



UNIVERSITÀ  
DEGLI STUDI  
FIRENZE

## FLORE

# Repository istituzionale dell'Università degli Studi di Firenze

### **Multistep Inhibition of $\alpha$ -Synuclein Aggregation and Toxicity in Vitro and in Vivo by Trodusquemine**

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

*Original Citation:*

Multistep Inhibition of  $\alpha$ -Synuclein Aggregation and Toxicity in Vitro and in Vivo by Trodusquemine / Perni M, Flagmeier P, Limbocker R, Cascella R, Aprile FA, Galvagnion C, Heller GT, Meisl G, Chen SW, Kumita JR, Challa PK, Kirkegaard JB, Cohen SIA, Mannini B, Barbut D, Nollen EAA, Cecchi C, Cremades N, Knowles TPJ, Chiti F, Zaslhoff M, Vendruscolo M, Dobson CM. - In: ACS CHEMICAL BIOLOGY. - ISSN 1554-8929. - STAMPA.

*Availability:*

The webpage <https://hdl.handle.net/2158/1132660> of the repository was last updated on 2018-08-20T15:53:50Z

*Published version:*

DOI: 10.1021/acscchembio.8b00466

*Terms of use:*

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

*Publisher copyright claim:*

La data sopra indicata si riferisce all'ultimo aggiornamento della scheda del Repository FloRe - The above-mentioned date refers to the last update of the record in the Institutional Repository FloRe

(Article begins on next page)

1 **Multistep Inhibition of  $\alpha$ -synuclein aggregation and toxicity *in vitro***  
2 **and *in vivo* by trodusquemine**

3  
4 Michele Perni<sup>1,2,+</sup>, Patrick Flagmeier<sup>1,2,+</sup>, Ryan Limbocker<sup>1,2,+</sup>, Roberta Cascella<sup>3</sup>,  
5 Francesco A. Aprile<sup>1,2</sup>, Céline Galvagnion<sup>1,4</sup>, Gabriella T. Heller<sup>1,2,+</sup>, Georg Meisl<sup>1,2</sup>, Serene W.  
6 Chen<sup>1,2</sup>, Janet R. Kumita<sup>1,2</sup>, Pavan K. Challa<sup>1,2</sup>, Julius B. Kirkegaard<sup>5</sup>, Samuel I. A. Cohen<sup>1,2</sup>,  
7 Benedetta Mannini<sup>1,2</sup>, Denise Barbut<sup>6</sup>, Ellen A. A. Nollen<sup>7</sup>, Cristina Cecchi<sup>3</sup>, Nunilo Cremades<sup>8</sup>,  
8 Tuomas P. J. Knowles<sup>1,2,9</sup>, Fabrizio Chiti<sup>3\*</sup>, Michael Zasloff<sup>6,10\*</sup>, Michele Vendruscolo<sup>1,2,\*</sup> and  
9 Christopher M. Dobson<sup>1,2,\*</sup>

10  
11 <sup>1</sup>*Department of Chemistry, University of Cambridge, Cambridge CB2 1EW, UK.*

12 <sup>2</sup>*Centre for Misfolding Diseases, Department of Chemistry, University of Cambridge, Cambridge*  
13 *CB2 1EW, UK.*

14 <sup>3</sup>*Department of Experimental and Clinical Biomedical Sciences,*  
15 *University of Florence, Florence 50134, Italy.*

16 <sup>4</sup>*German Centre for Neurodegenerative Diseases, DZNE, Sigmund-Freud-Strasse. 27, 53127,*  
17 *Bonn, Germany*

18 <sup>5</sup>*Department of Applied Mathematics and Theoretical Physics,*  
19 *University of Cambridge, Cambridge CB3 0WA, UK.*

20 <sup>6</sup>*Enterin Inc., 3624 Market Street, Philadelphia, PA 19104, USA.*

21 <sup>7</sup>*University Medical Centre Groningen, European Research Institute for the Biology of Aging,*  
22 *University of Groningen, Groningen 9713 AV, The Netherlands.*

23 <sup>8</sup>*Biocomputation and Complex Systems Physics Institute (BIFI)-Joint Unit BIFI-IQFR (CSIC),*  
24 *University of Zaragoza, 50018 Zaragoza, Spain.*

25 <sup>9</sup>*Department of Physics, Cavendish Laboratory, University of Cambridge,*  
26 *Cambridge CB3 0HE, UK.*

27 <sup>10</sup>*MedStar-Georgetown Transplant Institute, Georgetown University School of Medicine,*  
28 *Washington, DC 20010, USA.*

29  
30 <sup>+</sup>These authors made equal contributions

31 \*Corresponding authors: fabrizio.chiti@unifi.it, maz5@georgetown.edu, mv245@cam.ac.uk,  
32 cmd44@cam.ac.uk

33

34

35

### Abstract

36

37 **The aggregation of  $\alpha$ -synuclein, an intrinsically disordered protein that is highly abundant**  
38 **in neurons, is closely associated with the onset and progression of Parkinson's disease. We**  
39 **have shown previously that the aminosterol squalamine can inhibit the lipid induced**  
40 **initiation process in the aggregation of  $\alpha$ -synuclein and we report here that the related**  
41 **compound trodusquemine is capable of inhibiting not only this process but also the fibril-**  
42 **dependent secondary pathways in the aggregation reaction. We further demonstrate that**  
43 **trodusquemine can effectively suppress the toxicity of  $\alpha$ -synuclein oligomers in neuronal**  
44 **cells, and that its administration, even after the initial growth phase, leads to a dramatic**  
45 **reduction in the number of  $\alpha$ -synuclein inclusions in a *Caenorhabditis elegans* model of**  
46 **Parkinson's disease, eliminates the related muscle paralysis, and increases lifespan. On the**  
47 **basis of these findings, we show that trodusquemine is able to inhibit multiple events in the**  
48 **aggregation process of  $\alpha$ -synuclein, and hence to provide important information about the**  
49 **link between such events and neurodegeneration, as it is initiated and progresses. We**  
50 **suggest, in addition, that trodusquemine has the potential to be a therapeutic candidate in**  
51 **Parkinson's disease and related disorders, for these effects as well for its ability to cross the**  
52 **blood brain barrier and promote tissue regeneration.**

53

54

55

56

57 The aggregation of  $\alpha$ -synuclein, a 140-residue protein highly expressed in neuronal synapses<sup>1-5</sup>,  
58 is a hallmark of the pathogenesis of a variety of neurodegenerative disorders collectively known  
59 as  $\alpha$ -synucleinopathies, including Parkinson's disease (PD), PD with dementia, dementia with  
60 Lewy bodies, and multiple-system atrophy<sup>5-12</sup>. The mechanism of aggregation of  $\alpha$ -synuclein is  
61 highly complex and is modulated by a variety of environmental factors, such as pH, temperature,

62 ionic strength, and the presence of co-solvents, and by its interactions with a range of cellular  
63 components, including lipid membranes<sup>13-20</sup>. In addition, the aggregation process is highly  
64 heterogeneous and leads to the formation of multiple types of fibrillar and pre-fibrillar species,  
65 the degree of polymorphism of which also depends on the experimental conditions<sup>21-26</sup>.

66  
67 Although it is challenging to study the mechanistic events associated with  $\alpha$ -synuclein  
68 aggregation, a detailed understanding of this process is of considerable importance for the  
69 rational development and evaluation of potential therapeutics directed at reducing or eliminating  
70 the underlying sources of toxicity that lead to PD<sup>10,27,28, 29</sup>. The aggregation of  $\alpha$ -synuclein has  
71 been shown to be enhanced dramatically by its binding to lipid membranes<sup>14,30</sup>; disrupting such  
72 interactions with small molecules therefore, has the potential to provide new information about  
73 the molecular processes involved in pathogenicity, and could also represent the basis for an  
74 effective therapeutic strategy. In this context, we have recently found that the aminosterol  
75 squalamine<sup>31-33</sup> interferes with the binding of  $\alpha$ -synuclein to membranes, reduces the initiation of  
76 its aggregation *in vitro*, and decreases the toxicity associated with such aggregation in human  
77 neuroblastoma cells and in a *C. elegans* model of PD<sup>29</sup>.

78  
79 The existence of additional natural aminosterol compounds related to squalamine<sup>33</sup> prompted us  
80 to explore their effects on the formation and properties of  $\alpha$ -synuclein aggregates *in vitro* and *in*  
81 *vivo*. One such compound, trodusquemine (also known as MSI-1436, **Fig. S1a**)<sup>32-34</sup>, belongs to a  
82 class of cationic amphipathic aminosterols that have been widely studied in both animal models  
83 and in clinical trials in relation to the treatment of cancer<sup>35</sup>, anxiety<sup>36</sup>, and obesity<sup>37,38</sup>.  
84 Trodusquemine was initially isolated from dogfish shark liver as a minor aminosterol along with  
85 six other related compounds, including squalamine<sup>33</sup>. Moreover, it has since been shown to be  
86 able to cross the blood brain barrier, a property of considerable importance for any therapeutic  
87 molecule for the treatment of neuropathic disorders<sup>31</sup>. In addition, trodusquemine has been  
88 shown to be able to stimulate regeneration in a range of vertebrate tissues and organs following  
89 injury with no apparent effect on uninjured tissues<sup>39</sup>. Trodusquemine shares the same parent  
90 structure as squalamine, but with a spermine moiety replacing the spermidine on the side chain,  
91 resulting into an increased positive charge (**Fig. S1a**).

