

# A Novel “Molecular Tweezer” Inhibitor of $\alpha$ -Synuclein Neurotoxicity *in Vitro* and *in Vivo*

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**Summary** Aggregation of  $\alpha$ -synuclein ( $\alpha$ -syn) is implicated as being causative in the pathogenesis of Parkinson’s disease, multiple system atrophy, and dementia with Lewy bodies. Despite several therapies that improve symptoms in these disorders, none slow disease progression. Recently, a novel “molecular tweezer” (MT) termed CLR01 has been described as a potent inhibitor of assembly and toxicity of multiple amyloidogenic proteins. Here we investigated the ability of CLR01 to inhibit assembly and toxicity of  $\alpha$ -syn. *In vitro*, CLR01 inhibited the assembly of  $\alpha$ -syn into  $\beta$ -sheet-rich fibrils and caused disaggregation of pre-formed fibrils, as determined by thioflavin T fluorescence and electron microscopy.  $\alpha$ -Syn toxicity was studied in cell

cultures and was completely mitigated by CLR01 when  $\alpha$ -syn was expressed endogenously or added exogenously. To determine if CLR01 was also protective *in vivo*, we used a novel zebrafish model of  $\alpha$ -syn toxicity ( $\alpha$ -syn-ZF), which expresses human, wild-type  $\alpha$ -syn in neurons.  $\alpha$ -Syn-ZF embryos developed severe deformities due to neuronal apoptosis and most of them died within 48 to 72 h. CLR01 added to the water significantly improved zebrafish phenotype and survival, suppressed  $\alpha$ -syn aggregation in neurons, and reduced  $\alpha$ -syn-induced apoptosis.  $\alpha$ -Syn expression was found to inhibit the ubiquitin proteasome system in  $\alpha$ -syn-ZF neurons, resulting in further accumulation of  $\alpha$ -syn. Treatment with CLR01 almost completely

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mitigated the proteasome inhibition. The data suggest that CLR01 is a promising therapeutic agent for the treatment of Parkinson's disease and other synucleinopathies.

**Key Words** Parkinson's disease · zebrafish · synucleinopathy · amyloid · neuroprotection

## Introduction

Many neurodegenerative disorders are associated with accumulation and aggregation of misfolded proteins; in Parkinson's disease (PD), multiple system atrophy, and dementia with Lewy bodies, that protein is  $\alpha$ -synuclein ( $\alpha$ -syn). Several lines of evidence implicate  $\alpha$ -syn in the pathogenesis of PD. Rare mutations or duplication of the *SNCA* gene, which encodes  $\alpha$ -syn, cause dominantly inherited PD, and  $\alpha$ -syn is the major component of Lewy bodies and Lewy neurites, the hallmark lesions in the brain of patients with PD [1–5]. Increased concentration of wild-type (WT)  $\alpha$ -syn due to genetic or environmental insults appears to be sufficient for increasing the risk of developing PD, and in high enough levels it can cause PD [5–8].

CLR01 is a “molecular tweezer” [9] that inhibits the aggregation and toxicity of multiple amyloidogenic proteins, including those involved in Alzheimer's disease and type 2 diabetes, amyloid  $\beta$ -protein (A $\beta$ ), tau, and islet amyloid polypeptide, respectively [10]. The mechanism by which CLR01 inhibits the self-assembly process of amyloidogenic proteins is interfering with a combination of hydrophobic and electrostatic interactions by binding to Lys residues [10].  $\alpha$ -Syn contains 15 Lys residues (10.5% of the sequence), and therefore we expected that CLR01 would inhibit its aggregation and toxicity efficiently. We show several lines of evidence that CLR01 may be of therapeutic benefit in neurodegeneration due to toxic  $\alpha$ -syn assemblies. First, CLR01 inhibited the assembly of  $\alpha$ -syn fibrils and caused disaggregation of preformed fibrils. Second, the compound completely blocked  $\alpha$ -syn toxicity in cell lines when expressed endogenously or added exogenously. Third, in a novel  $\alpha$ -synuclein zebrafish ( $\alpha$ -syn-ZF) model of neurotoxicity, CLR01 reduced neuronal apoptosis, improved the abnormal phenotype, and extended survival.

## Methods

### Thioflavin T Fluorescence Assay

Recombinant  $\alpha$ -syn was purchased from rPeptide (Bogart, GA).  $\alpha$ -Syn (100  $\mu$ M) in phosphate-buffer saline (PBS) containing 10 mM sodium phosphate, pH 7.4, and 0.02% (w/v) sodium azide, was incubated at 37°C with

mechanical agitation in the presence or absence of different concentrations of CLR01 or CLR03. Ten- $\mu$ l aliquots of the aggregating solution were mixed with 100  $\mu$ l of 20- $\mu$ M thioflavin T (ThT) (Sigma-Aldrich, St. Louis, MO) in PB [11]. The fluorescence was measured following 5-minute incubation at  $\lambda_{ex}$ =452 nm and  $\lambda_{em}$ =485 nm, using a Hitachi F4500 spectrofluorometer (Hitachi Instruments, Rye, NH). To measure disaggregation,  $\alpha$ -Syn was incubated under the same conditions and 10-fold excess CLR01 were added on day 8, toward the end of the fibril elongation phase (disaggregation reaction D1), or on day 24 at which point elongation was no longer observed and the fibrils were assumed to be mature (D2).

### Electron Microscopy

Aliquots from the aggregation or disaggregation experiments were diluted 10-fold in water and 10  $\mu$ l were spotted on glow-discharged, carbon-coated Formvar grids (Electron Microscopy Science, Hatfield, PA), fixed with 5  $\mu$ L 2.5% glutaraldehyde and stained with 5  $\mu$ L 4% uranyl acetate. The samples were analyzed using a CX 100 transmission electron microscope (JEOL, Peabody, MA).

### Monitoring $\alpha$ -Syn Assembly by Gel Electrophoresis

$\alpha$ -Syn was treated with 1,1,1,3,3,3-hexafluoroisopropanol as previously described [12]. Dry protein films were kept at  $-20^{\circ}$ C until use. Films were reconstituted in 10 mM sodium phosphate, pH 7.4, containing 0.02% sodium azide at a final  $\alpha$ -syn concentration of 100  $\mu$ M in the absence or presence of 1 mM CLR01 or CLR03. Samples were agitated at 37°C for 11 days and aliquots were taken from each condition daily, flash frozen on dry ice, and stored at  $-80^{\circ}$ C. These aliquots were fractionated on 4 to 20% gradient Tris-Glycine native gels (Invitrogen, Eugene, OR) or on 10 to 20% gradient Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Invitrogen) and stained with SilverXpress (Invitrogen), according to the manufacturer's protocol.

### Analysis of $\alpha$ -Syn Incubated in the Presence of $^3$ H-CLR01

$\alpha$ -Syn was prepared as previously described. Films were reconstituted in sodium phosphate (pH 7.4) containing 0.02% sodium azide at a final  $\alpha$ -syn concentration of 100  $\mu$ M in the absence or presence of 25  $\mu$ Ci/ $\mu$ l  $^3$ H-CLR01 or 1  $\mu$ Ci/ $\mu$ l  $^3$ H $_2$ O as a control. Samples were agitated at 37°C for 11 days and then fractionated on a 10 to 20% gradient Tricine SDS/PAGE gel (Invitrogen). The gel was stained with Coomassie brilliant blue, scanned, and cut into sections, which were further cut in to small pieces and radioactivity was counted in Ultima Gold Liquid Scintillation Cocktail

(Perkin Elmer, Waltham, MA) using a Hidex Triathler Liquid Scintillation Counter (Hidex, Turku, Finland) (model 425-034). The data are expressed as disintegrations per minute.

