

## CEFTRIAXONE BLOCKS THE POLYMERIZATION OF $\alpha$ -SYNUCLEIN AND EXERTS NEUROPROTECTIVE EFFECTS IN VITRO

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### ABSTRACT:

The  $\beta$ -lactam antibiotic ceftriaxone was suggested as a therapeutic agent in several neurodegenerative disorders, either for its ability to counteract glutamate-mediated toxicity, as in cerebral ischemia, or for its ability to enhance the degradation of misfolded proteins, as in Alexander's disease. Recently, the efficacy of ceftriaxone in neuroprotection of dopaminergic neurons in a rat model of Parkinson's disease was documented. However, which characteristics of ceftriaxone mediate its therapeutic effects remains unclear. Since, at the molecular level, neuronal  $\alpha$ -synuclein inclusions and pathological  $\alpha$ -synuclein transmission play a leading role in initiation of Parkinson-like neurodegeneration, we thought of investigating, by circular dichroism spectroscopy, the capability of ceftriaxone to interact with  $\alpha$ -synuclein. We found that ceftriaxone binds with good affinity to  $\alpha$ -synuclein and blocks its in vitro polymerization. Considering this finding, we also documented that ceftriaxone exerts neuroprotective action in an in vitro model of Parkinson's disease. Our data, in addition to the findings on neuroprotective activity of ceftriaxone on Parkinson-like neurodegeneration in vivo, indicates ceftriaxone as a potential agent in treatment of Parkinson's disease.

**KEYWORDS:** Ceftriaxone,  $\alpha$ -synuclein, Parkinson's disease, circular dichroism, 6-OHDA, PC12 cells.

### I. INTRODUCTION

The  $\beta$ -lactam antibiotic ceftriaxone sodium (CAS number 104376-79-6, C<sub>18</sub>H<sub>16</sub>N<sub>8</sub>Na<sub>2</sub>O<sub>7</sub>S<sub>3</sub>·3.5H<sub>2</sub>O, Figure 1A) is a

safe and multipotent agent that has been used for decades as an antimicrobial. It is a white crystalline powder, which is readily soluble in water, and able to pass freely the blood-brain barrier.<sup>1</sup> In 2005, it was suggested as a therapeutic agent in some neurodegenerative disorders associated with glutamate-mediated toxicity, such as cerebral ischemia, amyotrophic lateral sclerosis, and epilepsy, based on its ability to increase the glutamate transporter subtype 1 (GLT-1) activity in astrocytes that are genetically impaired in their GLT-1 expression.<sup>2</sup> In 2010, reports from our group showed that ceftriaxone was also able to successfully eliminate the cellular toxic effects of misfolded glial fibrillary acidic protein (GFAP), in a cellular model of Alexander's disease (AxD).<sup>3</sup> Moreover, intravenous, cyclical ceftriaxone was used in a patient with adult-onset AxD, and a rapidly progressive clinical course.<sup>4</sup> A 4-year-long extension of the trial with ceftriaxone in this patient showed that the progression of neurodegeneration was halted and/or reversed by ceftriaxone, with a significant improvement of the quality of life of the patient.<sup>5</sup> In 2012, the efficacy of ceftriaxone in neuroprotection of dopaminergic neurons and amelioration of motor deficits in a rat model of Parkinson's disease (PD) was also documented.<sup>6</sup> However, which characteristics of ceftriaxone mediate its therapeutic effects in neurological disorders characterized by cellular accumulation of misfolded proteins, such as AxD and PD, it remains unclear. In particular, PD the second most common neurodegenerative disorder after Alzheimer's disease (AD), is essentially

characterized by both the intraneuronal accumulation of misfolded fibrillar  $\alpha$ -synuclein, in Lewy bodies and Lewy neuritis, and the selective degeneration of dopamine neurons in the substantia nigra pars compacta, which leads to bradykinesia, muscular rigidity, resting tremor, and postural instability.<sup>7</sup>  $\alpha$ -Synuclein is a small, soluble, 140 amino acid protein, which is widely expressed in the brain. The  $\alpha$ -synuclein sequence can be divided in three different regions: the N-terminal amphipathic region, which contains most of the sequence, repeats and the three point mutations linked to autosomal dominant early onset PD; the central

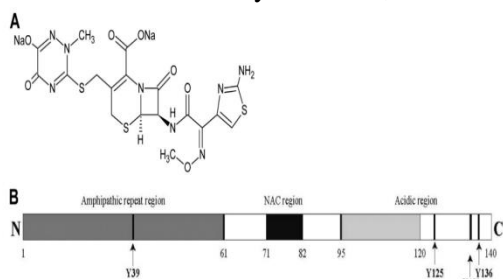


Figure 1. (A) Ceftriaxone disodium salt and (B)  $\alpha$ -synuclein structure.