92

93 Given the structural similarity between trodusquemine and squalamine, and previous reports  
94 suggesting that the more positively charged trodusquemine is likely to enhance its ability to  
95 reduce negative electrostatic surface charges on intracellular membranes<sup>32,40</sup>, we set out to  
96 characterise the effects of trodusquemine on the aggregation kinetics of  $\alpha$ -synuclein *in vitro*  
97 using experimental conditions previously used to study individual microscopic steps in the  
98 aggregation of  $\alpha$ -synuclein in the absence of any aminosterol<sup>15,41</sup>.

99

100 Our first studies of the effects of trodusquemine presented here were aimed to probe its effects  
101 on the initiation of  $\alpha$ -synuclein aggregation in the presence of lipid vesicles<sup>14,15,30,41</sup>, on fibril  
102 elongation<sup>13,15,41</sup> and on secondary nucleation<sup>13,15,41,42</sup>. We then explored the effects of  
103 trodusquemine on inhibiting the cytotoxicity of preformed oligomers of  $\alpha$ -synuclein towards  
104 neuronal cells<sup>43</sup>. We also evaluated the effects of trodusquemine using a well-established  
105 transgenic *C. elegans* model of PD, in which  $\alpha$ -synuclein forms inclusions over time in the large  
106 muscle cells leading to age-dependent paralysis<sup>29</sup>.

107

108

## 109 **Results and Discussion**

110

### 111 **Trodusquemine inhibits both the lipid-induced initiation and the fibril-induced** 112 **amplification steps of $\alpha$ -synuclein aggregation**

113

114 In order to characterize systematically the influence of trodusquemine on the microscopic events  
115 involved in the aggregation of  $\alpha$ -synuclein, we employed a previously described three-pronged  
116 chemical kinetics strategy<sup>13,15,41,42</sup>. This approach involves the study of  $\alpha$ -synuclein aggregation  
117 under a series of specifically designed conditions that make it possible to characterize separately  
118 the processes of heterogeneous primary nucleation<sup>14</sup>, fibril elongation<sup>13,15,41,42</sup>, and fibril  
119 amplification<sup>13,15,41,42</sup>, the latter including secondary processes such as fragmentation and  
120 surface-catalyzed secondary nucleation that result in the proliferation of aggregated forms of  $\alpha$ -  
121 synuclein.

122

123 First, we tested the influence of trodusquemine on the lipid-induced initiation of  $\alpha$ -synuclein  
124 aggregation<sup>14,15</sup>. Using DMPS vesicles at 30 °C<sup>14</sup>, we observed that increasing concentrations of  
125 trodusquemine resulted in an increase in the diameter of the vesicles to above 100 nm for  
126 trodusquemine-to-lipid ratios above 0.2, as monitored by dynamic light scattering (**Fig. S2a**). We  
127 have shown previously that variations in the size of the vesicles below 100 nm does not affect  
128 the kinetics of aggregation of  $\alpha$ -synuclein<sup>14</sup>, and so in the present study we carried out all kinetic  
129 and lipid-binding experiments at trodusquemine-to-lipid ratios below 0.2.  $\alpha$ -Synuclein was  
130 therefore incubated at a variety of concentrations, ranging from 20 to 100  $\mu$ M, in the presence of  
131 100  $\mu$ M DMPS under quiescent conditions at 30 °C, and in the presence of concentrations of  
132 trodusquemine ranging from 0 to 10  $\mu$ M. The aggregation reaction was monitored in real time  
133 using ThT fluorescence (**Figs. S3 and S4**), and we observed a dose-dependent inhibition of the  
134 lipid-induced aggregation of  $\alpha$ -synuclein by trodusquemine, which was very similar to that  
135 observed in the presence of squalamine<sup>29</sup>.

136

137 In the light of these results, we investigated the influence of trodusquemine on the lipid-binding  
138 properties of  $\alpha$ -synuclein using far-UV circular dichroism (CD) spectroscopy. We incubated 5  
139  $\mu$ M  $\alpha$ -synuclein in the presence of 250  $\mu$ M DMPS and increasing concentrations of  
140 trodusquemine (0-50  $\mu$ M) (**Fig. S2b-c**). At the protein-to-lipid ratio used in these experiments,  
141 effectively all the protein molecules in the absence of trodusquemine are bound to the surface of  
142 DMPS vesicles in an  $\alpha$ -helical conformation, as predicted from the binding constants previously  
143 determined for the  $\alpha$ -synuclein-DMPS system<sup>14</sup> (**Fig. S2b**). In the presence of increasing  
144 concentrations of trodusquemine, the CD signal of  $\alpha$ -synuclein measured at 222 nm increases  
145 from a value characteristic of an  $\alpha$ -helix to that of a random coil (**Fig. S2c**) indicating that  
146 trodusquemine, like squalamine, can displace  $\alpha$ -synuclein from the surfaces of vesicles.

147

148 We then analyzed these data using the competitive binding model that was used to describe the  
149 displacement of  $\alpha$ -synuclein from vesicles by  $\beta$ -synuclein<sup>13,15,41</sup> and by squalamine<sup>29</sup>, in which  $\alpha$ -  
150 synuclein and  $\beta$ -synuclein or squalamine compete for binding sites at the surface of the DMPS  
151 vesicles. This analysis allowed us to determine both the dissociation constant ( $K_{D,T}$ ) and

152 stoichiometry ( $L_T$ ) associated with the trodusquemine-DMPS system,  $K_{D,T} = 1.62 \times 10^{-8}$  M and  
153  $L_T = 5.1$  (**Fig. S2c**). In comparable experiments conducted with the squalamine-DMPS system,  
154 we found  $K_{D,S} = 6.7 \times 10^{-8}$  M and  $L_S = 7.3$ , respectively<sup>29</sup>; the higher affinity for the anionic  
155 phospholipids by trodusquemine compared to squalamine is expected on the basis of the higher  
156 net positive charge of the former zwitterion. To characterize this inhibition in more detail we  
157 analyzed the early time points of the aggregation reaction (see **Materials and Methods** for  
158 details) by globally fitting a single-step nucleation model to the kinetic traces as previously  
159 described for squalamine<sup>14</sup> (**Figs. 1a,b, S4 and S5**). This analysis is described in the **SI** and  
160 indicates that trodusquemine inhibits  $\alpha$ -synuclein lipid induced aggregation via a somewhat more  
161 complex mechanism than that reported for squalamine. In particular, our analysis suggests that  
162 the mechanism involves not only the displacement of monomeric  $\alpha$ -synuclein from the  
163 membrane, as observed for squalamine, but also the interaction with intermediate species on the  
164 aggregation pathway; indeed, such interactions could also contribute to the ability of  
165 trodusquemine to suppress the interaction of  $\alpha$ -synuclein oligomers with cell membranes as  
166 described below.

167  
168 We next explored the influence of trodusquemine on fibril elongation and secondary nucleation  
169 using experiments carried out in the presence of pre-formed fibrils at neutral and acidic pH  
170 <sup>13,15,41</sup>. We studied fibril elongation by performing experiments with monomeric  $\alpha$ -synuclein  
171 (20-100  $\mu$ M) in the presence of pre-formed fibrils of the protein at a concentration of 5  $\mu$ M  
172 (monomer equivalents) under quiescent conditions at pH 6.5 and 37 °C (**Fig. 1c and S6**); under  
173 these conditions, the kinetics of  $\alpha$ -synuclein aggregation have been found to be dominated by the  
174 rate of fibril elongation<sup>13,15</sup>. The kinetic profiles of the aggregation reaction were then acquired  
175 in the presence of various concentrations of trodusquemine [0-10  $\mu$ M]. In all cases, the ThT  
176 fluorescence was found to decrease slowly during the apparent plateau reached at the end of the  
177 reaction. This behaviour is often observed in such measurements, primarily because fibrils  
178 formed during the reaction tend to assemble into large assemblies with reduced exposed surface  
179 area available for ThT interaction<sup>13,15,41</sup>. For each trodusquemine concentration, we extracted the  
180 elongation rate through linear fits of the early time points of the kinetic traces<sup>13,15</sup>, and found that  
181 trodusquemine does not detectably influence fibril elongation (**Figs. 1d and S7**). We then  
182 explored the influence of trodusquemine on fibril-catalysed secondary nucleation by incubating

183 monomeric  $\alpha$ -synuclein (60-100  $\mu$ M) at 37 °C in the presence of pre-formed fibrils at a  
184 concentration of 50 nM (monomer equivalents) with increasing concentrations of trodusquemine  
185 (0-10  $\mu$ M) under quiescent conditions at pH 4.8<sup>13,15,41</sup> (**Figs. 1e,f, S8, S9**). We then analyzed the  
186 change in fibril number concentration as previously described<sup>15</sup>, and found that the rate of  
187 secondary nucleation decreased significantly with increasing concentrations of trodusquemine.

188  
189 The observation that trodusquemine can inhibit secondary nucleation processes is likely to be  
190 associated with its ability to bind to the surfaces of amyloid fibrils, as such a situation has been  
191 found for molecular chaperones that show similar abilities<sup>44</sup>. We therefore probed the binding of  
192 trodusquemine to  $\alpha$ -synuclein fibrils by incubating pre-formed fibrils at a concentration of 10  
193  $\mu$ M (monomer equivalents) overnight with equimolar concentrations of trodusquemine, followed  
194 by an ultracentrifugation step<sup>29</sup>. We determined the concentration of trodusquemine in the  
195 supernatant before and after incubation with fibrils using mass-spectrometry (**Fig. S10**), and  
196 found that approximately 70% of the trodusquemine molecules were associated with the fibrils,  
197 consistent with its ability to bind to their surfaces and inhibit the secondary nucleation of  $\alpha$ -  
198 synuclein. In summary, therefore, we observed that trodusquemine inhibits both the lipid-  
199 induced initiation and the fibril-induced amplification (secondary nucleation) step, in the process  
200 of  $\alpha$ -synuclein aggregation (**Fig. 1g**). This behavior can be attributed, at least in large part, to its  
201 ability to displace the protein from the surface of both lipid vesicles and fibrils.