#### Analysis of $\alpha$ -Synuclein Incubated in the Presence of “Molecular Tweezers” by Mass Spectrometry

$\alpha$ -Syn was prepared as previously described. Films were reconstituted in sodium phosphate (pH 7.4) containing 0.02% sodium azide at a final  $\alpha$ -syn concentration of 100  $\mu$ M in the absence or presence of 1 mM CLR01 or CLR03. The molecular weight of the protein in each sample was measured by liquid-chromatography–electrospray-ionization mass spectrometry using a C18 reversed-phase high-performance liquid chromatography (HPLC) column interfaced to either an electrospray ionization (ESI)-time-of-flight (Agilent Technologies, Inc., LC-TOF, Santa Clara, CA) or a Fourier-transform ion cyclotron resonance (Bruker Daltonics Inc., 15T solariX FT-ICR, Billerica, MA) mass spectrometer. The measured average molecular mass (14,460 Da) of the native protein is consistent with the calculated mass (14,460.11). The mass of the protein incubated in the presence of CLR01 or CLR03 was identical to that of  $\alpha$ -syn alone within experimental error. These results strongly suggest that MT do not bind covalently to the protein.

#### Toxicity of Endogenous, Conditionally Expressed $\alpha$ -Syn

Human embryonic kidney (HEK) cells were grown in DMEM/F12 supplemented with 10% fetal bovine serum and 1% Pen/Strep. HEK cells were transfected with pTet-On (Clontech, Mountain View, CA) expressing rTA and grown with neomycin for selection. Human  $\alpha$ -syn cDNA was cloned into pTRE-Tight-BI-DsRed Express (pTRE-syn-DsRed; Clontech). Cells were grown to 50% confluency and transfected with pTRE-syn-DsRed using SuperFect (Qiagen Inc., Valencia, CA). Twenty-four hours after transfection, doxycycline (0.1  $\mu$ g/ml) was added to the media to induce expression of both  $\alpha$ -syn and DsRed under the control of the bidirectional tetracycline response element (TRE) promoter. Expression of  $\alpha$ -syn was confirmed by Western blot (WB) and DsRed by epifluorescence. Three days after induction, cell numbers and cell death (measured using propidium iodide) were determined using a Beckman Coulter XL-MCL flow cytometer (Beckman Coulter, Inc., Brea, CA) as previously described [13].

#### Toxicity Assay Using Exogenous $\alpha$ -Syn

Rat pheochromocytoma (PC-12) cells were maintained in F-12 nutrient mixture with Kaighn’s modification (F-12K) (Gibco BRL, Carlsbad, CA) supplemented with 15% heat-inactivated horse serum and 2.5% fetal bovine serum at 37°C in an atmosphere of 5% CO<sub>2</sub>. For cell viability assays, cells were plated in

96-well plates at a density of 25,000 cells per well in differentiation media (F-12K, 0.5% FBS, 100  $\mu$ M nerve growth factor) and maintained for 48 h.  $\alpha$ -Syn (200  $\mu$ M) was incubated in culture media in the presence or absence of CLR01 or CLR03 for 24 h at 37°C prior to adding to the cells. Then 10  $\mu$ L of the solution were added to the cells at final  $\alpha$ -syn concentration 20  $\mu$ M. Cell viability was assessed by the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) after 24 h of incubation as previously described [14].

#### Zebrafish (*Danio rerio*) Maintenance and cDNA Injections

Zebrafish (ZF) were maintained and bred in tanks with recirculating water at 28°C on a regular light-dark cycle [15]. The  $\alpha$ -syn gene construct was composed of a T2A bicistronic configuration expressing monomeric DsRed driven by the ZF neuron-specific *HuC* promoter (accession #AF173984) (Fig. S4). The T2A sequence is an 18-amino acid peptide that is self-cleaving in all eukaryotic cells tested [16]. The *HuC* promoter was cloned into a modified pBluescript vector (Agilent Technologies, Inc., LC-TOF) containing a *Xho* I-*Afl*III fragment of the pEGFP-N1 vector [17]. T2A cDNA was cloned into pDsRed-Monomer N1 vector (Clontech) using polymerase chain reaction (PCR) and *Bam*HI and *Age*I restriction sites. The T2A-DsRed cDNA was ligated into the *HuC*-vector and human  $\alpha$ -syn was cloned into the *HuC*-T2A-DsRed construct using *Sac* II and *Xma* I restriction sites. The *HuC*-GFP<sup>u</sup> construct was made using a GFP<sup>u</sup> fragment provided by Bence et al. [18].

Circular plasmid DNA (50 ng/ $\mu$ l) diluted in elution buffer (Qiagen, MD) containing 1% phenol red was injected into ZF eggs at the 1 cell stage using a microinjector. When two constructs were co-injected, each construct was diluted to 30 ng/ $\mu$ l to limit toxicity.

#### Immunohistochemistry

Embryos were dechorionated with forceps and fixed with 4% paraformaldehyde in PBS at 4°C overnight. They were washed twice, immersed in 30% sucrose, embedded in OCT, and cryosectioned (10- $\mu$ m sections). Sectioned slides were fixed with 4% paraformaldehyde, washed twice, dried, and stored at -20°C until staining. Sections were thawed, sequentially incubated in 50% methanol/PBS, 100% methanol, and 50% methanol/PBS followed by 2 washes. They were blocked in 10% normal goat serum, incubated with mouse anti- $\alpha$ -syn antibody (BD Transduction Laboratories, San Jose, CA) overnight at 4°C. The sections were then washed twice, incubated with Alexa-Fluor 488-conjugated goat anti-mouse secondary antibody for 2 h, washed, and cover slipped with mounting medium containing 4'-6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA).

## Acridine Orange Staining

Embryos were manually dechorionated at 24 h post-fertilization (hpf) and incubated with 10  $\mu\text{g/ml}$  acridine orange (Invitrogen) for 1 h. They were then washed, anesthetized with tricaine, and imaged.

## WBs

ZF embryos (48 hpf) were de-yolked in fish ringers buffer and lysed in 1% triton high-salt lysis buffer. Protein concentrations were measured using the DC Protein assay/Lowry method (Bio-Rad Laboratories, Hercules, CA). SDS-PAGE and WB analysis were performed as previously described [19]. Antigen-antibody complexes were visualized using chemiluminescence reagent (Perkin Elmer, Waltham, MA) and bands were normalized to actin using an anti-actin antibody (Sigma-Aldrich).

## RNA Quantification

ZF embryos were injected with *Hu*C-T2A-DsRed plasmid DNA, and treated with CLR01 or vehicle from 8 to 72 hpf by addition to the fish water. Embryos were lysed and total RNA was isolated using the RNAqueous-4PCR kit (Life Technologies/Ambion, Carlsbad, CA). Reverse transcription polymerase chain reaction using primers specific for DsRed (F: 5'-AACGGCCACTACTTCGAGAT-3'; R: 5'-CTTGGAGCCGTACTGGA-3'), ZF  $\beta$ -actin (accession # AF057040), and ZF 18S RNA (accession # BX296557) [20] was performed using the AgPath-ID One-Step RT-PCR kit (Life Technologies/Ambion). Resulting cDNA products were analyzed by electrophoresis on a 2% agarose gel with ethidium bromide and photographed using a digital gel imaging system (Fotodyne, Hartland, WI). Band intensity was quantified using National Institutes of Health (NIH) imaging software. *Hu*C abundance was measured relative to both ZF  $\beta$ -actin and ZF 18S. Results were obtained from 3 fully independent experiments.