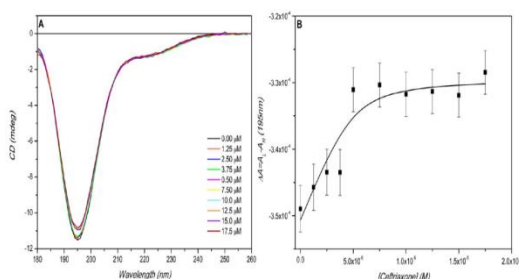


Figure 2. (A) Far-UV SRCD spectra of  $\alpha$ -synuclein in the presence of increasing amount of ceftriaxone.  $\alpha$ -Synuclein was 5.0  $\mu$ M in 5 mM Tris-HCl buffer, pH 6.8; ceftriaxone was either 100 or 200  $\mu$ M in 2:8 v/v EtOH/5 mM Tris-HCl buffer, pH 6.8. Spectra were recorded at 25  $^{\circ}$ C. CD spectra were corrected by subtraction of the far-UV CD spectra of equivalent amount of ceftriaxone. The  $\Delta A$  values were measured in the presence of increasing amount of ceftriaxone (indicated). (B) Determination of the  $K_d$  value by CD spectroscopy analyses. The  $\Delta A$  values at 195 nm, measured as a function of different

ligand/AS molar ratios, are plotted versus the ceftriaxone concentration. Errors are less than 1.0%.

region (NAC), which encompasses the most hydrophobic residues and promotes aggregation; and the acidic C-terminal portion, which contains three of the four Tyr residues and tends to decrease protein aggregation (Figure 1B).<sup>7</sup> Notably, although ceftriaxone seems to have a definite role in counteracting neurodegeneration in several neurodegenerative disorders, its molecular interaction with  $\alpha$ -synuclein has not been reported. In our opinion, in virtue of their accessibility, well-known and safe drugs such as ceftriaxone, potentially able to bind to proteins involved in the formation of amyloid-like aggregates, and to inhibit their production, are of immense interest as potential leads for therapeutic intervention in PD and other degenerative disorders caused by intracellular accumulation and aggregation of misfolded proteins.<sup>8</sup>

Since, at the molecular level, neuronal  $\alpha$ -synuclein inclusions and pathological  $\alpha$ -synuclein transmission play a leading role in initiation and progression of Parkinson-like neurodegeneration,<sup>9,10</sup> and on account of the capability of ceftriaxone to reduce the intracytoplasmic aggregates of mutant GFAP,<sup>3</sup> we thought of investigating, by circular dichroism (CD) spectroscopy, the capability of ceftriaxone to interact with  $\alpha$ -synuclein.

Moreover, we studied the protective effect of ceftriaxone on PC12 cells exposed to the neurotoxic compound 6-hydroxydopamine (6-OHDA). PC12 cells are catecholaminergic cells widely used as a highly informative in vitro model of PD, also useful for testing potential neuroprotective drugs for PD treatment.<sup>11</sup>

## II. RESULTS AND DISCUSSION

In the present study, we used CD spectroscopy to estimate the binding affinity of ceftriaxone to  $\alpha$ -synuclein and to probe the capability of this  $\beta$ -lactam antibiotic to interfere with the  $\alpha$ -synuclein

aggregation process. Spectroscopy tools have been used to investigate the biochemical key events involved in the formation of protein aggregates. In particular, CD has been used for assessing the conformational transition of amyloid species proteins<sup>12,13</sup> and to gain insight both into the interaction of various small molecules with native protein and into the effects on amyloid aggregation.<sup>14</sup>

In order to evaluate the capability of ceftriaxone to interact with monomeric  $\alpha$ -synuclein, we examined the far-UV spectrum of  $\alpha$ -synuclein in a range between 190 and 250 nm. In the absence of ligand, the far-UV CD spectrum of  $\alpha$ -synuclein displayed a negative band at about 199 nm (Figure 2A), suggesting that in aqueous solution  $\alpha$ -synuclein is essentially unstructured and displays a rapidly interconverting ensemble of structural conformations. The addition of increasing amounts of ceftriaxone slightly modified the far-UV spectrum of  $\alpha$ -synuclein. In presence of the ligand, the CD spectrum of  $\alpha$ -synuclein, obtained by subtraction of the ligand's contribution from the spectrum of the  $\alpha$ -synuclein-ligand complex, is characterized by a decrease of the intensity of the negative band. These changes confirm the binding of ceftriaxone with the monomeric form of  $\alpha$ -synuclein, though this interaction did not induce any stable  $\alpha$ -helix or  $\beta$ -sheet conformations of  $\alpha$ -synuclein.

The binding affinity of ceftriaxone toward  $\alpha$ -synuclein can be clearly determined by the spectral changes in the far-UV CD spectra of  $\alpha$ -synuclein (Figure 2). To improve the signal-to-noise level, synchrotron radiation circular dichroism (SRCD) experiments have been performed at the Diamond beamline B23 module end station B. The  $\alpha$ -synuclein solution was titrated with increasing amount of ceftriaxone dissolved in 20% EtOH in Tris-HCl buffer. The value of the apparent dissociation constant  $K_d$  was determined by the nonlinear regression analysis of the  $\Delta A$  values of the CD

spectra at 195 nm with increasing ceftriaxone/ $\alpha$ -synuclein molar ratios. The stoichiometry of the ceftriaxone/AS complex was determined by Job's plot (data not shown).<sup>15</sup> This revealed that at low ceftriaxone concentrations two molecules of AS interacted with one molecule of ligand, while in the presence of one or more equivalents of ceftriaxone an equimolar binding was estimated. In this condition, the  $K_d$  value determined for the ceftriaxone/AS complex was  $0.53 \pm 0.2 \mu\text{M}$ .