202  
203 In order to exclude the probability that any contribution to the observed effects was a result of  
204 the quenching of ThT by trodusquemine, we incubated preformed  $\alpha$ -synuclein fibrils with ThT in  
205 presence or absence of trodusquemine, and could see that the compound did not affect the ThT  
206 signal to any detectable extent (**Figure S11**). In addition, because it has been found in some  
207 cases that a small molecule can inhibit the aggregation by sequestering proteins in a non-specific  
208 manner as micelles or larger aggregates<sup>45</sup>, we investigated the behavior of trodusquemine itself  
209 under the conditions used here (20 mM phosphate buffer, pH 6.5, 30 °C), using 1D <sup>1</sup>H and 2D  
210 <sup>1</sup>H diffusion ordered spectroscopy (DOSY) experiments (**Figure S12-S13**). The comparison of  
211 the trodusquemine intensities with those of an internal standard with the same concentration  
212 indicated that trodusquemine is very largely (>95%) monomeric under these conditions (**Figure**  
213 **S12**). Moreover, a diffusion coefficient of  $2.42 \cdot 10^{-10} \text{ m}^2 \text{ s}^{-1}$ , which is typical of a small

214 molecule of this size in an aqueous solution, was determined for trodusquemine under these  
215 conditions, ruling out its self-assembly in solution (**Figure S13**). This result is supported by the  
216 absence of significant effects in the elongation step described above (**Figure 1**), which would be  
217 decreased if the concentration of free monomeric trodusquemine were reduced.

218

### 219 **Trodusquemine suppresses the toxicity of $\alpha$ -synuclein oligomers in human neuroblastoma** 220 **cells by inhibiting their binding to the cell membrane**

221

222 In an additional series of experiments we explored the effect of trodusquemine on the  
223 cytotoxicity associated with the aggregation of  $\alpha$ -synuclein<sup>43,46,47</sup>. We prepared samples of toxic  
224 type B\* oligomers based on recently developed protocols<sup>46,47</sup> and added them to the cell culture  
225 medium of human Sh-Sy5Y neuroblastoma cells at a concentration of 0.3  $\mu$ M (monomer  
226 equivalents of  $\alpha$ -synuclein). The MTT assay, which provides a measure of cellular viability (see  
227 **Methods** for details), confirms previous results that these oligomers are toxic to cells (**Fig. 2a**).  
228 We then treated the cells with these oligomers (0.3  $\mu$ M) in the presence of increasing  
229 concentrations of trodusquemine (0.03, 0.1 and 0.3  $\mu$ M), and observed that the toxicity was  
230 markedly reduced, particularly at the highest trodusquemine concentration (0.3  $\mu$ M) where  
231 essentially complete protection was observed (**Fig. 2a**). In addition, these  $\alpha$ -synuclein oligomers  
232 (0.3  $\mu$ M) in the absence of trodusquemine were shown to induce an increase in the levels of  
233 reactive oxygen species (ROS) in this cell model, indicating their ability to inflict cellular  
234 damage (**Fig. 2b**). Repeating the experiments with increasing concentrations of trodusquemine  
235 (0.03, 0.3 and 3.0  $\mu$ M), however, resulted in a marked decrease in the degree of ROS-derived  
236 fluorescence, showing a well-defined dose dependence and virtually complete inhibition of  
237 intracellular ROS production at a protein-to-trodusquemine ratio of 1:10 (**Fig. 2b**).

238

239 We next investigated the mechanism by which trodusquemine inhibits  $\alpha$ -synuclein oligomer  
240 toxicity by probing the interactions between the oligomers (0.3  $\mu$ M) and human SH-SY5Y cells  
241 at increasing concentrations of trodusquemine (0.03, 0.3 and 3.0  $\mu$ M) using anti- $\alpha$ -synuclein  
242 antibodies in conjunction with confocal microscopy. The images were scanned at apical planes to  
243 detect oligomers (green) interacting with cellular surfaces (red) by confocal microscopy (**Fig.**  
244 **2c**). Following the addition of the  $\alpha$ -synuclein oligomers to the cell culture medium, a large

245 number of these species were observed to be associated with the plasma membranes of the cells,  
246 but their number was significantly and progressively decreased as the trodusquemine  
247 concentration was increased, showing a well-defined dose dependence (**Fig. 2c**). We have shown  
248 previously that the toxicity caused by protein oligomers, which are membrane disruptive<sup>43,48,49</sup>,  
249 correlates with the affinity of membrane binding<sup>50</sup>. This protective effect can therefore be  
250 attributed to the reduced ability of  $\alpha$ -synuclein oligomers to interact with the cell membranes in  
251 the presence of trodusquemine. Such protection is likely to result from the ability of  
252 trodusquemine to bind to the cellular membranes and displace the oligomers from them, and  
253 potentially through its interactions with the oligomeric species themselves as suggested by the *in*  
254 *vitro* experiments on lipid binding discussed above.

255

### 256 **Trodusquemine inhibits formation of the $\alpha$ -synuclein inclusion in a *C. elegans* model of PD,** 257 **and increases both fitness and longevity of the PD worms**

258

259 To examine whether or not trodusquemine shows the effects observed in cell cultures in a living  
260 organism, we used a well-established *C. elegans* model in which  $\alpha$ -synuclein is over expressed in  
261 the large muscle cells ('PD worms') that shows age-dependent inclusion formation and related  
262 toxicity, which can be measured by a decrease in the number of body bends per minute (BPM),  
263 an increase in paralysis rate and a decrease in speed of movement<sup>29</sup>. As described previously for  
264 squalamine, we first carried out experiments aimed at optimizing the treatment profile of the  
265 worms<sup>29</sup>. We evaluated different treatment schedules by administering trodusquemine as a  
266 single, continuous dose either at an early stage of the life of the animals (L4 larval stage) or late  
267 in the adulthood (D5, adulthood stage) (**Fig. 3a**).

268

269 We then investigated PD worms expressing  $\alpha$ -synuclein fused to the yellow fluorescent protein  
270 (YFP), and also control worms expressing only YFP, in conjunction with fluorescence  
271 microscopy in order to probe the number and distribution of  $\alpha$ -synuclein inclusions<sup>51,52</sup>. The  
272 expression of YFP in the large muscle cells of the control animals was uniform and did not lead  
273 to the formation of visible inclusions at D12 of adulthood; furthermore, the YFP expression  
274 pattern was not significantly affected by the administration of trodusquemine and was constant  
275 with age (**Fig. 3b**). By contrast, PD worms in the absence of trodusquemine were found to have

276 large numbers of inclusions at D12, with the number decreasing significantly (up to 50%) after  
277 treatment with trodusquemine either at the L4 larval stage ( $p < 0.001$  at D12) or at D5 of  
278 adulthood ( $p < 0.001$  at D12). Indeed, treatment of the PD worms at the L4 larval stage, prior to  
279 the appearance of  $\alpha$ -synuclein aggregates, significantly reduced the rate of formation of visible  
280  $\alpha$ -synuclein inclusions as the animals progressed through adult stages (**Fig. 3c**). In addition,  
281 initiation of treatment of the PD worms as adults on D5, after  $\alpha$ -synuclein inclusions had already  
282 appeared in the animals, also significantly decreased the subsequent rate of formation of  
283 inclusions as the animals aged (**Fig. 3c**).

284  
285 In an additional series of experiments, the functional behaviour of the worms was found to be  
286 correlated with the observed effects on inclusion formation. We observed that PD worms treated  
287 at the L4 larval stage showed a strongly increased frequency of body bends per time unit ( $p <$   
288  $0.001$  at D14), an increased speed of movement ( $p < 0.001$  at D14), and a decreased rate of  
289 paralysis ( $p < 0.001$  at D14) in comparison to untreated PD worms. Indeed, the behaviour of the  
290 PD worms treated with  $10 \mu\text{M}$  trodusquemine was comparable to that of the healthy control  
291 worms (**Fig. 3d**). Accordingly, trodusquemine appears to share similarities in its mode of action  
292 with compounds that inhibit the primary nucleation events in protein aggregation such as  
293 squalamine<sup>29</sup>. We observed in addition, however, that PD worms treated at D5 of adulthood also  
294 showed a greatly decreased fraction of paralyzed animals ( $p < 0.001$  at D14) in combination with  
295 increased body bends per time unit ( $p < 0.001$  at D14) and speed of movement ( $p < 0.001$  at  
296 D14) (**Fig. 3d**). Taken together, these results suggest strongly that trodusquemine has the ability  
297 to inhibit secondary nucleation as observed *in vitro* (**Fig. 1**). Thus, in this animal model of PD,  
298 trodusquemine exhibits both prophylactic and therapeutic efficacy with respect to the formation  
299 of  $\alpha$ -synuclein aggregates. By calculating the ‘total fitness’ score, which is a linear combination  
300 of the various types of behaviour of the worms, including frequency of body bend, speed of  
301 movement and paralysis rate<sup>29</sup>, the significant increase in the health of PD worms treated with  
302 trodusquemine at L4 ( $p < 0.001$  at D14) and D5 ( $p < 0.001$  at D14) in comparison to untreated  
303 PD worms is clearly evident (**Fig. 3e**).