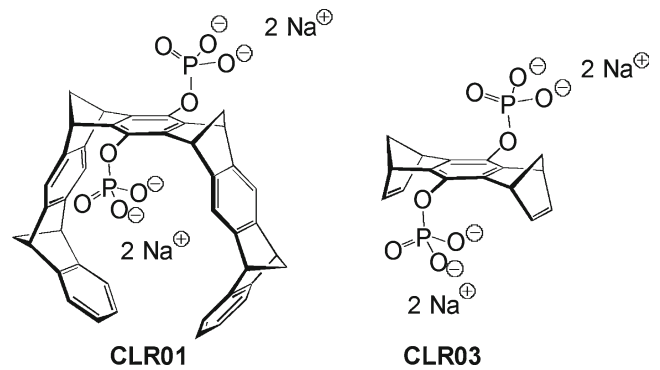
## Statistical Analysis

All statistical analysis was performed using Student's *t* test or analysis of variance as appropriate, with a minimum significance level set at  $p < 0.01$ .

## Results

### CLR01 Inhibits $\alpha$ -Syn Aggregation

Initial tests examined whether CLR01 (Fig. 1) could inhibit the assembly of  $\alpha$ -syn into fibrils [11]. In the absence of



**Fig. 1** Structure of the molecular tweezers CLR01 and CLR03.

CLR01, following a lag phase of  $\sim 100$  h, ThT fluorescence increased monotonously up to  $\sim 250$  h, after which the fluorescence signal reached a plateau. CLR01 was found to inhibit  $\alpha$ -syn fibrillogenesis in a dose-dependent manner. Nearly complete inhibition of  $\beta$ -sheet formation was observed at a 1:1  $\alpha$ -syn:CLR01 concentration ratio and partial inhibition was observed at 10:1  $\alpha$ -syn:CLR01 ratio (Fig. 2a), suggesting that CLR01 inhibited both the nucleation and the elongation of  $\alpha$ -syn fibrils. In contrast, 10-fold molar excess of the control compound, CLR03, an inactive MT derivative (Fig. 1), had no effect on  $\alpha$ -syn assembly (Fig. 2a). At the end of the reaction, samples were examined by electron microscopy. The control samples showed abundant fibrils, whereas in the presence of CLR01, but not CLR03, only nonfibrillar morphology was observed (Fig. 2b).

### CLR01 Disaggregates $\alpha$ -Syn Fibrils

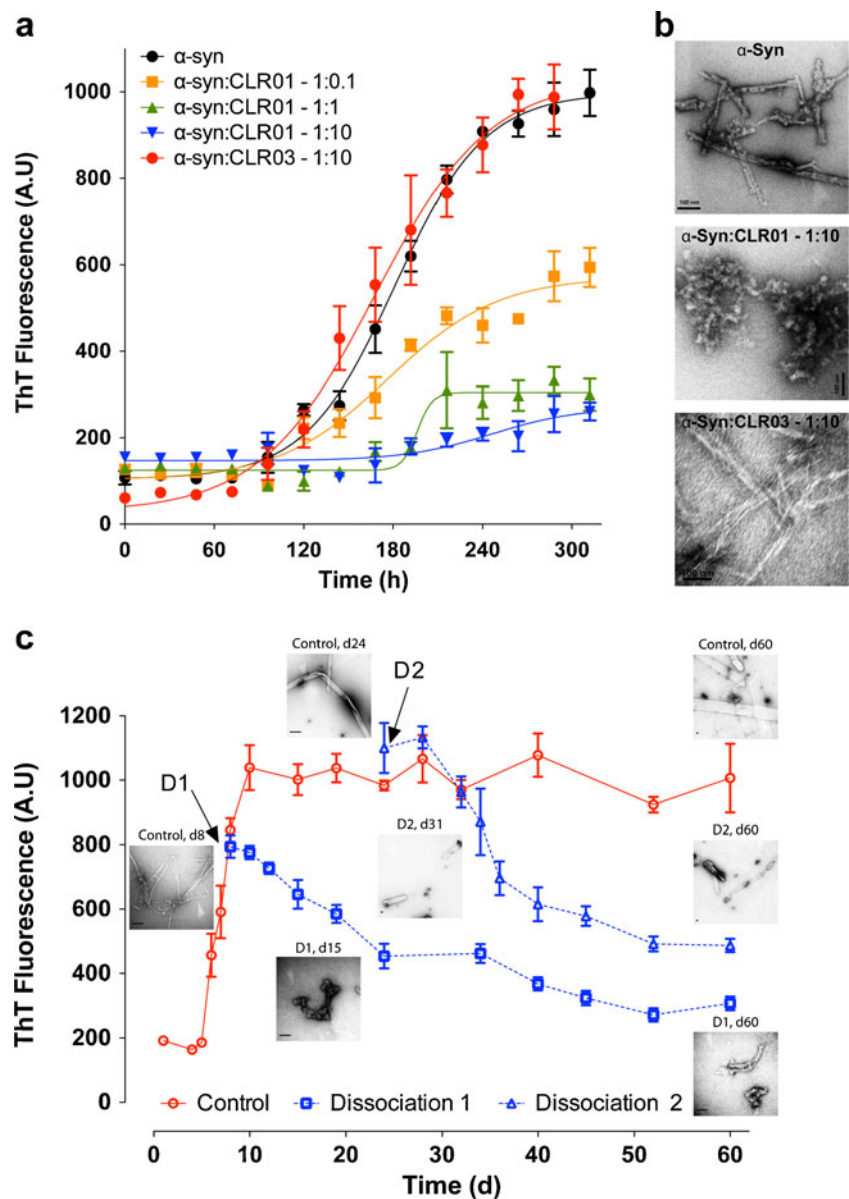
To test whether CLR01 could disaggregate pre-formed  $\alpha$ -syn fibrils,  $\alpha$ -syn was allowed to aggregate for 60 days (Fig. 2c) and 10-fold excess CLR01 were added on day 8, in the middle of the growth phase (D1), or on day 24, when the ThT signal had been stable for more than a week (D2). In both disaggregation reactions, following addition of CLR01, a gradual decrease in the ThT fluorescence signal was observed, suggesting the arrest of fibril growth and dissociation of existing fibrils. Morphological examination confirmed the disappearance of fibrils and appearance of nonfibrillar aggregates. These results demonstrate that CLR01 not only inhibits aggregation of  $\alpha$ -syn but also dissociates existing fibrils.

### CLR01 Accelerates $\alpha$ -Syn Oligomerization

To determine the state of soluble  $\alpha$ -syn in the presence of CLR01, we subjected purified  $\alpha$ -syn to PAGE.  $\alpha$ -Syn migrated as a predominant band of 127 kD on Clear native



**Fig. 2** CLR01 inhibits  $\alpha$ -synuclein ( $\alpha$ -syn) assembly and disaggregates  $\alpha$ -syn *in vitro*. **(a)**  $\beta$ -Sheet formation in  $\alpha$ -syn in the absence or presence of different concentrations of CLR01 or CLR03 was followed by measuring thioflavin T (ThT) (Sigma-Aldrich) fluorescence. **(b)** Electron micrographs of  $\alpha$ -syn in the presence or absence of 10-fold molar excess of CLR01 or CLR03 at the end of the reactions shown in panel A. Scale bars denote 100 nm. **(c)** Disaggregation of  $\alpha$ -syn fibrils by CLR01 was initiated at 2 time points: on day 8 (dissociation reaction D1) or on day 24 (D2), and the reactions were monitored using ThT fluorescence. Electron micrographs were obtained periodically and show the morphology of  $\alpha$ -syn at the indicated time points. Scale bars: 100 nm. The data are an average of 3 independent experiments.



gels, presumably corresponding to an oligomer. In addition, there was a band of 238 kD and a higher molecular weight smear of approximately 700 to 900 kD, presumably corresponding to higher molecular weight oligomers (Fig. S1A–S1C). In the presence of CLR01 (Fig. S1B), a similar pattern to that of  $\alpha$ -syn alone or with the inactive MT CLR03 was observed, but the higher molecular weight smear was much more prominent. These data suggest that CLR01 promotes the formation and/or stabilization of high molecular weight oligomers.

