).

On the other hand, Lee and co-workers have shown that DAQ inhibits α-syn fibrillization by inducing structural changes in the protein, which is mediated by interaction of DAQ with the ^{125}YEMPS^{129} motif of the protein and, most importantly, this process is reversible (Norris et al., 2005). Interestingly, Follmer et al. (2007) have demonstrated that α-syn protofibrils formed in the presence of dopamine are capable to evolving to fibrils when incubated for a long period of time. Fibrillar structures are also present in α-syn incubated with vitamins K or 1,4-NQ, beside amorphous aggregates.

The conflicting data about the effect of DAQ on α-syn fibrillization might be ascribed to the fact of the experiments were usually performed by incubating α-syn with dopamine instead of its oxidized form DAQ. Dopamine in aqueous solution is readily oxidized to DAQ, generating ROS, which in turn might promote the oxidation of certain residues, primarily Met residues (Leong et al., 2009b). Furthermore, although DAQ may be a stable oxidation product, it is still capable of undergoing oxidation with time in route to forming melanin. Thus α-syn oligomerization mediated by dopamine will be greatly dependent on the oxidation state of dopamine (Pham et al., 2009). Therefore, misleading conclusions might arise from the wide range of products generated from dopamine oxidation (dopamine *ortho*-quinone, dopaminechrome, leucodopaminechrome and until melanin) when dopamine is incubated in the presence of α-syn.

3.6. Potential targets for 1,4-NQ and Vitamins K in the brain

Recent evidences have indicated that vitamins K play an important role in the brain (Carrié et al., 2004), including sphingolipid synthesis and activation of the protein Gas6, this latter involved in protection against apoptosis and cell proliferation (Ferland et al., 1992). Low levels of vitamin K have been correlated with the apolipoprotein Eε4 allele, a risk factor associated with AD (Allison, 2001). Interestingly, it was demonstrated that Gas6 can rescue cortical neurons from amyloid-β induced apoptosis besides a protective role against oxidative damage in developing neurons (Yagami et al., 2002). Additionally, it has been reported that patients with early stage AD consume less vitamin K than healthy subjects (Presse et al., 2008). AD is the condition most commonly associated with secondary α-syn aggregation (Lippa et al., 2005) and one-half of the patients with familiar forms of AD have numerous fibrillar α-syn aggregates in the form of Lewy bodies in their amygdale (Lippa et al., 1998), making α-syn an important target in the development of therapies not only for PD but also for AD.

The action of vitamins K on α-syn fibrillization seems to be associated to 1,4-NQ scaffold since 1,4-NQ itself exhibits anti-fibrillogenic and fibrils dissolving activities, reinforcing the idea of these and other structurally related 1,4-NQs are lead compounds for the development of new therapies targeting α-syn aggregation. Interestingly, our previous studies have suggested that 1,4-NQ is a potential scaffold for designing new reversible MAO inhibitors. In case of menadione, it acts as a competitive and reversible inhibitor of human MAO with 60-fold selectivity for MAO-B (Ki = 0.4 μM) in relation to MAO-A (Ki = 26 μM), which makes it as selective as rasagiline (Coelho-Cerqueira et al., 2011). Since MAO catalyzes the oxidative deamination of biogenic and exogenous amines and its inhibitors have been used in several neurological conditions including affective disorders, stroke, neurodegenerative diseases and aging (Youdim and Bakhle, 2006), 1,4-NQ might represent a multitarget pharmacophore acting on both MAO inhibition and α-syn fibrillization.

4. Conclusions

Although several molecules have been described to inhibit α-syn fibrillization, many of them behave as promiscuous anti-fibrillogenic agents, interacting with α-syn through non-specific hydrophobic interactions. In contrast with other lipophilic vitamins, we demonstrated that the interaction of vitamins K with α-syn is mediated by specific residues located at N-terminus portion of the protein, notably Lys32, leading to the formation of short fibrils and amorphous aggregates rather than oligomers. The highly hydrophobic region of the protein involved in the aggregation process (NAC domain) does not participate of this interaction. Taken together, our data pointed out that 1,4-NQ, found in all forms of vitamins K and many other bioactive molecules, might represent a potential scaffold for designing drugs targeting α-syn fibrillization, even though the *in vivo* effect of these compounds remains unknown.

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