304  
305 In addition to such protective actions on the effects of aggregation, exposure of trodusquemine at  
306 either L4 ( $p < 0.001$  at D20) or D5 ( $p < 0.001$  at D20) significantly increased the longevity of the

307 PD worms, related to untreated animals (**Fig. 3f**). The lifespan (described here as the age at  
308 which there is the 50% mortality of the population) of untreated PD worms was about  $17\pm 1$  days,  
309 similar to that of control individuals (**Fig. 3f**). Treatment of the PD worms at L4 extended  
310 longevity to  $24\pm 1$  days, and at D5, to  $20\pm 1$  days, in each case exceeding the longevity of healthy  
311 control animals. Intriguingly, trodusquemine administration also improved the survival of control  
312 animals (**Fig. 3g**), although to a lesser extent, suggesting that the effect of trodusquemine on  
313 lifespan is enhanced in the presence of tissue injury caused by the accumulation of  $\alpha$ -synuclein  
314 inclusions. Longevity in *C. elegans* has been studied extensively and the roles of multiple  
315 pathways including insulin/insulin-like growth factor (IGF)-1 signalling, and dietary restriction,  
316 has been shown to influence lifespan<sup>53-58</sup>. Of particular significance in the context of our  
317 observations is the report that trodusquemine can stimulate regeneration in various vertebrate  
318 tissues and organs following injury, including those of adult mice, through mobilization of stem  
319 cells, with no apparent effect on the growth of uninjured tissues<sup>39</sup>. The electrostatic interactions  
320 between trodusquemine and membrane-associated phosphatidylinositol 3,4,5-triphosphate  
321 (PIP3), driven by the spermine moiety, could also play an important role in this phenomenon, as  
322 the displacement of specific PIP3 binding proteins has been shown to increase fitness and  
323 longevity in wild type and PD models in *C. elegans*<sup>59</sup>. Further experiments will, however, be  
324 required to define in detail how trodusquemine extends, in particular, the lifespan of the PD  
325 worms and its potential relevance to human subjects.

326

327

328

## 329 **Conclusions**

330

331 We have shown that the aminosterol trodusquemine inhibits the lipid-induced initiation of  
332 aggregation of  $\alpha$ -synuclein *in vitro*, whereas it has no detectable effect on the rate of elongation  
333 of fibrils. As with the related aminosterol squalamine, trodusquemine inhibits the initiation of  
334 aggregation, but we have also found, however, that it inhibits the secondary nucleation of  $\alpha$ -  
335 synuclein, thereby reducing the proliferation of the aggregates. The reduction by trodusquemine  
336 of both these nucleation processes will reduce substantially the number of potentially toxic  
337 aggregates, providing an explanation for the observation of the substantial protective effects of

338 trodusquemine observed both in cultured cells and in a *C. elegans* model of PD. As with  
339 squalamine<sup>29</sup>, trodusquemine appears to be able to displace  $\alpha$ -synuclein and its oligomers from  
340 the membrane, inhibiting both the lipid-induced initiation of the aggregation process and the  
341 ability of the oligomers to disrupt the integrity of membranes. In addition, these molecules may  
342 interact directly with the oligomers to reduce their inherent toxicity. Intriguingly, administration  
343 of trodusquemine, also extends the longevity of the PD worms, beyond even that of control  
344 animals and to an extent larger than that observed with squalamine. Taken together these results  
345 suggest that trodusquemine, which can cross the blood brain barrier, could have multiple benefits  
346 in the context of human  $\alpha$ -synucleinopathies, ranging from an increased understanding of the  
347 nature and progression of these conditions to disease modification and tissue regeneration.

348

349

350

## 351 **Materials and Methods**

352

353 **Reagents.** 1,2-Dimyristoyl-sn-glycero-3-phospho-L-serine (sodium salt; DMPS) was purchased  
354 from Avanti Polar Lipids (Alabaster, AL, USA). Trodusquemine (as the chloride salt) was  
355 synthesized as previously described<sup>31</sup> and was greater than 97% pure as evaluated by mass  
356 spectrometry. Trodusquemine powder was used immediately after dilution.

357

358 **Protein expression and lipid preparation.** Wild type human  $\alpha$ -synuclein was recombinantly  
359 expressed and purified as described previously<sup>14,15</sup>. For concentration measurements, we used an  
360 extinction coefficient of 5600 M<sup>-1</sup> cm<sup>-1</sup> at 275 nm. After the final size-exclusion chromatography  
361 step (20 mM phosphate buffer, pH 6.5), the protein was snap frozen in liquid nitrogen in the  
362 form of 1 ml aliquots and stored at -80 °C. These aliquots were used without further treatment,  
363 except for the aggregation experiments at low pH where the pH was adjusted to the desired value  
364 with small volumes of 100 mM NaOH or HCl. The lipids were dissolved in 20 mM phosphate  
365 buffer (NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>), pH 6.5, 0.01% NaN<sub>3</sub> and stirred at ca. 45 °C for 2 h. The solution  
366 was then frozen and thawed five times using dry ice and a water bath at 45 °C. The preparation  
367 of vesicles was carried out on ice by means of sonication (3 x 5 min, 50% cycles, 10% maximum  
368 power on ice) using a Bandelin Sonopuls HD 2070 (Bandelin, Berlin, Germany). After

369 centrifugation, the sizes of the vesicles were checked using dynamic light scattering (Zetasizer  
370 Nano ZSP, Malvern Instruments, Malvern, UK) and were shown to consist of a distribution  
371 centered on a diameter of 20 nm.

372

### 373 *Circular dichroism (CD) spectroscopy.*

374 *Data Acquisition:* Samples were prepared as described previously<sup>29</sup> by incubating 5  $\mu\text{M}$   $\alpha$ -  
375 synuclein with 250  $\mu\text{M}$  DMPS vesicles in 20 mM phosphate buffer, pH 6.5, 0.01%  $\text{NaN}_3$  and 0-  
376 50  $\mu\text{M}$  trodusquemine. Far-UV CD spectra were recorded on a JASCO J-810 instrument (Tokyo,  
377 Japan) equipped with a Peltier thermally controlled cuvette holder at 30  $^\circ\text{C}$ . Quartz cuvettes with  
378 path lengths of 1 mm were used, and the CD signal was measured in each case at 222 nm by  
379 averaging 60 individual measurements with a bandwidth of 1 nm, a data pitch of 0.2 nm, a  
380 scanning speed of 50 nm/min and a response time of 1 s. The signal of the buffer containing  
381 DMPS and the different concentrations of trodusquemine was subtracted from that of the protein.

382 *Data Analysis:* The concentration of  $\alpha$ -synuclein bound to DMPS vesicles ( $[\alpha_b]$ ) when 5  $\mu\text{M}$   $\alpha$ -  
383 synuclein was incubated in the presence of 250  $\mu\text{M}$  DMPS and increasing concentrations of  
384 trodusquemine ( $[T]$ ) was calculated from the CD signal measured at 222 nm as described  
385 previously<sup>14</sup>. The change in  $[\alpha_b]$  with increasing  $[T]$  was then analyzed as described previously<sup>29</sup>  
386 using the standard solution of the cubic equation:

387

$$388 \quad K_{D,\alpha} = \frac{([DMPS] - L_T[T_b] - L_\alpha[\alpha_b])([\alpha] - [\alpha_b])}{L_\alpha[\alpha_b]} \quad \text{Eq. 1}$$

$$389 \quad [T_b] = \frac{[DMPS] - L_\alpha[\alpha_b] + K_{D,T}L_T + L_T[T] - \sqrt{4L_T([\alpha_b]L_\alpha[T] - [DMPS][T]) + ([DMPS] - [\alpha_b]L_\alpha + K_{D,T}L_T + L_T[T])^2}}{2L_T} \quad \text{Eq. 2}$$

390 where  $K_{D,\alpha}$  and  $K_{D,T}$  are the binding constant of  $\alpha$ -synuclein and trodusquemine, respectively;  
391  $L_\alpha$  and  $L_T$  are the stoichiometries at which DMPS binds to  $\alpha$ -synuclein and trodusquemine, i.e.  
392 the number of lipid molecules interacting with one molecule of either  $\alpha$ -synuclein or  
393 trodusquemine, respectively;  $[DMPS]$ ,  $[\alpha]$  and  $[T]$  are the total concentrations of DMPS,  $\alpha$ -  
394 synuclein and trodusquemine, respectively;  $[\alpha_b]$  and  $[T_b]$  are the concentrations of  $\alpha$ -synuclein  
395 and trodusquemine bound to DMPS vesicles, respectively. The best fit is shown in Figure S2  
396 with  $K_{D,T} = 1.62 \times 10^{-8}$  M and  $L_T = 5.1$ . Further details of the analysis see the **Supplementary**  
397 **Materials.**

398

399 ***Dynamic light scattering (DLS) measurements.***

400 Measurements of vesicle size distributions in the absence and presence of the indicated  
401 concentrations of trodusquemine were carried out by dynamic light scattering (DLS) experiments  
402 using a Zetasizer Nano ZSP Instrument (Malvern Instruments, Malvern, UK) with backscatter  
403 detection at a scattering angle of 173°. The concentration of the vesicles was 0.1 mM in  
404 phosphate buffer (20 mM, pH 6.5) and the experiments were carried out at a temperature of  
405 25 °C.