In SDS-PAGE gels,  $\alpha$ -syn migrated as a predominant band corresponding to a monomer of 17 kD. In addition, adjacent bands, above and below the monomer band, were observed with increasing time of incubation (Fig. S1D–S1F). The slower migrating band likely represents a dimer and the

faster migrating bands likely correspond to breakdown products. The abundance of these breakdown products increased with time and were unlikely produced by bacteria, because the protein was incubated in the presence of 0.02% sodium azide. In the presence of CLR01, but not CLR03, additional higher molecular weight (MW) bands appeared after 3 days of incubation representing higher (MW) oligomers (Fig. S1E). These data from SDS-PAGE suggest that CLR01 stabilizes small, nontoxic  $\alpha$ -syn oligomers, consistent with our previous findings that CLR01 promotes formation of similar, nontoxic A $\beta$  oligomers [10]. However, whether or not these bands represent actual oligomers *in vivo* is difficult to determine using SDS-PAGE [21], and they are clearly different than the size of the oligomers seen on native gels.

### CLR01 Does Not Covalently Bind to or Modify $\alpha$ -Syn

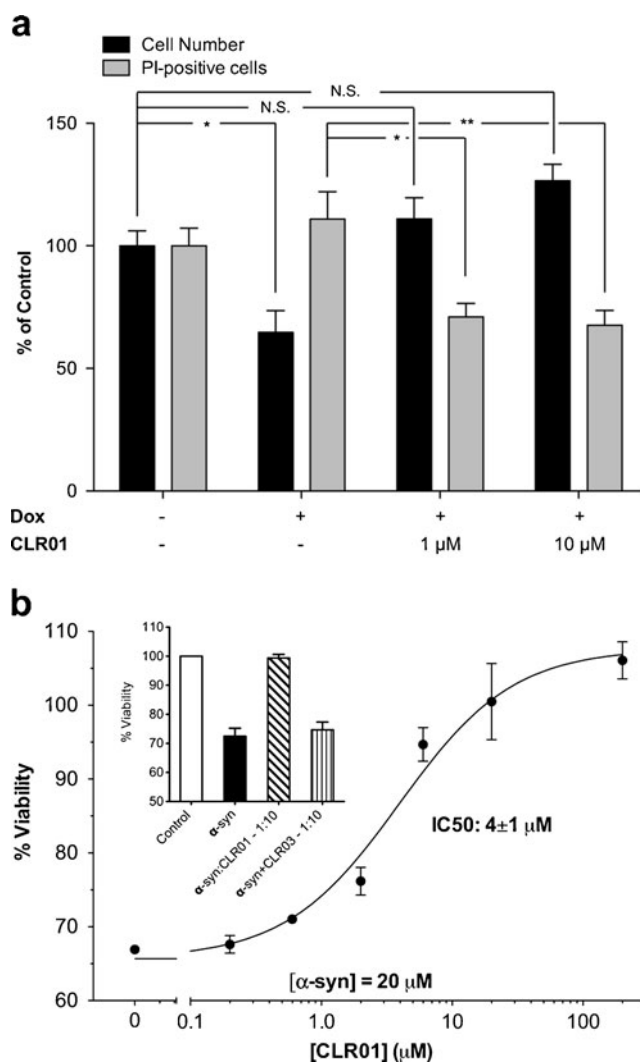
We performed SDS-PAGE on purified  $\alpha$ -syn that had been incubated with  $^3\text{H}$ -CLR01 or  $^3\text{H}_2\text{O}$  as a control (Fig. S2) to determine if CLR01 covalently binds to  $\alpha$ -syn. The results clearly demonstrate that CLR01 is not stably associated with  $\alpha$ -syn under these conditions (Fig. S2). Furthermore, we performed mass spectrometry on  $\alpha$ -syn incubated in the presence of MTs and found no modifications when it was incubated with CLR01 or CLR03 (Fig. S3).

### CLR01 Inhibits $\alpha$ -Syn Toxicity in Cell Culture

To determine if CLR01 attenuated intracellular  $\alpha$ -syn toxicity, we used HEK cells conditionally expressing human, wild-type  $\alpha$ -syn. There was no detectable  $\alpha$ -syn expression before induction in these cells, but robust expression was found 3 h after adding doxycycline as determined by WB analysis (Fig. S5). Induction of  $\alpha$ -syn expression for 2 days resulted in a decrease in HEK cell number ( $p < 0.0003$ ) of almost 40%, but no change in propidium iodide staining (Fig. 3a). This suggested that  $\alpha$ -syn expression either killed the cells by necrosis and/or reduced cell proliferation. Therefore, we measured proliferation using bromodeoxyuridine (BrdU) incorporation and found that it was lowered by  $34 \pm 6\%$  when  $\alpha$ -syn was expressed ( $p < 0.007$ ), indicating cell proliferation was reduced. When CLR01 was simultaneously added with induction of  $\alpha$ -syn expression, cell numbers did not decrease at  $1 \mu\text{M}$  CLR01 and they even increased slightly at  $10 \mu\text{M}$  CLR01 (Fig. 3a). The number of propidium iodide (PI)-positive cells significantly decreased by the addition of CLR01, but the numbers of BrdU-positive cells remained unchanged ( $66\%$  vs  $72\%$  of controls;  $p = 0.6$ ). Taken together, CLR01 ameliorates  $\alpha$ -syn toxicity in this model by reducing cell death without altering proliferation.

Until recently, a toxicity assay in which  $\alpha$ -syn is added exogenously to cultured cells would have been dismissed as physiologically irrelevant because  $\alpha$ -syn was believed to be strictly intracellular. However, recent demonstrations that extracellular  $\alpha$ -syn oligomers can induce intracellular  $\alpha$ -syn aggregation in neurons and that  $\alpha$ -syn can be transmitted from cell-to-cell to cause neurotoxicity [22–24] suggest that toxicity assays using exogenous  $\alpha$ -syn are relevant. The advantage of using such assays is that the concentration and assembly state of  $\alpha$ -syn can be controlled more accurately than with endogenously expressed  $\alpha$ -syn.

Twenty  $\mu\text{M}$   $\alpha$ -syn incubated for 24 h at  $37^\circ\text{C}$  caused  $\sim 35\%$  decrease in cell viability measured by the MTT reduction assay (Fig. 3b), whereas freshly dissolved  $\alpha$ -syn at the same concentration was not toxic, suggesting that the 24-h incubation promoted formation of neurotoxic  $\alpha$ -syn oligomers. CLR01 demonstrated dose-dependent inhibition of toxicity induced



**Fig. 3** CLR01 inhibits  $\alpha$ -synuclein ( $\alpha$ -syn) toxicity in cell culture. **(a)** CLR01 inhibits endogenously expressed  $\alpha$ -syn toxicity in HEK293 cells.  $\alpha$ -Syn expression was induced by adding doxycycline (Dox) in the absence or presence of CLR01. Cell numbers and cell death measured using propidium iodide were determined by flow cytometry ( $N = 12$  per condition;  $*p < 0.0003$ ;  $**p < 0.007$ ). **(b)** Differentiated PC-12 cells were treated with  $20 \mu\text{M}$   $\alpha$ -syn incubated in the absence or presence of increasing concentrations of CLR01 for 48 h and cell viability was measured using the MTT assay. Inset: Viability of PC-12 cells treated similarly with  $\alpha$ -syn in the presence or absence of 10-fold molar excess of CLR01 or CLR03. The data are an average of at least 3 independent experiments with 6 wells per condition. Not significant (NS) = xxx; PI = xxx.

by  $20\text{-}\mu\text{M}$   $\alpha$ -syn with half-maximal inhibition ( $\text{IC}_{50}$ ) value of  $4 \pm 1 \mu\text{M}$  (Fig. 3b). In contrast, 10-fold molar excess CLR03 had no effect, as expected (Fig. 3b inset).