To confirm the interaction of ceftriaxone with  $\alpha$ -synuclein, we also examined the near-UV CD spectrum of  $\alpha$ -synuclein in the 260–375 nm region. In this region, an absorption band attributable to the Lb transition, according to the Platt notation, of the Tyr side chain is observable at about 280 nm.<sup>16</sup> This band is highly sensitive to the environment and conformational changes of the Tyr side chains that even minor structural perturbations register a change in the CD signal as shown in Figure 3.

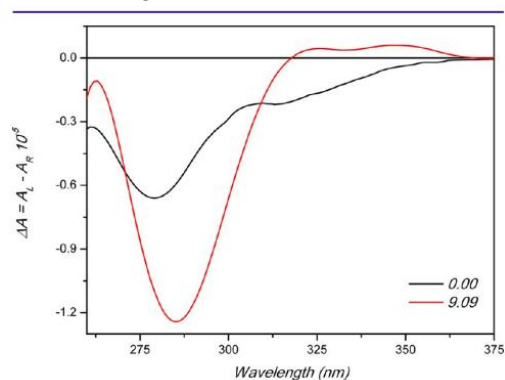


Figure 3. Near-UV CD spectra of  $\alpha$ -synuclein (5.01  $\mu\text{M}$ ).  $\alpha$ -Synuclein and ceftriaxone were dissolved in 5 mM Tris-HCl buffer, pH 6.8. Black line =  $\alpha$ -synuclein alone, red line =  $\alpha$ -synuclein in the presence of ceftriaxone (indicated). The CD spectrum of the  $\alpha$ -synuclein- ceftriaxone complex was corrected by subtraction of the near-UV CD spectra of equivalent amount of ceftriaxone.

CD spectroscopy has been also applied to study the secondary structure transition of amyloidogenic proteins from soluble monomers

to small oligomers.<sup>12,13</sup> This approach was used to analyze the capacity of ceftriaxone to inhibit the  $\alpha$ -synuclein aggregation.

The kinetic of  $\alpha$ -synuclein aggregation strongly depends on the composition and pH of medium as well as on the protein concentration. Aggregation studies were performed in phosphate buffered saline (PBS) solution. Phosphate buffers are the most suitable media for assessing protein conformation using CD,<sup>17</sup> while high concentration of chloride ions strongly absorb below 195 nm, hindering exploration of the far-UV region. With respect to concentration, we found that 6  $\mu$ M  $\alpha$ -synuclein exhibited a conformational transition from native to amyloid species within several days when incubated in a 0.1 cm path length CD quartz cells at 37 °C. The strong accelerating effect of surface in the fibril production was first described by Zhu and co-workers.<sup>18</sup> Independent preparations of  $\alpha$ -synuclein produced consistent data on the initial and final conformation of protein, as well as to the kinetics of the conformational transition. Importantly, the  $\alpha$ -synuclein concentration used in aggregation studies was comparable to the concentration used in the binding studies.

A solution of  $\alpha$ -synuclein (6.31  $\mu$ M, in 10 mM PBS, pH 6.8) was placed in two different 0.1 cm path length quartz cells (200  $\mu$ L), and 10  $\mu$ L of either ceftriaxone solution in PBS buffer or buffer (control) were added in the different cells. The cells were incubated at 37 °C for 11 days.

Freshly prepared  $\alpha$ -synuclein solutions, alone or in the presence of ceftriaxone, gave a far-UV CD spectra characteristic of the unfolded protein conformation (Figure 4). The far-UV CD spectrum of  $\alpha$ -synuclein alone, recorded during the 11 days of incubation, showed a gradual change in the shape: the intensity of negative band at about 200 nm decreased in time-dependent manner, and on the seventh day a positive band at about 210 nm and a negative band at about 230 nm appeared, indicating the

presence of partially folded structure with a significant contribution of the  $\beta$ -sheet structure (Figures 4A). The intensity of these two bands is strongly lower compared to the negative band in the starting solution: this is linked to the decrease of protein concentration due to the appearance of insoluble fibrils.

By plotting the CD values at 200 nm against time, a characteristic sigmoid curve was obtained, where three kinetic steps are present: a lag phase, an exponential growth phase, and a plateau phase (Figure 5).

Conversely, the CD spectrum of the  $\alpha$ -synuclein incubated in the presence of ceftriaxone is only initially modified by the addition of the inhibitor. Any further changes have been observed during the incubation time (Figures 4B and 5). The CD spectrum of  $\alpha$ -synuclein recorded at different times and corrected for the contribution of ligand resembled those of an unfolded protein conformation, in which the monomer is the predominant species in solution. The presence of ceftriaxone inhibits the exponential-growth phase with the formation of higher-molecular-weight protofibrils, and then fibrils.