406

407 ***Aggregation kinetics in the presence of lipid vesicles.***

408 *Data Acquisition:*  $\alpha$ -Synuclein was incubated at a concentration of 20-100  $\mu$ M in 20 mM sodium  
409 phosphate, pH 6.5, 0.01% NaN<sub>3</sub>, in the presence of 50  $\mu$ M ThT, 100  $\mu$ M DMPS vesicles and  
410 increasing concentrations of trodusquemine (0 to 10  $\mu$ M)<sup>29</sup>. The stock solution of trodusquemine  
411 was prepared by dissolving the molecule in 20 mM phosphate buffer to a final concentration of  
412 100  $\mu$ M. The change in the ThT fluorescence signal with time was monitored using a Fluostar  
413 Optima or a Polarstar Omega (BMG Labtech, Aylesbury, UK) fluorimeter under quiescent  
414 conditions at 30 °C<sup>29</sup>. Corning 96-well plates with half-area (black/clear bottom) nonbinding  
415 surfaces (Sigma Aldrich, St. Louis, MO, USA) were used for each experiment.

416 *Data Analysis:* The early times of the aggregation curves of  $\alpha$ -synuclein in the presence of  
417 DMPS and different concentrations of trodusquemine were fitted using the one-step nucleation  
418 model described previously<sup>14</sup> and the following equation:

419

$$420 \quad M(t) = \frac{K_M k_+ m(0)^{n+1} k_n [DMPS] t^2}{2(K_M + m(0)) L_\alpha} \quad \text{Eq. 3}$$

421

422 where  $t$  is the time,  $M(t)$  is the aggregate mass,  $m(0)$  is the free monomer concentration,  $K_M$  is  
423 the saturation constant of the elongation process (125  $\mu$ M<sup>14</sup>,  $k_n$  and  $k_+$  are the rate constants of  
424 nucleation and elongation, respectively, and  $\frac{[DMPS]}{L_\alpha}$  is the concentration of protein-binding sites at  
425 the surface of the membrane. First, the early times of the kinetic traces measured for  $\alpha$ -synuclein  
426 in the presence of 100  $\mu$ M DMPS and in the absence of trodusquemine were fitted using Eq. 3,  
427 with  $k_n k_+$ , and  $n$  being global fitting parameters (see fits in **Fig. S4a**). The global fit yields  $n =$

428 0.745, and  $k_n k_+ = 8.6 \times 10^{-3} \text{ M}^{-(n+1)} \cdot \text{s}^{-2}$ . We then fixed  $n$  to 0.745 and fitted the early times of the  
 429 data in the presence of trodusquemine using Eq. 3, with  $k_n k_+$ , being the only global fitting  
 430 parameters (see **Fig. S4 b-f**). This way we obtain the effective rate of lipid-induced aggregation  
 431 of  $\alpha$ -synuclein relative to that in the absence of trodusquemine, at all  $\alpha$ -synuclein and  
 432 trodusquemine concentrations (see **Fig. S5**):

$$433 \quad r_{eff} = \frac{k_n k_+'}{k_n k_+} \quad \text{Eq. 4}$$

434 where  $k_n k_+'$  is the product of the nucleation and elongation rate constants at a given  
 435 [trodusquemine]:[ $\alpha$ -synuclein] ratio. If trodusquemine were to inhibit  $\alpha$ -synuclein lipid-induced  
 436 aggregation via the same mechanism as that reported previously for squalamine<sup>29</sup> and  $\beta$ -  
 437 synuclein<sup>14,41</sup>, the effective rate should scale with a power of the coverage, i.e.  $r_{eff} = \theta_\alpha^{n_b}$ ,  
 438 where  $\theta_\alpha$  is the fractional coverage of a lipid vesicle in  $\alpha$ -synuclein and  $n_b$  is the reaction order  
 439 of lipid-induced aggregation with respect to  $\theta_\alpha$ . In the absence of trodusquemine,  $\theta_\alpha = 1$ , as we  
 440 are in a regime where the vesicles are saturated. We determined  $\theta_\alpha$  for the different  
 441 [trodusquemine]:[ $\alpha$ -synuclein] ratios used in our study using a simplified competitive binding  
 442 model with the values of  $K_{D,T}$  and  $L_T$  determined in this study and  $K_{D,\alpha}$  and  $L_\alpha$  determined  
 443 previously<sup>14</sup>, using  $n_b = 5.5$ , as determined previously<sup>29,41</sup> and the following equations:

$$444 \quad \theta_\alpha = \frac{[\alpha_b] L_\alpha}{[DMPS]} \quad \text{Eq. 5}$$

$$446 \quad [\alpha_b] = \frac{[DMPS] L_\alpha \kappa - [DMPS] L_T - [\alpha] L_\alpha L_T - L_\alpha L_T [T] \kappa}{2(L_\alpha^2 \kappa - L_\alpha L_T)} \quad \text{Eq. 6}$$

$$449 \quad + \frac{\sqrt{4[\alpha][DMPS] L_T (L_\alpha^2 \kappa - L_\alpha L_T) + ([DMPS] L_T + [\alpha] L_\alpha L_T - [DMPS] L_\alpha \kappa + L_\alpha L_T [T] \kappa)^2}}{2(L_\alpha^2 \kappa - L_\alpha L_T)}$$

$$450 \quad \text{where } \kappa = \frac{K_{D,\alpha}}{K_{D,T}}.$$

451  
 452 We found that the values of  $r_{eff}$  obtained at different [trodusquemine]:[ $\alpha$ -synuclein] ratios do  
 453 not scale simply as  $\theta_\alpha^{n_b}$ , with the coverage  $\theta_\alpha$  calculated using Eqs. 5-6 at the corresponding  
 454 ratios, suggesting that trodusquemine inhibits  $\alpha$ -synuclein lipid-induced aggregation via a more

455 complex mechanism than that described for squalamine (see **Fig. S5**), perhaps resulting from a  
456 direct interaction with the oligomeric species resulting in an inhibition of their inherent toxicity.

457  
458 **Seed fibril formation.** Seed fibrils were produced as previously described<sup>15,41</sup>. Briefly, we  
459 incubated 500  $\mu$ l solutions of  $\alpha$ -synuclein at concentrations between 500 and 800  $\mu$ M in 20 mM  
460 phosphate buffer at pH 6.5 for 48 - 72 h at ca. 40 °C with maximal stirring with a Teflon bar on  
461 an RCT Basic Heat Plate (IKA, Staufen, Germany). The fibrils were divided into aliquots, frozen  
462 in liquid N<sub>2</sub> and stored at -80 °C until required. For aggregation experiments, the seed fibrils  
463 were diluted to 200  $\mu$ M monomer equivalents into the specific buffer to be used in the  
464 experiment and sonicated 3 times for 10 s using a Bandelin Sonopuls HD 2070 probe sonicator  
465 (Bandelin, Berlin, Germany), using 10% maximum power and 30% cycles.

466  
467 **Seeded aggregation kinetics.** The seeded experiments were performed as described  
468 previously<sup>15,41</sup>. Briefly, to probe fibril elongation, preformed seeds fibrils (5  $\mu$ M monomer  
469 equivalents) were added to solutions of monomeric  $\alpha$ -synuclein (20-100  $\mu$ M) in 20 mM  
470 phosphate buffer (pH 6.5) with 50  $\mu$ M ThT, under quiescent conditions, at 37 °C. For  
471 experiments to probe the influence on secondary nucleation, seeds (50 nM monomer equivalents)  
472 were added to monomeric  $\alpha$ -synuclein (60-100  $\mu$ M) in 20 mM phosphate buffer (pH 4.8) and in  
473 the presence of 50  $\mu$ M ThT, under quiescent conditions, also at 37 °C. The increase in ThT  
474 fluorescence was monitored in low binding, clear-bottomed half-area Corning 96 well plates  
475 (Sigma Aldrich, St. Louis, MO, USA) that were sealed with tape, and using either a Fluostar  
476 Optima, Polarstar Omega (BMG Labtech, Aylesbury, UK) or an M1000 (Tecan Group Ltd.,  
477 Männedorf, Switzerland) fluorescence plate-reader in bottom reading mode. All experiments  
478 were performed under quiescent conditions (i.e. without shaking).

479  
480 **Data analysis.** Data were analyzed as previously described<sup>15,41</sup>.

481  
482 **Mass spectrometry.** Experiments were carried out as previously described<sup>29</sup>. Briefly, fibrils of  $\alpha$ -  
483 synuclein at a concentration of 10  $\mu$ M (monomer equivalents) were incubated with 10  $\mu$ M  
484 trodusquemine in 20 mM Tris, pH 7.4, 100 mM NaCl overnight under quiescent conditions at  
485 room temperature. The samples were then centrifuged at 100.000g for 30 min and the

486 supernatant then removed for analysis. Samples for the analysis by mass spectrometry were  
487 prepared as described<sup>29</sup> and the experiments were run using a Waters Xevo G2-S QTOF  
488 spectrometer (Waters Corporation, MA, USA).