### CLR01 Protects ZF Embryos from $\alpha$ -Syn Neurotoxicity

To test the effect of CLR01 on  $\alpha$ -syn neurotoxicity *in vivo*, we used a novel ZF model that expresses human, wild-type  $\alpha$ -syn in its neurons. ZF are vertebrates and have a well-

developed central nervous system similar to that of mammals [25]. The embryos develop quickly and are transparent, facilitating monitoring of protein expression and other cellular processes using fluorescent reporter genes. We devised a construct comprising *HuC*, a neuronal ZF promoter, human  $\alpha$ -syn, monomeric DsRed as a reporter gene, and a T2A peptide inserted between the  $\alpha$ -syn and DsRed sequence, which is cleaved post-translationally releasing the native sequence of each protein (*HuC*- $\alpha$ -syn-T2A-DsRed) (Fig. S4) [16]. Using this construct,  $\alpha$ -Syn was expressed in neurons as a fusion protein with DsRed, but it rapidly cleaved to release native  $\alpha$ -syn. This allowed us to monitor expression of  $\alpha$ -syn in living embryos without altering the structure or function of the protein.

*HuC*- $\alpha$ -syn-T2A-DsRed was expressed in neurons starting at ~12 hpf and resulted in decreased survival compared to *HuC*-T2A-DsRed injected fish (Fig. 4). The survival of the ZF injected with *HuC*-T2A-DsRed was lower than that of the uninjected embryos due to the somewhat invasive nature of the microinjection. Surviving embryos injected with *HuC*- $\alpha$ -syn-T2A-DsRed showed robust  $\alpha$ -syn expression/DsRed fluorescence. Most of these embryos were deformed and almost all died by 240 hpf (Fig. 4). The phenotype of the  $\alpha$ -syn-expressing ZF was somewhat variable, ranging from a modest bend in the tail to grossly deformed embryos (Fig. 4b, c, e, f) compared to normal appearing *HuC*-T2A-DsRed expressing embryos (Fig. 4a and d). Because the *HuC* promoter leads to expression only in neurons, the deformation was due to dysfunction and death of critical neurons, especially in the spine, and fluorescent cells, which were never seen distal to the bend. The phenotype was not due to death of dopaminergic cells as the *HuC* promoter was not active in these cells at this stage of development. The high rate of deformity and lethality was not seen in the control embryos expressing *HuC*-T2A-DsRed and it was nearly identical in embryos injected with a *HuC*- $\alpha$ -syn construct lacking T2A and DsRed (Fig. 4a and d), indicating that the toxicity was caused by  $\alpha$ -syn expression.

CLR01 was added to the water 8 hpf and markedly reduced  $\alpha$ -syn toxicity in a dose-dependent manner (Fig. 5). Ten  $\mu$ M CLR01 improved survival by 3-fold at 72 hpf and 13-fold at 240 hpf ( $p=5.3 \times 10^{-15}$ ). In addition, the fraction of normal-appearing embryos in those that survived also dramatically increased following addition of CLR01.

#### Aggregated $\alpha$ -Syn-Induced Neuronal Apoptosis is Rescued by CLR01

The deformities in the  $\alpha$ -syn-expressing embryos correlated with loss of DsRed positive neurons. To determine if the

loss of these neurons reflected apoptotic cell death, we stained the embryos with acridine orange.  $\alpha$ -Syn expression induced a marked increase in apoptosis as early as 24 hpf (Fig. 5b), but was suppressed in the presence of CLR01.  $\alpha$ -syn-induced apoptosis was confirmed by immunohistochemistry using an anti-active caspase 3 antibody (data not shown) [26].

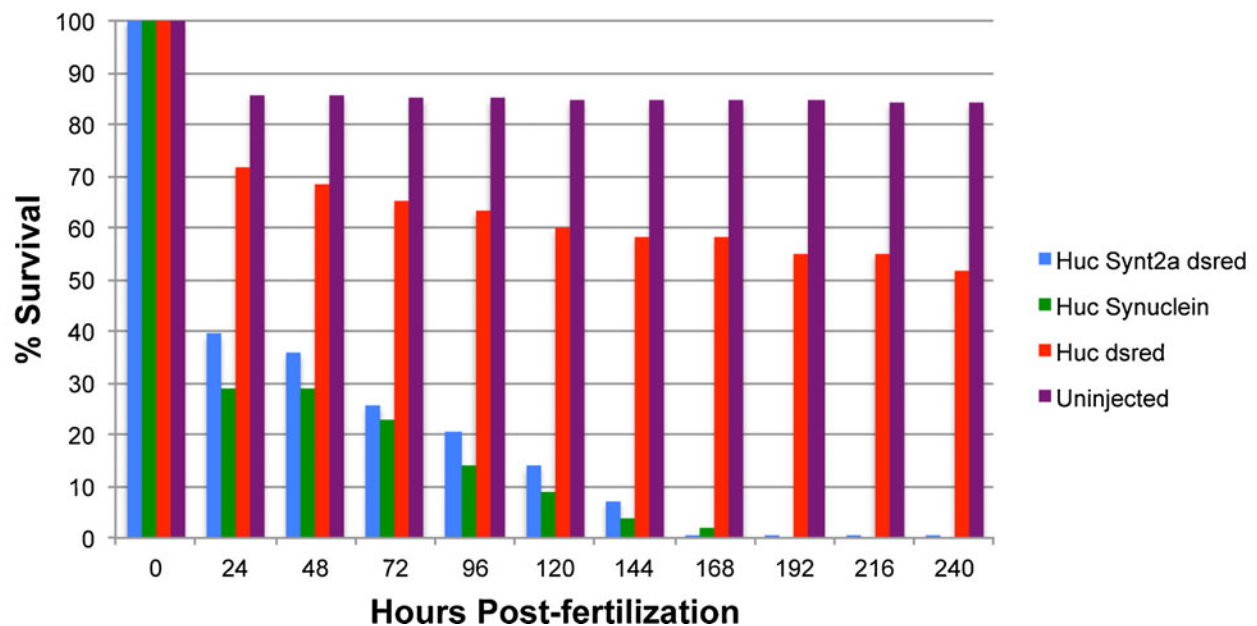
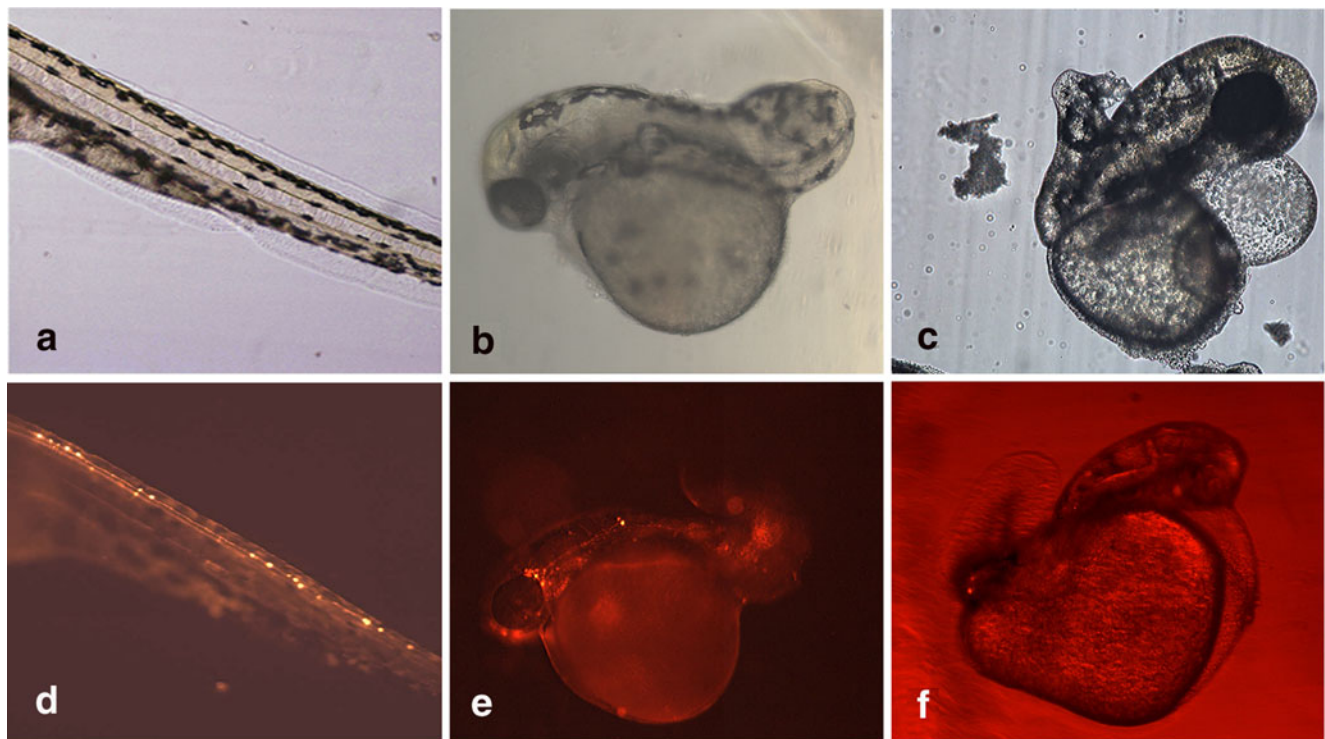
Because aggregated  $\alpha$ -syn is a pathological hallmark of PD, we asked whether similar aggregates could be detected in  $\alpha$ -syn-expressing ZF. We used immunohistochemistry with antibodies specific for human  $\alpha$ -syn and found that DsRed positive neurons had abundant small clumps that reacted with these antibodies (Fig. 6a and b). In contrast,  $\alpha$ -syn-expressing ZF embryos developing in the presence of CLR01 showed strong  $\alpha$ -syn reactivity, but this reactivity appeared completely soluble in all cases and aggregates or clumps were not detected (Fig. 6c–e). WB analysis confirmed complete cleavage of  $\alpha$ -syn and DsRed with and without CLR01 demonstrating that the change in  $\alpha$ -syn staining was not due to altered cleavage at the T2A site (Fig. 6f).