Whether ceftriaxone prevents fibrillation by inhibiting protofibril assembly, by destabilizing protofibrils, or both, it is at the moment a topic of investigation. Our results suggest that ceftriaxone inhibits the aggregation process of  $\alpha$ -synuclein, probably acting in the protofibrils generation. In particular, it is believed that interactions between the C-terminus and the central portion of  $\alpha$ -synuclein can prevent or minimize its aggregation and fibril formation.<sup>19</sup> Moreover, the three Tyr residues in the C-terminus of  $\alpha$ -synuclein play a crucial role in fibril formation in vitro.<sup>20</sup> Indeed, this was completely inhibited when the three Tyr residues were replaced with Ala.<sup>20</sup> Importantly, it has been recently shown that binding of ceftriaxone to bovine serum albumin (BSA) is sufficient to perturb the environment in the proximity of Tyr

residues, making the latter one from nonpolar to slightly polar, with a slight increase of the hydrophilicity around Tyr residues.<sup>21</sup> By analogy, we may speculate that in our experimental model, the binding of ceftriaxone to  $\alpha$ -synuclein may have tempered the hydrophobicity related to Tyr residues in the C-terminus of  $\alpha$ -synuclein. This may lead to an increased compactness of monomeric  $\alpha$ -synuclein, which has been linked to its inhibition of fibril formation.<sup>22</sup>

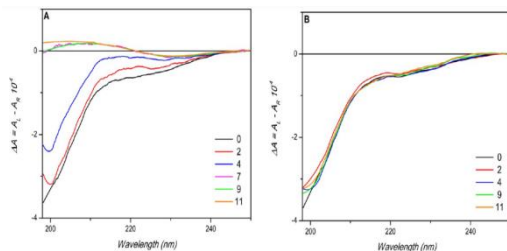


Figure 4. Far-UV CD spectra of  $\alpha$ -synuclein in 10 mM phosphate saline buffer, pH 6.8, alone (A) or in the presence of 5.0 equiv of ceftriaxone (B). Each solution was incubated at 37 °C for 11 days. The spectra were recorded at different times (days) as indicated.

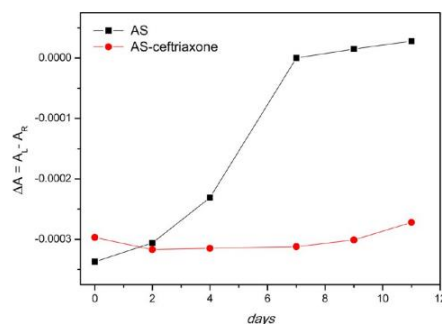


Figure 5. Time-course of the intensity of the CD band at 200 nm of  $\alpha$ -synuclein alone or in the presence of 5.0 equiv of ceftriaxone.

To characterize at molecular level the interactions between ceftriaxone and  $\alpha$ -synuclein, a computational docking study was carried out. The molecular mechanisms underlying the effectiveness of ceftriaxone in inhibiting  $\alpha$ -synuclein aggregation are unknown. We hypothesized that ceftriaxone should bind to a region involved in amyloid fibril formation. While the hydrophobic NAC region of  $\alpha$ -

synuclein is the most important site in the formation of amyloid-like fibrils, some of the residues present in the hydrophilic tail of the C-terminus, such as Tyr125, Tyr133, and Tyr136, are predicted to play a crucial role in fibril formation.<sup>20</sup> Thus, the binding to this region of a putative inhibitor should affect  $\alpha$ -synuclein aggregation and prevent intracellular accumulation of misfolded protein. Accordingly, although the protein topology presents a micelle binding state, the unfolded region of the protein on the hydrophilic tail (Asp98-Ala140) can be well suited as a docking target (Figure 6). Ceftriaxone was docked to the hydrophilic tail within residues Asp98-Ala140 using the MOE-Dock default parameters based on the simulated annealing protocol, and crucial protein–ligand interactions were analyzed. The coordinates for  $\alpha$ -synuclein protein (PDB code: 1XQ8) were used for computational docking.

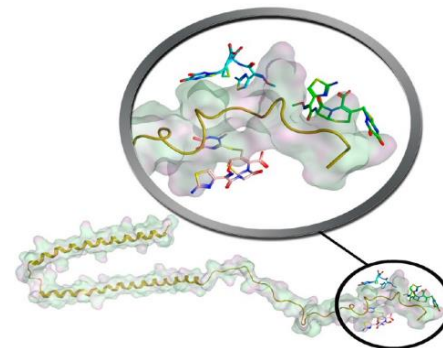


Figure 6. Overall topology of the micelle-bound  $\alpha$ -synuclein. Among the helix-N and helix-C, connected by a short linker, the unstructured tail spanning the hydrophobic region is shown. The  $\alpha$ -synuclein protein is represented by cartoon and molecular surfaces, whereas the three poses of ceftriaxone are shown in green, cyan, and pink sticks. The enlarged view (in circle) of the active site shares disposition of the three poses between the residues involved in molecular recognition.