489  
490

491 **Nuclear magnetic resonance (NMR) spectroscopy.** H<sub>2</sub>O was removed from trodusquemine by  
492 three cycles of lyophilisation and the compound was resoluted in 100% D<sub>2</sub>O (Sigma Aldrich). It  
493 was then diluted into 20 mM phosphate buffer, pH 6.5 in 100% D<sub>2</sub>O to a final concentration of  
494 10 μM. All NMR measurements were performed at 30 °C on a Bruker AVANCE-500  
495 spectrometer, operated at a <sup>1</sup>H frequency of 500.13 MHz, equipped with a cryogenic probe.  
496 Measurements of diffusion coefficients were performed using 2D <sup>1</sup>H diffusion  
497 ordered spectroscopy (DOSY) experiments<sup>60</sup>. These spectra were acquired using the standard  
498 ‘*ledbpgppr2s*’ Bruker pulse program, using a bipolar gradient pulse pair-stimulated echo  
499 sequence incorporating a longitudinal eddy current delay<sup>61</sup>, with a diffusion time of (Δ) 100 ms,  
500 a gradient pulse length (δ) of 3 ms, and increasing the gradient strength between 4.8 < *g* < 38.5  
501 Gcm<sup>-1</sup>. The values of (Δ) and (δ) were chosen based on measurements of 1.5 mM trodusquemine  
502 in water, in which it is very soluble. To remove the signal from residual H<sub>2</sub>O in the sample,  
503 presaturation was used. Data for 1.5 mM trodusquemine in 100% D<sub>2</sub>O were collected with 8  
504 scans and 16 gradient steps, while data for 10 μM trodusquemine in 20 mM phosphate buffer  
505 were collected with 400 scans and 12 gradient steps. Individual rows of the pseudo-2D diffusion  
506 data were phased and baseline corrected. DOSY spectra were processed using the TopSpin 2.1  
507 software (Bruker). The diffusion dimension was generated using the intensities (I) of resolved  
508 peaks between 3.0 and -0.5 ppm according to the Stejskal-Tanner equation<sup>62</sup>:

509

$$\frac{I}{I_0} = e^{-\gamma^2 g^2 \delta^2 D (\Delta - \frac{\delta}{3})}$$

510 where  $\gamma$  is the gyromagnetic ratio and using the DynamicsCenter 2.5.3 software (Bruker). 1D <sup>1</sup>H  
511 NMR spectra of 10 μM trodusquemine alone in 20 mM phosphate buffer, pH 6.5, 30 °C, made  
512 up in 100% D<sub>2</sub>O or in the presence of equimolar 4,4-dimethyl-4-silapentane-1-sulfonic acid  
513 (DSS, Sigma Aldrich) as an internal concentration standard. These spectra were acquired using

514 the standard ‘*noesypr1d*’ Bruker pulse program with presaturation remove the signal from  
515 residual H<sub>2</sub>O in the sample. Spectra were processed using TopSpin 2.1.

516  
517 ***ThT binding to trodusquemine.*** Pre-formed fibrils of  $\alpha$ -synuclein (5  $\mu$ M) were added to  
518 monomeric  $\alpha$ -synuclein at a concentration of 100  $\mu$ M and incubated in 96-well half-area, low-  
519 binding polyethylene glycol coating plate (Corning 3881, Kennebuck ME, USA) at 37 °C in 20  
520 mM phosphate buffer (pH 6.5) under quiescent conditions for 24 h. Then, the resulting longer  
521 fibrils were diluted to 50  $\mu$ M  $\alpha$ -synuclein with 50  $\mu$ M ThT and the absence of presence of the  
522 indicated concentration of trodusquemine in 20 mM phosphate buffer (pH 6.5) and incubated at  
523 37 °C for 12 h. The fluorescence intensity of the different samples was measured using a plate  
524 reader (Fluostar Optima, BMG Labtech, Ortenberg, Germany).

525  
526 ***Preparation of oligomers.*** Samples of  $\alpha$ -synuclein type B\* oligomers were prepared as  
527 previously described<sup>47</sup>. Briefly, monomeric  $\alpha$ -synuclein was lyophilized in Milli-Q water and  
528 subsequently resuspended in PBS, pH 7.4, to give a final concentration of *ca.* 800  $\mu$ M (12  
529 mg/mL). The resulting solutions were passed through a 0.22  $\mu$ m cut-off filter before incubation  
530 at 37 °C for 20 - 24 h under quiescent conditions<sup>47</sup>. Very small amounts of fibrillar species  
531 formed during this time were removed by ultracentrifugation for 1h at 90,000 rpm (using a TLA-  
532 120.2 Beckman rotor, 288,000 g). Excess monomeric protein and any small oligomeric species  
533 were then removed by multiple cycles of filtration using 100 kDa cut-off membranes. The final  
534 concentration of the prepared oligomers was estimated using  $\epsilon_{275} = 5600 \text{ M}^{-1} \text{ cm}^{-1}$ .

535  
536 ***Neuroblastoma cell cultures.*** Human SH-SY5Y neuroblastoma cells (A.T.C.C., Manassas, VA,  
537 USA) were cultured in DMEM, F-12 HAM with 25 mM HEPES and NaHCO<sub>3</sub> (1:1) and  
538 supplemented with 10% FBS, 1 mM glutamine and 1.0% antibiotics. Cell cultures were  
539 maintained in a 5% CO<sub>2</sub> humidified atmosphere at 37 °C and grown until they reached 80%  
540 confluence for a maximum of 20 passages<sup>63</sup>.

541  
542 ***MTT reduction assay.***  $\alpha$ -Synuclein oligomers (0.3  $\mu$ M monomer equivalents) were incubated  
543 without or with increasing concentrations (0.03, 0.1 and 0.3) of trodusquemine for 1 h at 37 °C

544 under shaking conditions and then added to the cell culture medium of SH-SY5Y cells seeded in  
545 96-well plates for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
546 (MTT) reduction assay was performed as previously described<sup>64</sup>.

547  
548 **Measurement of intracellular ROS.** SH-SY5Y cells were seeded on glass coverslips and treated  
549 for 15 min with  $\alpha$ -synuclein oligomers (0.3  $\mu$ M) and increasing concentrations (0.03, 0.3 and 3  
550  $\mu$ M) of trodusquemine. After incubation, the cells were washed with PBS and loaded with 10  
551  $\mu$ M 2',7'-dichlorodihydrofluorescein diacetate (CM-H<sub>2</sub>DCFDA) (Life Technologies, CA, USA)  
552 as previously described<sup>63</sup>. The fluorescence of the cells was then analyzed by means of a TCS  
553 SP5 scanning confocal microscopy system (Leica Microsystems, Mannheim, Germany)  
554 equipped with an argon laser source, using the 488 nm excitation line. A series of 1.0  $\mu$ m thick  
555 optical sections (1024 x 1024 pixels) was taken through the cells for each sample using a Leica  
556 Plan Apo 63 oil immersion objective.

557  
558 **Oligomer binding to cellular membranes.** SH-SY5Y cells were seeded on glass coverslips and  
559 treated for 15 min with  $\alpha$ -synuclein oligomers (0.3  $\mu$ M) and increasing concentrations (0.03, 0.3  
560 and 3  $\mu$ M) of trodusquemine. After incubation, the cells were washed with PBS and  
561 counterstained with 5.0  $\mu$ g/ml Alexa Fluor 633-conjugated wheat germ agglutinin (Life  
562 Technologies, CA, USA) to label fluorescently the cellular membrane<sup>29</sup>. After washing with  
563 PBS, the cells were fixed in 2% (w/v) buffered paraformaldehyde for 10 min at room  
564 temperature (20 °C). The presence of oligomers was detected with 1:250 diluted rabbit  
565 polyclonal anti- $\alpha$ -synuclein antibodies (Abcam, Cambridge, UK) and subsequently with 1:1000  
566 diluted Alexa Fluor 488-conjugated anti-rabbit secondary antibodies (Life Technologies, CA,  
567 USA). Fluorescence emission was detected after double excitation at 488 nm and 633 nm by the  
568 scanning confocal microscopy system described above and three apical sections were projected  
569 as a single composite image by superimposition.

570  
571 **C. elegans media.** Standard conditions were used for the propagation of *C. elegans*<sup>65</sup>. Briefly, the  
572 animals were synchronized by hypochlorite bleaching, hatched overnight in M9 (3 g/l KH<sub>2</sub>PO<sub>4</sub>, 6  
573 g/l Na<sub>2</sub>HPO<sub>4</sub>, 5 g/l NaCl, 1  $\mu$ M MgSO<sub>4</sub>) buffer, and subsequently cultured at 20 °C on nematode  
574 growth medium (NGM) plates (1 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 5  $\mu$ g/ml cholesterol, 250  $\mu$ M

575 KH<sub>2</sub>PO<sub>4</sub>, pH 6, 17 g/L Agar, 3g/l NaCl, 7.5g/l casein) seeded with the *E. coli* strain OP50.  
576 Saturated cultures of OP50 were grown by inoculating 50 mL of LB medium (10g/l tryptone,  
577 10g/l NaCl, 5g/l yeast extract) with OP50 cells and incubating the culture for 16 h at 37 °C.  
578 NGM plates were seeded with bacteria by adding 350 µl of saturated OP50 cells to each plate  
579 and leaving the plates at 20 °C for 2-3 days. On day 3 after synchronization, the animals were  
580 placed on NGM plates containing 75 µM 5-fluoro-2'-deoxy-uridine (FUDR).

581

582 **Strains.** The following strains were used: zgIs15 [P(unc-54):: $\alpha$ -syn::YFP]IV (OW40), in which  
583  $\alpha$ -synuclein fused to YFP relocates to inclusions, which are visible as early as day 2 after  
584 hatching and increase in number and size during the ageing of the animals, up to late adulthood  
585 (Day 17)<sup>29,66</sup>. and rmIs126 [P(unc-54)Q0::YFP]V (OW450). In OW450, YFP alone is expressed  
586 and remains diffusely localized throughout ageing<sup>29,66</sup>.

587

588 **Trodusquemine-coated plates.** Plates were prepared as previously described<sup>29</sup>. Briefly, aliquots  
589 of NGM media were autoclaved, poured and seeded with 350 µL OP50 culture, and grown  
590 overnight at RT. After incubating for up to 3 d at room temperature, aliquots of trodusquemine  
591 dissolved in water at different concentrations were added. NGM plates containing FUDR (75  
592 µM, unless stated otherwise) were seeded with aliquots of the trodusquemine dissolved in water,  
593 at the appropriate concentration. The plates were then placed in a laminar flow hood at room  
594 temperature to dry and the worms were transferred to plates coated with trodusquemine at larval  
595 stage L4.