#### CLR01 Prevents $\alpha$ -Syn Inhibition of the Ubiquitin-Proteasome System

Aggregated proteins, such as those involved in neurodegenerative disorders, have been shown to inhibit protein degradation [18]. To determine if  $\alpha$ -syn degradation was altered in  $\alpha$ -syn-expressing ZF due to ubiquitin-proteasome system (UPS) inhibition, and whether CLR01 affected this system, we measured relative levels of  $\alpha$ -syn using WB in the presence or absence of CLR01 and/or the UPS inhibitor lactacystin. Embryos expressing *HuC*- $\alpha$ -syn-T2A-DsRed showed the expected  $\alpha$ -syn band at ~19 kDa and a DsRed band at 40 kDa (Fig. 6f). Complete cleavage of the fusion protein was confirmed by the lack of a 59 kDa band using either anti- $\alpha$ -syn or anti-DsRed antibodies.

$\alpha$ -Syn-expressing embryos treated with CLR01 contained significantly less  $\alpha$ -syn than untreated embryos, whereas DsRed levels remained unchanged (Fig. 6f). These data were consistent with the hypothesis that aggregated  $\alpha$ -syn decreased  $\alpha$ -syn degradation by the UPS and by preventing aggregation, CLR01 enabled clearance of  $\alpha$ -syn. This interpretation was supported by the observation that incubating CLR01-treated embryos with the UPS inhibitor lactacystin attenuated the degradation of  $\alpha$ -syn (and DsRed) and resulted in  $\alpha$ -syn levels similar to those in untreated embryos (Fig. 6f). Furthermore, we tested whether CLR01 altered expression of the *HuC* promoter using reverse transcription polymerase chain reaction and found that it had no effect relative to actin or 18S RNA (expression of CLR01 treated:untreated was  $1.01 \pm 0.07$  using actin as a reference and  $1.04 \pm 0.13$  using 18S RNA as a reference; N=3).





**Fig. 4** Overexpression of  $\alpha$ -synuclein ( $\alpha$ -syn) in neurons causes dysmorphic zebrafish (ZF) embryos and death. (a–f) DNA constructs were injected into ZF eggs at the 1 cell stage and embryos imaged at 48 hpf. Bright-field images are shown in (a–c) and fluorescent images in (d–f). *HuC*- $\alpha$ -syn-T2A-dsRed injected embryos were clearly dysmorphic with reduced numbers of DsRed and  $\alpha$ -syn

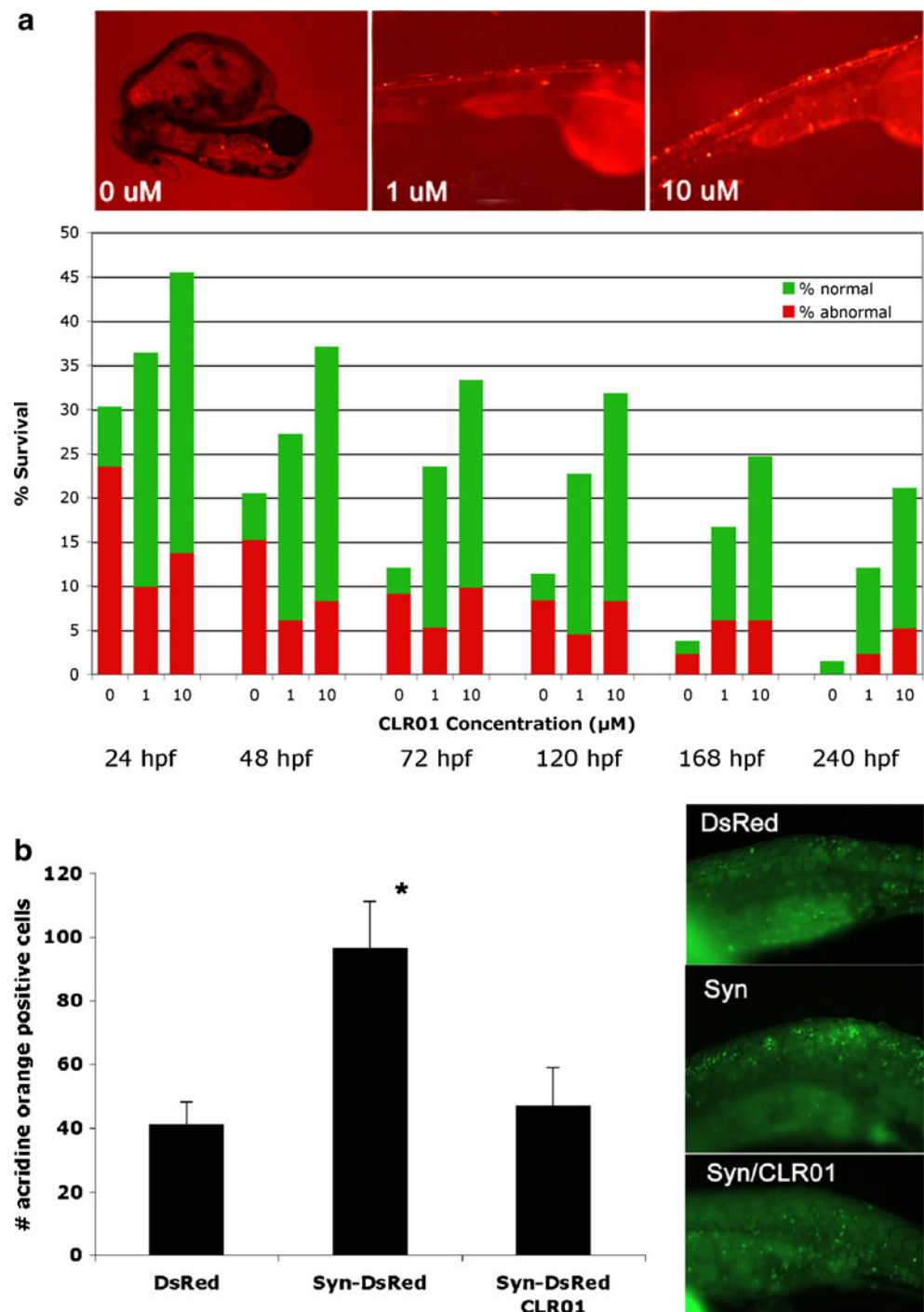
expressing neurons (b, c, e, and f). *HuC*-dsRed control injected embryos were larger, normal appearing, with abundant DsRed-expressing neurons (a and d). (g) Survival of *HuC*- $\alpha$ -syn-T2A-dsRed injected embryos was nearly identical to *HuC*- $\alpha$ -syn injected embryos confirming that the toxicity was caused by  $\alpha$ -syn expression.