Analysis of the results showed similar binding modes for 30 best-scoring poses in the docking cluster, within the amino acid pocket located in the carboxy-terminus of the peptide chain



(COOH hairpin). In particular, top-ranked energy poses of ceftriaxone bind to three main sites of the protein (Figures 7 and 8) within residues 122–139. Comparable estimated free binding energy values ( $\Delta G_b \sim -18$  kcal/mol) for these three conformations of ceftriaxone would support the affinity for this region.

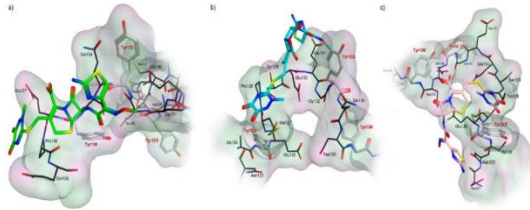


Figure 7. In silico docking of three independent top-ranked energy poses of ceftriaxone to the hydrophilic tail of the  $\alpha$ -synuclein. The protein is represented by molecular surfaces, and all the amino acid residues and ligand which are involved in mutual molecular interactions are shown as wireframes and sticks, respectively.

(a–c) Hypothetical disposition and proposed binding modes of ceftriaxone within the putative binding pockets of  $\alpha$ -synuclein target protein. Ceftriaxone binds to three sites (depicted in a–c)

located within the residues 122–139. Key tyrosine amino acid triad is indicated in red.

Most important H-bond interactions among protein residues and ceftriaxone are highlighted in dashed yellow/gray lines.

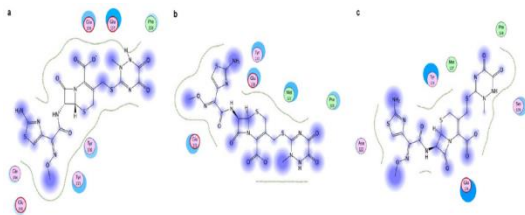


Figure 8. Interaction for the poses with the most favorable binding energies of ceftriaxone (a–c)

onto the  $\alpha$ -synuclein hydrophilic tail binding site. Amino acid residues (circles) are shown as follows: hydrophobic residues (green interior), polar residues (light purple), basic residues (blue ring), and acidic residues (red ring). Differences in solvent accessible surface area for ceftriaxone and for protein residues are plotted as a blue smudge and a violet halo, respectively. The

border of the binding pocket is marked by a dashed green line.

As far as the binding modes on target sites are concerned, the first conformation places the thiazol-2-amine moiety in close proximity to Glu131, Tyr133, Gln134, Tyr136, Glu137, Pro138, and Glu139. The amide nitrogen of ceftriaxone interacts by hydrogen bond with the key residue Tyr136, whereas favorable van der Waals interaction with Glu131 was observed by the methoxyimino functionality (Figures 7a and 8a). Otherwise, the orientation of the 1,2-dihydro-1,2,4-triazine-5,6-dione ring away from the pocket shows important H-bond contacts between the triazine N–H and Glu137.

Ceftriaxone also binds to  $\alpha$ -synuclein in an alternate conformation, where the triazine-dione backbone is reversed of  $180^\circ$  compared to the previous pose. More specifically, the hypothetical disposition of the 1,2,4-triazine-5,6-dione is predicted to establish good affinities within the pocket that includes Met127, Pro128, Glu130, Glu131, and Tyr133. (Figures 7b and 8b). In the third conformation, the triazine-5,6-dione behaves similarly to that of the second pose, since this ring system results in significant contacts with amino acid residues within the pocket formed by Asn122, Tyr125, Glu126, Met127, Pro128, and Ser129 (Figures 7c and 8c). More specifically, both the amidic and the triazine-5,6-dione nitrogens of the ceftriaxone are engaged in consistent H-bonds with Glu126 and Met127, respectively.

It is worth nothing that since the residues Tyr125, Tyr133, and Tyr136 are predicted to play a role in AD, interfering with these amino acids would be relevant for designing novel inhibitors able to prevent  $\alpha$ -helical and  $\beta$ -strands formation. It seems increasingly likely that the early oligomers or protofibrils during fibrillization process are actually the major cytotoxic species responsible for neuronal cell death, whereas the mature fibrils, such as Lewy bodies, are the inert metabolic products that may

protect cells against degeneration. This implies that the reduction of the oligomeric intermediate by inhibiting protein oligomerization is the goal for therapeutic use in neurodegenerative diseases. Therefore, our finding that ceftriaxone can interact with  $\alpha$ -synuclein, inhibiting its aggregation, could be of potential utility in the treatment of PD. During the preparation of this paper, Leung and co-workers<sup>6</sup> reported the efficacy of ceftriaxone in neuroprotection of dopaminergic neurons and amelioration of motor deficits in a rat model of Parkinson's disease. The authors associated these effects to an upregulation of GLT1 expression and/or to a redistribution of GLT1 as responses of ceftriaxone treatment in the Parkinson model. Other researchers demonstrated that ceftriaxone increased not only GLT1 expression but also its function.<sup>23,24</sup> Ceftriaxone shows other neuroprotective effects that may be related to its side chain, D- $\alpha$ -aminoadipic acid. This moiety was readily carbonylated upon oxidative damage, preventing the carbonylation of endogenous target,<sup>25</sup> such as DJ-1 protein, which was linked to Parkinson's and Alzheimer's diseases.<sup>26,27</sup> The D- $\alpha$ -aminoadipic acid is an antagonist of the NMDA receptors.<sup>28,29</sup> Therefore, ceftriaxone might also function as a noncompetitive antagonist of the NMDA receptors and thus attenuate glutamate excitotoxicity. Additionally, the capability of ceftriaxone to reduce inflammation and apoptosis has been recently demonstrated.<sup>30</sup>