596

597 **Automated motility assay.** All *C. elegans* populations were cultured at 20 °C and  
598 developmentally synchronized from a 4 h egg-lay. At 64-72 h post egg-lay (time zero)  
599 individuals were transferred to FUDR plates, and body movements were assessed over the times  
600 indicated. At different ages, the animals were washed off the plates with M9 buffer and spread  
601 over an OP-50 unseeded 6 cm plate, after which their movements were recorded at 30 fps using a  
602 recently developed microscopic procedure for 30 s or 1 min<sup>29</sup>. Up to 200 animals were counted  
603 in each experiment unless otherwise stated. One experiment that is representative of the three  
604 measured in each series of experiments is shown and videos were analyzed using a custom made  
605 tracking code<sup>29,67</sup>.

606

607 **Lifespan assays.** Lifespan analysis was carried out as previously described<sup>68</sup>. On day 4 after  
608 synchronization the animals were placed on NGM plates containing FUDR. On L4 or D5, they  
609 were manually transferred to plates seeded with 10  $\mu$ M trodusquemine. Experiments were  
610 performed at 20 °C. Lifespan experiments were performed with 75 animals per condition. At  
611 each time point, the number of surviving animals, determined by movement and response to nose  
612 touch, was counted. Animals that crawled out of the plates during the assay were excluded.  
613 Three independent experiments were carried out in each case and one representative is shown.  
614 Analysis was performed using GraphPad Prism (GraphPad Software).

615

616 **Quantification of inclusions.** Individual animals were mounted on 2% agarose pads, containing  
617 40 mM NaN<sub>3</sub> as an anesthetic, on glass microscope slides for imaging<sup>29</sup>. Only the frontal region  
618 of the worms was considered<sup>29,66</sup>. The numbers of inclusions in each animal were quantified  
619 using a Leica MZ16 FA fluorescence dissection stereomicroscope (Leica Microsystems, Wetzlar,  
620 Germany) at a nominal magnification of 20X or 40X, and images were acquired using an  
621 Evolve 512 Delta EMCCD Camera, with high quantum efficiency (Photometrics, Tucson, AZ,  
622 USA). Measurements on inclusions were performed using ImageJ software<sup>29</sup>. All experiments  
623 were carried out in triplicate and the data from one representative experiment are shown in the  
624 figure. The Student's t-test was used to calculate p values, and all tests were two-tailed unpaired  
625 unless otherwise stated. At least 50 animals were examined per condition, unless stated  
626 otherwise<sup>29</sup>.

627

628

### 629 **Associated Content**

630 The Supporting information is available free of charge on the ACS Publications website at  
631 <http://pubs.acs.org>. This file includes experimental procedures and characterization including,

632

633 The structure of trodusquemine and sequence of  $\alpha$ -synuclein, dynamic light scattering (DLS),  
634 circular dichroism (CD), lipid induced aggregation, global analysis of kinetic traces, effective  
635 rate of  $\alpha$ -synuclein lipid-induced aggregation, fibril elongation and relative rates, fibril

636 amplification and relative rates, mass spectrometry, ThT binding, nuclear magnetic resonance  
637 (NMR).

638

639

640

#### 641 **Acknowledgments**

642 This work was supported by the Boehringer Ingelheim Fonds (P.F.), the Studienstiftung des  
643 Deutschen Volkes (P.F.), Gates Cambridge Scholarships (R.L. and G.T.H) and a St. John's  
644 College Benefactors' Scholarship (R.L.), the UK Biotechnology and Biochemical Sciences  
645 Research Council (M.V. and C.M.D.), a Senior Research Fellowship award from the  
646 Alzheimer's Society, UK, (F. A. A.), the Wellcome Trust (C.M.D., M.V. and T.P.J.K.), the  
647 Frances and Augustus Newman Foundation (T.P.J.K.), the Regione Toscana - FAS Salute -  
648 Supremal project (R.C., C.C. and F.C.), a Marie Skłodowska-Curie Actions – Individual  
649 Fellowship (C.G.), Sidney Sussex College Cambridge (G.M.), the Spanish Government –  
650 MINECO (N.C.) and by the Cambridge Centre for Misfolding Diseases (M.P., P.F., R.L.,  
651 F.A.A., C.G., G.T.H., S.W.C., J.R.K., T.P.J.K., M.V. and C.M.D). The authors would like to  
652 thank E. Klimont for her assistance with the expression and purification of  $\alpha$ -synuclein and N.  
653 Fernando for assistance with the *C. elegans* experiments. We acknowledge the NMR Service at  
654 the Chemistry Department of the University of Cambridge for helpful discussions, particularly P.  
655 Grice and D. Howe and the UK EPSRC for Core Capabilities funding (EP/K039520/1).

656

657

#### 658 **Author contributions**

659 M.P., P.F., R.L., F.A., C.G., T.P.J.K., D.B., M.Z., M.V., F.C., C.C. and C.M.D. were involved in  
660 the design of the study. M.P. and R.L. performed the *C. elegans* experiments. P.F. and C.G.  
661 carried out the *in vitro* experiments. P.F., C.G. and G.M. analyzed the kinetic data of  $\alpha$ -synuclein  
662 aggregation. R.C. carried out the cell experiments. G.T.H. performed the NMR experiments. S.C.  
663 purified the  $\alpha$ -synuclein oligomers. M.P., P.F., R.L., M.Z., M.V., F.C. and C.M.D. wrote the  
664 paper, and all the authors were involved in the analysis of the data and editing of the paper.

665

666

667 **Competing financial interests**

668 M.Z. and D.B. hold patents for the use of trodusquemine in the treatment of Parkinson's disease  
669 (PD).

670

671

672

673 **References**

674

675 (1) Bodner, C. R., Dobson, C. M., and Bax, A. (2009) Multiple tight phospholipid-binding  
676 modes of alpha-synuclein revealed by solution NMR spectroscopy. *J Mol Biol* 390, 775–790.

677

678 (2) Wilhelm, B. G., Mandad, S., and Truckenbrodt, S. (2014) Composition of isolated synaptic  
679 boutons reveals the amounts of vesicle trafficking proteins. *Science* 344, 1023–1028.

680

681 (3) Maroteaux, L., Campanelli, J. T., and Scheller, R. H. (1988) Synuclein: a neuron-specific  
682 protein localized to the nucleus and presynaptic nerve terminal. *J Neurosci* 8, 2804–2815.

683

684 (4) Takeda, A., Mallory, M., Sundsmo, M., Honer, W., Hansen, L., and Masliah, E. (1998)  
685 Abnormal accumulation of NACP/alpha-synuclein in neurodegenerative disorders. *Am J Pathol*  
686 152, 367–372.

687

688 (5) Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M., and Goedert, M. (1998) alpha-  
689 Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with  
690 lewy bodies. *Proc Natl Acad Sci U S A* 95, 6469–6473.

691

692 (6) Dettmer, U., Selkoe, D., and Bartels, T. (2016) New insights into cellular  $\alpha$ -synuclein  
693 homeostasis in health and disease. *Curr Opin Neurobiol* 36, 15–22.

694

695 (7) Dawson, T. M., and Dawson, V. L. (2003) Molecular pathways of neurodegeneration in  
696 Parkinson's disease. *Science* 302, 819–822.

697

698 (8) Knowles, T. P. J., Vendruscolo, M., and Dobson, C. M. (2014) The amyloid state and its  
699 association with protein misfolding diseases. *Nat Rev Mol Cell Biol* 15, 384–396.

700

701 (9) Chiti, F., and Dobson, C. M. (2006) Protein misfolding, functional amyloid, and human  
702 disease. *Annu Rev Biochem* 75, 333–366.

703

704 (10) Lee, V. M. Y., and Trojanowski, J. Q. (2006) Mechanisms of Parkinson's disease linked to  
705 pathological alpha-synuclein: new targets for drug discovery. *Neuron* 52, 33–38.

706

707 (11) Tong, J., Wong, H., Guttman, M., Ang, L. C., Forno, L. S., Shimadzu, M., Rajput, A. H.,  
708 Muentert, M. D., Kish, S. J., Hornykiewicz, O., and Furukawa, Y. (2010) Brain  $\alpha$ -synuclein

709 accumulation in multiple system atrophy, Parkinson's disease and progressive supranuclear  
710 palsy: a comparative investigation. *Brain* 133, 172–188.

711

712 (12) Chiti, F., and Dobson, C. M. (2017) Protein misfolding, amyloid formation, and human  
713 disease: a summary of progress over the last decade. *Annu Rev Biochem* 86, 27–68.

714

715 (13) Buell, A. K., Galvagnion, C., Gaspar, R., Sparr, E., Vendruscolo, M., Knowles, T. P. J.,  
716 Linse, S., and Dobson, C. M. (2014) Solution conditions determine the relative importance of  
717 nucleation and growth processes in  $\alpha$ -synuclein aggregation. *Proc Natl Acad Sci U S A* 111,  
718 7671–7676.

719

720 (14) Galvagnion, C., Buell, A. K., Meisl, G., Michaels, T. C. T., Vendruscolo, M., Knowles, T.  
721 P. J., and Dobson, C. M. (2015) Lipid vesicles trigger  $\alpha$ -synuclein aggregation by stimulating  
722 primary nucleation. *Nat Chem Biol* 11, 229–234.

723

724 (15) Flagmeier, P., Meisl, G., Vendruscolo, M., Knowles, T. P. J., Dobson, C. M., Buell, A. K.,  
725 and Galvagnion, C. (2016) Mutations associated with familial Parkinson's disease alter the  
726 initiation and amplification steps of  $\alpha$ -synuclein aggregation. *Proc Natl Acad Sci U S A* 113,  
727 10328–10333.