The effect of  $\alpha$ -syn expression on UPS activity was also measured directly *in vivo* using a fluorescent degron (*HuC*-GFP<sup>u</sup>). Under normal conditions, this protein is rapidly degraded and green fluorescence

due to GFP accumulation is not detected. In contrast, when UPS degradation is impaired, GFP accumulates and can be detected by measuring its green fluorescence.



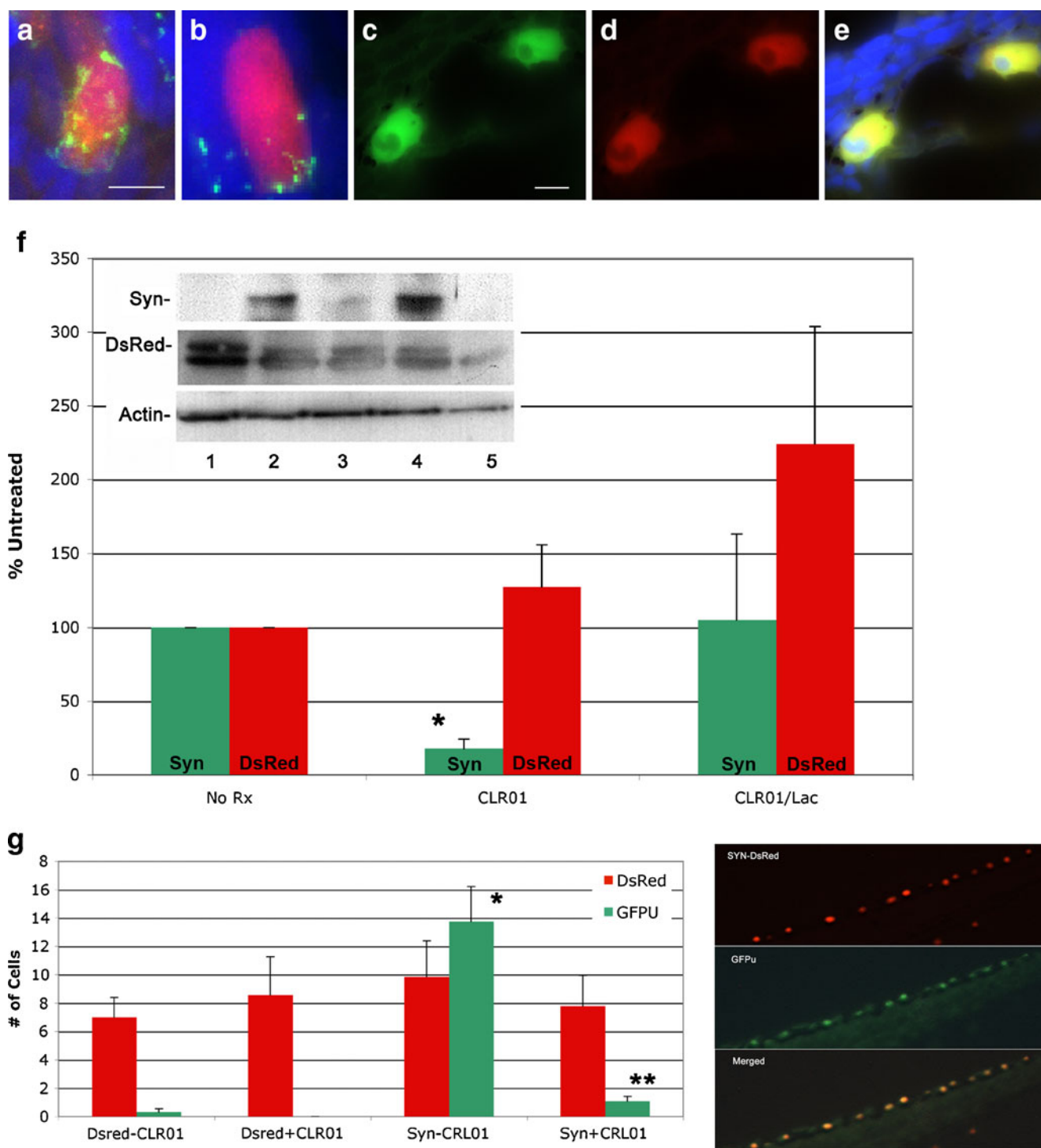
**Fig. 5** CLR01 ameliorates  $\alpha$ -synuclein ( $\alpha$ -syn) neurotoxicity in zebrafish (ZF). **(a)** ZF embryos were treated with CLR01 at 8 hpf and were monitored for abnormal appearance and survival. Bright-field and fluorescent overlay images were taken at 72 hpf (top). Green bars represent normal-appearing embryos and red bars represent abnormal embryos (N=132/condition). **(b)** CLR01 prevents  $\alpha$ -syn-induced apoptosis. ZF embryos expressing DsRed or  $\alpha$ -syn were incubated in acridine orange 24 hpf and apoptotic cells were counted (N=6 per condition). Ten  $\mu$ M CLR01 reduced  $\alpha$ -syn-induced apoptosis to control levels (\* $p$ <0.007 Syn-DsRed vs Syn-DsRed/CLR01 and DsRed control). Representative images are shown on the right.



Co-injection of *HuC*-GFP<sup>u</sup> with *HuC*-DsRed resulted in observation of only red neurons at 48 hpf and later, confirming an active UPS. When *HuC*-GFP<sup>u</sup> was co-injected with *HuC*- $\alpha$ -syn-T2A-DsRed, both green and red neurons were prevalent indicating inhibition of UPS by  $\alpha$ -syn. In contrast, *HuC*- $\alpha$ -syn-T2A-DsRed-expressing fish treated with CLR01 showed only red fluorescence in their neurons, suggesting that CLR01 prevented  $\alpha$ -syn-induced UPS inhibition and enabled the UPS to resume normal function (Fig. 6g) [27].