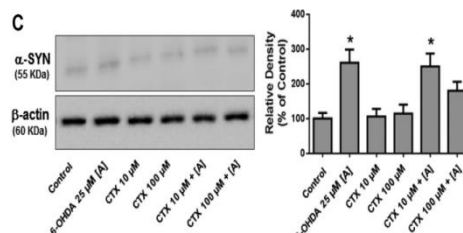
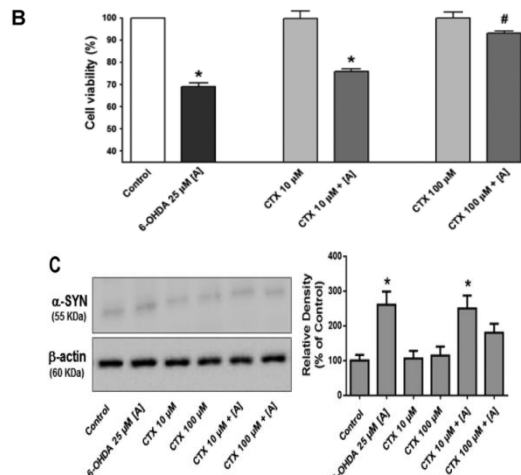
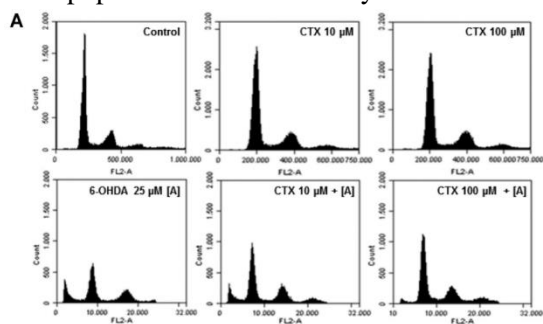


Figure 9. Flow cytometry analysis (A), MTT assay (B), and  $\alpha$ -synuclein ( $\alpha$ -SYN) immunoreactivity (Western Blot analysis (C)) of PC12 cells exposed to 6-hydroxydopamine (6-OHDA) and ceftriaxone (CTX). PC12 cells were treated with 10 or 100  $\mu$ M CTX 30 min before exposure to 6-OHDA (25  $\mu$ M, [A]). The analyses were performed 24 h after CTX administration. \* $p < 0.05$  vs control; #  $p < 0.05$  vs 6-OHDA.

Considering the Leung groups's experimental findings in an animal model of PD<sup>6</sup> and our data on the molecular interaction between ceftriaxone and  $\alpha$ -synuclein, we thought to study the in vitro effects of ceftriaxone on viability and  $\alpha$ -synuclein expression of PC12 cells exposed to 6-OHDA. Indeed, it is known that the treatment of neural cells with 6-OHDA reduces cell viability,<sup>31</sup> induces apoptosis,<sup>32–34</sup> and increases  $\alpha$ -synuclein immunoreactivity.<sup>32,33</sup> In particular, in our in vitro model, the exposition of PC12 cells to 25  $\mu$ M 6-OHDA increased the hypodiploid (apoptotic) DNA content (Figure 9A) and reduced cell availability of about 30% (Figure 9B). Ceftriaxone at the concentration of 10  $\mu$ M was unable both to prevent apoptosis and to reduce cell death. Instead, at the concentration of 100  $\mu$ M, it was able to significantly revert cell apoptosis and restore PC12 viability. Even higher concentrations of ceftriaxone (up to 1 mM) failed to completely revert 6-OHDA-

induced apoptosis in PC12 cells (data not shown). Our studies on PC12 cell expression of  $\alpha$ -synuclein (Figure 9C), instead, showed that the exposure of PC12 cells to 25  $\mu$ M 6-OHDA significantly increased the  $\alpha$ -synuclein signal while ceftriaxone alone (10 and 100  $\mu$ M) did not modify the cellular  $\alpha$ -synuclein expression. The exposure of PC12 cells to 6-OHDA 25  $\mu$ M, after ceftriaxone pretreatment at 100  $\mu$ M, resulted in a not significant increase of  $\alpha$ -synuclein expression, when compared to control group, while a lower dosage of ceftriaxone (10  $\mu$ M) failed to reduce the 6-OHDA-induced increase of  $\alpha$ -synuclein immunoreactivity.