## Discussion

Several groups have reported inhibitors of  $\alpha$ -syn assembly and toxicity, including *N*-methylated peptides [28] dopamine and L-dopa [29], rifampicin [30], curcumin [31], and various other compounds [32–34]. In most cases, the compounds have been shown to inhibit  $\alpha$ -syn aggregation *in vitro*, whereas inhibition of toxicity was not studied. A few studies have reported assembly inhibitors, including



**Fig. 6** CLR01 prevents  $\alpha$ -synuclein ( $\alpha$ -syn) aggregation and proteasome inhibition. (a–e) ZF embryos expressing  $\alpha$ -syn-DsRed (72 hpf) were subjected to immunohistochemistry (IHC). Green represents anti- $\alpha$ -syn antibody binding, red is DsRed, and blue is DAPI-stained nuclei. (a, b) Representative neurons in untreated ZF. (c, d) CLR01-treated embryos. (e) Merged image of panels (c and d). (f)  $\alpha$ -Syn expression inhibits the UPS in ZF embryos. Embryos were lysed and proteins subjected to WB analysis (10 embryos per condition, N=4 experiments). Lane 1, DsRed control; lane 2,  $\alpha$ -syn-DsRed untreated; lane 3,  $\alpha$ -syn-DsRed CLR01 treated; lane 4,  $\alpha$ -syn-DsRed CLR01 and lactocystin treated; lane 5, wild-type untreated embryos. Optical densities for  $\alpha$ -syn

(green bars) and DsRed (red bars) were normalized to actin and expressed as the percentage of untreated controls ( $*p<0.0002$ ). (g) CLR01 prevents  $\alpha$ -syn aggregate-induced inhibition of the ubiquitin-proteasome system (UPS). *HuC*-syn-DsRed (N=19) or *HuC*-DsRed (N=13, control) constructs were co-injected with *HuC*-GFP<sup>U</sup> to determine UPS activity in neurons overexpressing  $\alpha$ -syn. Values represent the mean number of fluorescent cells in the tail region per embryo at 48 hpf. Green bars represent the number of GFP<sup>U</sup>-positive cells (low UPS activity), and red bars represent the number of DsRed-positive cells (high UPS activity). Representative images are shown on the right. ( $*p<0.0001$  vs DsRed-CLR01;  $**p<0.0002$  vs  $\alpha$ -syn-CLR01).

peptides and small molecules [35] that protected cells against  $\alpha$ -syn-induced toxicity, and encouraging results were reported with the green tea-derived polyphenol (–) epigallocatechin gallate [36]. A difficult problem has been to rationally design or select effective inhibitors with desired druggable characteristics. In most cases, the mechanism of action of the inhibitors used has been poorly understood. The importance of understanding the mechanism of inhibition has recently been highlighted [37] following a study suggesting that many inhibitors of fibrillogenesis may act nonspecifically, likely making them unsuitable for treating aberrant protein assembly-related diseases [38].

Here, we used CLR01, a general inhibitor of amyloidogenic protein assembly with a well-defined mechanism of action, which was selected based on first principles. We demonstrate that CLR01 interferes with  $\alpha$ -syn aggregation and mitigates its toxicity. Importantly, CLR01 was found to block the toxicity of both extracellular and intracellular  $\alpha$ -syn at similar concentrations to those needed for inhibiting  $\alpha$ -syn aggregation *in vitro*, supporting the hypothesis that CLR01 protected cells against  $\alpha$ -syn toxicity by inhibiting  $\alpha$ -syn aggregation. One novel aspect of this report is the generation of a ZF model of  $\alpha$ -syn neurotoxicity. We used a T2A bicistronic construct leading to  $\alpha$ -syn expression as a fusion protein with DsRed, which rapidly is cleaved, producing native  $\alpha$ -syn and at the same time facilitating the monitoring of the expression of  $\alpha$ -syn. After CLR01 treatment,  $\alpha$ -syn concentrations in  $\alpha$ -syn-ZF embryos were reduced, whereas DsRed levels did not change. These observations suggested that CLR01 led to improved clearance of  $\alpha$ -syn rather than affecting its expression. Indeed, in living embryos we found that the UPS was inhibited in neurons expressing  $\alpha$ -syn, and that this inhibition was prevented in ZF treated with CLR01.

The neurotoxicity of  $\alpha$ -syn is well established, but the mechanism of this toxicity and the pathogenic species that confer toxicity are less clear. Proposed mechanisms include impaired protein degradation (both UPS and autophagy), toxicity to synaptic terminals, inflammation, and induction of mitochondrial dysfunction [39]. Although  $\alpha$ -syn fibrils have been found in both humans and animal models of synucleinopathies, several lines of evidence suggest that nonfibrillar soluble oligomers of  $\alpha$ -syn are the most toxic species [40, 41].

Inhibition of the UPS system has been reported not only for  $\alpha$ -syn oligomers [42, 43], but also with toxic assemblies of other amyloidogenic proteins, including A $\beta$  [44] and islet amyloid polypeptide [45, 46]. A recent report demonstrated that overexpression of polyglutamine results in transient UPS inhibition in mice, but the activity returned when large inclusions formed [47]. Thus, amyloidogenic protein oligomers appear to act in a vicious manner akin to the human immunodeficiency virus, shutting down the very

system expected to rid them. Here, we demonstrate for the first time in an *in vivo* vertebrate model that  $\alpha$ -syn overexpression leads to UPS inhibition and that preventing  $\alpha$ -syn aggregation preserves UPS activity.

Several lines of evidence support a role for UPS dysfunction and the development of PD. Two known genetic causes of PD involve aspects of UPS function (Parkin and UCH-L1), and  $\alpha$ -syn is a substrate for the UPS. Reduced UPS activity has been found in brains of sporadic PD patients [48, 49] and some investigators have found that administration of UPS inhibitors to rodents can recreate some of the features of PD, although these models remain controversial [50–55]. Finally, we have found that several commonly used pesticides inhibit the UPS and are associated with an increased risk of developing PD [7, 13].

The finding that CLR01 promotes  $\alpha$ -syn clearance by maintaining  $\alpha$ -syn in a soluble, nonaggregated form, and alleviating UPS inhibition is also important when potential toxicity of CLR01 is considered. Ostensibly, Lys-specific MTs, including CLR01, might bind to exposed Lys residues in proteins other than those they are expected to inhibit. MTs were selected as inhibitors based on the hypothesis that the molecular interactions leading to formation of oligomers and nuclei of amyloidogenic proteins were sufficiently weak (hence, nucleus formation is rare and oligomer structure is metastable) to be inhibited by compounds that bind with moderate affinity. As the structure of naturally folded proteins has been optimized by evolution and is substantially more stable, this allows for less strict specificity requirements, because binding with moderate (micromolar) affinity to structurally stable proteins is not expected to affect their structure or activity, whereas labile binding of MTs to naturally unstructured proteins, such as  $\alpha$ -syn inhibits their aberrant assembly and prevents their toxicity. Because proteasomal degradation depends on attachment of ubiquitin to free Lys residues, the results presented here demonstrate that despite binding specifically to Lys, CLR01 does not prevent ubiquitination of  $\alpha$ S, supporting our hypothesis.

It has also been reported that  $\alpha$ -syn could impair autophagy, especially in dopamine-producing cells [56]. Inhibition of autophagy is unlikely the cause of toxicity in the ZF model used here because treatment of the embryos with an UPS inhibitor along with CLR01 restored  $\alpha$ -syn levels. In addition,  $\alpha$ -syn requires the interaction with dopamine for inhibiting chaperone-mediated autophagy, and here  $\alpha$ -syn was expressed primarily in nondopamine-producing neurons [56].

In summary, we show here that CLR01 inhibits  $\alpha$ -syn aggregation and toxicity both *in vitro* and *in vivo*. Encouragingly, CLR01 dramatically improved clearance of  $\alpha$ -syn in a vertebrate model by restoring UPS activity, suggesting that maintaining  $\alpha$ -syn in a benign, unaggregated form may be sufficient for alleviating its neurotoxic effects.

Our findings have important therapeutic implications because  $\alpha$ -syn aggregation is believed to be central to the pathogenesis of PD and other synucleinopathies, and CLR01 inhibits this process without any apparent adverse effects.

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