In particular, in our study, 6-OHDA was able to upregulate the PC12 cellular expression of an oligomeric species of  $\alpha$ -synuclein, which migrated in gel electrophoresis at an apparent molecular mass of 55 kDa. This 55 kDa  $\alpha$ -synuclein has a molecular weight considerably higher than that of the natively unfolded  $\alpha$ -synuclein monomer (14.5 kDa) frequently reported in cells and brain tissue.<sup>34</sup> Recent findings indicate that the 55 kDa  $\alpha$ -synuclein likely represent a helically folded, tetramer.<sup>34</sup>

The biochemical mechanisms related to the neurotoxic effects of 6-OHDA on PC12 catecholaminergic cells are only partially understood. Taken together, our molecular interaction data and our in vitro findings documenting that pretreatment with ceftriaxone induces a down-regulation of  $\alpha$ -synuclein expression in PC12 cells treated with 6-OHDA, and consequently a decrease in the probability of oligomer and fibril formation,<sup>35</sup> suggest a possible, direct molecular interaction between ceftriaxone and the 55 kDa  $\alpha$ -synuclein with consequent reduction of  $\alpha$ -synuclein pathogenicity. Nevertheless, how ceftriaxone exactly interacts with  $\alpha$ -synuclein to reduce cellular  $\alpha$ -synuclein expression remains unclear and needs to be further evaluated.

In conclusion, we found that ceftriaxone binds with good affinity to  $\alpha$ -synuclein, blocks its in

vitro polymerization, and partially protects PC12 cells against 6-OHDA-induced damage. The data, in addition to the findings on the neuroprotective activity of ceftriaxone on Parkinson-like neurodegeneration, indicates that the treatment with ceftriaxone, or other molecules with similar functional properties, may be a novel and highly effective approach to the treatment of PD and other neurodegenerative disorders related to intracellular accumulation and aggregation of misfolded proteins.

### III. METHODS

**Expression of  $\alpha$ -Synuclein.**  $\alpha$ -Synuclein was expressed in Escherichia coli strain BL21(DE3) from a pET-28 plasmid according to the procedure described by Huang et al.<sup>36</sup> The  $\alpha$ -synuclein concentration was determined by absorption spectroscopy ( $\epsilon = 5960 \text{ M}^{-1} \text{ cm}^{-1}$  at 280 nm).

**Circular Dichroism and  $\alpha$ -Synuclein Titration.** CD spectra were obtained using a nitrogen flushed Jasco J-715 spectropolarimeter (Tokyo, Japan) using either a 1.0 cm (near-UV) or a 0.1 cm (far-UV) quartz cell (Hellma Ltd.). SRCD spectra from 180 to 260 nm were collected at the beamline B23 module end station B, with bandwidth of 1.2 nm, integration time of 1s, 1 nm digital resolution, 39 nm/min scan speed, and two repeated scans per spectrum, using Suprasil cells (Hellma Ltd.) with 0.1 cm path length. Titration was performed in aqueous buffer (5 mM Tris-HCl, pH 6.8) at 25 °C by addition of small aliquots of ceftriaxone stock solution in 2:8 v/v EtOH/Tris-HCl buffer. The contribution of the ceftriaxone alone, evaluated by control CD titrations, was subtracted from the CD spectra of the  $\alpha$ -synuclein-ceftriaxone complex. Ceftriaxone concentration was determined by absorption spectroscopy ( $\epsilon_{\text{ceftriaxone}} = 34\,000 \text{ M}^{-1} \text{ cm}^{-1}$  at 272 nm). The data are the mean of three different titrations, and the calculated error, as percentage difference, was less than 1%. The plot was constructed using OriginPro 8 software.



The dissociation constant  $K_d$  ( $K_d = 1/K_a$ ) of the complex was determined by analyzing the CD data at a single wavelength by a nonlinear least-squares computer fitting to the equation based on 1:1 binding stoichiometry:

$$\Delta A = \left\{ \left[ (\Delta \epsilon_1 - \Delta \epsilon_0)(1 + K_a P + K_a L) - \sqrt{(1 + K_a P + K_a L)^2 - 4K_a^2 PL} \right] / 2K_a \right\} + \Delta \epsilon_0 P$$

where  $\Delta \epsilon_0$  and  $\Delta \epsilon_1$  are the differential molar extinction coefficients ( $M^{-1} \text{ cm}^{-1}$ ) either of protein alone or bound to ligand, respectively.  $L$  and  $P$  represent the total concentration ( $\mu M$ ) of ligand and protein, respectively, and  $K_a$  is the association constant ( $M^{-1}$ ) for the ligand-protein interaction.<sup>37</sup>

**Aggregation Studies.** A solution of  $\alpha$ -synuclein  $6.3 \mu M$  in  $10 \text{ mM}$  PBS,  $\text{pH } 6.8$ , was prepared and added with  $0.03\%$   $\text{NaN}_3$  to avoid any bacterial growth. Solution was filtered through a  $0.1 \mu m$  filter to remove any aggregated material, and  $200 \mu L$  of this solution was placed in two different  $0.1 \text{ cm}$  quartz cells. Ceftriaxone was dissolved in PBS and added to one cell containing the  $\alpha$ -synuclein solution to achieve a final concentration of  $30 \mu M$  in a total volume of  $210 \mu L$ . In the other cell,  $10 \mu L$  of buffer was added. Samples were incubated in  $37^\circ \text{C}$  for 11 days. At different times, the far-UV CD spectrum of each solution was recorded in the  $190\text{--}250 \text{ nm}$  range.

**Computational Methods.** Ligand docking studies were performed using Molecular Operating Environment (MOE), 2009.10.<sup>38</sup> The NMR structure of  $\alpha$ -synuclein used for docking experiment was retrieved from PDB Data Bank (<http://www.rcsb.org/>, PDB code: 1XQ8).<sup>39</sup> AMBER99 force field was selected, and partial charges and protonation state were assigned with a generalized Born solvation model, considering the ionization state of the amino acid residues involved in the binding pocket at  $\text{pH} \sim 7$ . The structure of ceftriaxone was built in MOE and minimized before the docking using the MMFF94x force field, with

the systematic algorithms, until a RMSD gradient of  $0.001 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$  was reached. A systematic conformational search was carried out and 120 different conformations were stored in a search database. Since the experimentally determined first protonation constant (for the carboxylic group of ceftriaxone) is  $\text{pK}_a 1 = 2.37,40$  we presumed that it predominantly exists in the monodeprotonated form under  $\text{pH} \sim 7$  or in physiological conditions, and the compound was modeled for docking in this way. Rigid receptor-flexible ligand docking calculation was performed using the docking simulation feature MOE-dock by setting grid sizes that included the entire macromolecules. The triangle matcher was used as placement method to generate docking poses,<sup>41</sup> and the London  $\Delta G_{\text{binding}}$  scoring function that estimates the free energy of binding (in kilocalories per mole,  $\text{kcal/mol}$ ) was used to rank hit candidates, after a force field based refinement and rescoring.

**Cell Culture and Treatments.** Rat pheochromocytoma PC12 cells were grown in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM-F12) containing 10% horse serum, 5% fetal bovine serum (FBS), and 1% penicillin ( $5000 \text{ U/mL}$ )/streptomycin ( $5000 \mu\text{g/mL}$ ) at  $37^\circ \text{C}$  under humidified 5%  $\text{CO}_2/\text{air}$ . Cells were exposed to increasing concentrations of ceftriaxone ( $10\text{--}100 \mu M$ ) alone or associated with a 6-OHDA ( $25 \mu M$ ) for 24 h. For each experiment,  $100 \times 10^3$  PC12 cells/ $\text{cm}^2$  were plated and treated 24 h later.

**Flow Cytometric Analysis.** Cells undergoing apoptosis were detected by staining with propidium iodide (PI) in the dark according to the manufacturer's instructions (Sigma). PC12 cells were seeded at a density of  $1.5 \times 10^6$  cells in a 6-well plate. After 24 h incubation of cells in a humidified 5%  $\text{CO}_2$  air incubator and  $37^\circ \text{C}$ , cells were exposed to different concentration of ceftriaxone ( $10$  and  $100 \mu M$ ) and 6-OHDA ( $25 \mu M$ ). After 24 h, culture medium and PC12

cells were harvested and washed twice by centrifugation (3000 rpm, 5 min) in ice-cold PBS. Cell pellets were suspended in 250  $\mu$ L of 10  $\mu$ g/mL PI/RNase (RNA with a ribonuclease) in PBS and stored at 4 °C in the dark. The stained cells were analyzed after 1 h using flow cytometry (Accuri C6, BD Biosciences).

**MTT Assay.** PC12 cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) reduction assay. The metabolic dye MTT is reduced by viable cells to form blue formazan crystals. In brief, the cells were incubated with 1 mg of MTT per milliliter of medium, and the cultures were allowed to incubate at 37 °C for 4 h. The MTT was removed, and the cells were rinsed with PBS and centrifuged at 4000 rpm for 15 min. Thereafter, the supernatant was discarded, and the pellet was dissolved in 2 mL of isopropanol; after centrifugation at 4000 rpm for 5 min, the color was read at 600 nm using a Bauty Diagnostic microplate reader. Experiments were done in triplicate. A standard curve was constructed using different concentrations of cells at the start of every experiment.

**Western Blot Analysis.** After rinsing cell cultures with ice-cold PBS, cell lysis was performed in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP40, 150 mM NaCl, 1 mM EDTA) containing 1 mM PMSF and a protease inhibitor cocktail (Sigma). A clear supernatant was obtained by centrifugation of lysates at 12 000g for 10 min. Protein content was determined using Bradford protein assay. Equal amounts of protein extracts were resolved by standard SDS/PAGE. Samples were electroblotted onto Protran nitrocellulose membranes (Schleicher & Schuell GmbH). Membranes were incubated with 3% low-fat milk in 1 $\times$  PBS-Tween 0.05% solution with the indicated antibody for 16 h at 4 °C. Anti-rabbit IgG (whole molecule) peroxidase antibody was used to reveal

immunocomplexes by enhanced chemiluminescence (Pierce).

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