728

729 (16) Fink, A. L. (2006) The aggregation and fibrillation of alpha-synuclein. *Acc Chem Res* 39,  
730 628–634.

731

732 (17) Munishkina, L. A., Phelan, C., Uversky, V. N., and Fink, A. L. (2003) Conformational  
733 behavior and aggregation of alpha-synuclein in organic solvents: modeling the effects of  
734 membranes. *Biochemistry* 42, 2720–2730.

735

736 (18) Munishkina, L. A., Henriques, J., Uversky, V. N., and Fink, A. L. (2004) Role of protein-  
737 water interactions and electrostatics in alpha-synuclein fibril formation. *Biochemistry* 43, 3289–  
738 3300.

739 (19) Uversky, V. N., Li, J., Souillac, P., Millett, I. S., Doniach, S., Jakes, R., Goedert, M., and  
740 Fink, A. L. (2002) Biophysical properties of the synucleins and their propensities to fibrillate:  
741 inhibition of alpha-synuclein assembly by beta- and gamma-synucleins. *J Biol Chem* 277,  
742 11970–11978.

743

744 (20) Uversky, V. N., Li, J., and Fink, A. L. (2001) Evidence for a partially folded intermediate in  
745 alpha-synuclein fibril formation. *J Biol Chem* 276, 10737–10744.

746

747 (21) Heise, H., Hoyer, W., Becker, S., Andronesi, O. C., Riedel, D., and Baldus, M. (2005)  
748 Molecular-level secondary structure, polymorphism, and dynamics of full-length  $\alpha$ -synuclein  
749 fibrils studied by solid-state NMR, *Proc Natl Acad Sci U S A* 102(44):15871-6.

750

751 (22) Vilar, M., Chou, H.-T., Lührs, T., Maji, S. K., Riek-Loher, D., Verel, R., Manning, G.,  
752 Stahlberg, H., and Riek, R. (2008) The fold of alpha-synuclein fibrils. *Proc Natl Acad Sci U S A*  
753 105, 8637–8642.

754

755 (23) Comellas, G., Lemkau, L. R., Nieuwkoop, A. J., Kloepper, K. D., Lador, D. T., Ebisu, R.,  
756 Woods, W. S., Lipton, A. S., George, J. M., and Rienstra, C. M. (2011) Structured regions of  $\alpha$ -  
757 synuclein fibrils include the early-onset Parkinson's disease mutation sites. *J Mol Biol* 411, 881–  
758 895.

759  
760 (24) Gath, J., Bousset, L., Habenstein, B., Melki, R., Böckmann, A., and Meier, B. H. (2013)  
761 Unlike twins: an NMR comparison of two  $\alpha$ -synuclein polymorphs featuring different toxicity.  
762 *PLoS ONE* 9, e90659–e90659.

763  
764 (25) Gath, J., Bousset, L., Habenstein, B., Melki, R., Meier, B. H., and Böckmann, A. (2014) Yet  
765 another polymorph of  $\alpha$ -synuclein: solid-state sequential assignments. *Biomol NMR Assign* 8,  
766 395–404.

767  
768 (26) Tuttle, M. D., Comellas, G., Nieuwkoop, A. J., Covell, D. J., Berthold, D. A., Kloepper, K.  
769 D., Courtney, J. M., Kim, J. K., Barclay, A. M., Kendall, A., Wan, W., Stubbs, G., Schwieters,  
770 C. D., Lee, V. M. Y., George, J. M., and Rienstra, C. M. (2016) Solid-state NMR structure of a  
771 pathogenic fibril of full-length human  $\alpha$ -synuclein. *Nat Struct Mol Biol* 23, 409–415.

772  
773 (27) Tóth, G., Gardai, S. J., Zago, W., Bertocini, C. W., Cremades, N., Roy, S. L., Tambe, M.  
774 A., Rochet, J.-C., Galvagnion, C., Skibinski, G., Finkbeiner, S., Bova, M., Regnstrom, K., Chiou,  
775 S.-S., Johnston, J., Callaway, K., Anderson, J. P., Jobling, M. F., Buell, A. K., Yednock, T. A.,  
776 Knowles, T. P. J., Vendruscolo, M., Christodoulou, J., Dobson, C. M., Schenk, D., and  
777 McConlogue, L. (2014) Targeting the intrinsically disordered structural ensemble of  $\alpha$ -synuclein  
778 by small molecules as a potential therapeutic strategy for Parkinson's disease. *PLoS ONE* 9,  
779 e87133–11.

780  
781 (28) Kakish, J., Lee, D., and Lee, J. S. (2015) Drugs which bind to alpha-synuclein.  
782 Neuroprotective or neurotoxic? *ACS Chem Neurosci* 16;6(12):1930-40.

783  
784 (29) Perni, M., Galvagnion, C., Maltsev, A., Meisl, G., Muller M. B. D., Challa, P. K.,  
785 Kirkegaard, J. B., Flagmeier, P., Cohen, S. I. A., Cascella, R., Chen, S. W., Limboker, R., Pietro  
786 Sormanni, Heller, G. T., Aprile, F. A., Cremades, N., Cecchi, C., Chiti, F., Nollen, E. A. A.,  
787 Knowles, T. P. J., Vendruscolo, M., Bax, A., Zaslhoff, M., and Dobson, C. M. (2017) A natural  
788 product inhibits the initiation of  $\alpha$ -synuclein aggregation and suppresses its toxicity.  
789 *114(6):E1009-E1017*.

790  
791 (30) Galvagnion, C., Brown, J. W. P., Ouberai, M. M., Flagmeier, P., Vendruscolo, M., Buell, A.  
792 K., Sparr, E., and Dobson, C. M. (2016) Chemical properties of lipids strongly affect the kinetics  
793 of the membrane-induced aggregation of  $\alpha$ -synuclein. *Proc Natl Acad Sci U S A* 113, 7065–  
794 7070.

795  
796 (31) Zaslhoff, M., Williams, J. I., Chen, Q., Anderson, M., Maeder, T., Holroyd, K., Jones, S.,  
797 Kinney, W., Cheshire, K., and McLane, M. (2001) A spermine-coupled cholesterol metabolite  
798 from the shark with potent appetite suppressant and antidiabetic properties. *Int J Obes Relat*  
799 *Metab Disord* 25, 689–697.

800

801 (32) Zasloff, M., Adams, A. P., Beckerman, B., Campbell, A., Han, Z., Luijten, E., Meza, I.,  
802 Julander, J., Mishra, A., Qu, W., Taylor, J. M., Weaver, S. C., and Wong, G. C. L. (2011)  
803 Squalamine as a broad-spectrum systemic antiviral agent with therapeutic potential. *Proc Natl*  
804 *Acad Sci U S A* 108, 15978–15983.

805  
806 (33) Rao, M. N., Shinnar, A. E., Noecker, L. A., Chao, T. L., Feibush, B., Snyder, B.,  
807 Sharkansky, I., Sarkahian, A., Zhang, X., Jones, S. R., Kinney, W. A., and Zasloff, M. (2000)  
808 Aminosterols from the Dogfish Shark *Squalus acanthias*. *J Nat Prod* 63, 631–635.

809  
810 (34) Sills, A. K., Williams, J. I., Tyler, B. M., Epstein, D. S., Sipos, E. P., Davis, J. D., McLane,  
811 M. P., Pitchford, S., Cheshire, K., Gannon, F. H., Kinney, W. A., Chao, T. L., Donowitz, M.,  
812 Lattera, J., Zasloff, M., and Brem, H. (1998) Squalamine inhibits angiogenesis and solid tumor  
813 growth in vivo and perturbs embryonic vasculature. *Cancer Res* 58, 2784–2792.

814  
815 (35) Krishnan, N., Koveal, D., Miller, D. H., Bin Xue, Akshinthala, S. D., Kragelj, J., Jensen, M.  
816 R., Gauss, C.-M., Page, R., Blackledge, M., Muthuswamy, S. K., Peti, W., and Tonks, N. K.  
817 (2014) Targeting the disordered C terminus of PTP1B with an allosteric inhibitor. *Nat Chem Biol*  
818 10, 558–566.

819  
820 (36) Qin, Z., Pandey, N. R., Zhou, X., Stewart, C. A., Hari, A., Huang, H., Stewart, A. F. R.,  
821 Brunel, J. M., and Chen, H.-H. (2015) Functional properties of Claramine: a novel PTP1B  
822 inhibitor and insulin-mimetic compound. *Biochem Biophys Res Commun.* 458, 21–27.

823  
824 (37) Lantz, K. A., Hart, S. G. E., Planey, S. L., Roitman, M. F., Ruiz-White, I. A., Wolfe, H. R.,  
825 and McLane, M. P. (2010) Inhibition of PTP1B by Trodusquemine (MSI-1436) Causes Fat-  
826 specific Weight Loss in Diet-induced Obese Mice. *Obesity* 18, 1516–1523.

827  
828 (38) Ahima, R. S., Patel, H. R., Takahashi, N., Qi, Y., Hileman, S. M., and Zasloff, M. A. (2002)  
829 Appetite suppression and weight reduction by a centrally active aminosterol. *Diabetes* 51, 2099–  
830 2104.

831  
832 (39) Smith, A. M., Maguire-Nguyen, K. K., Rando, T. A., Zasloff, M. A., Strange, K. B., and  
833 Yin, V. P. (2017) The protein tyrosine phosphatase 1B inhibitor MSI-1436 stimulates  
834 regeneration of heart and multiple other tissues. *NPJ Regen Med* 2, 57.

835  
836 (40) Yeung, T., Gilbert, G. E., Shi, J., Silvius, J., Kapus, A., and Grinstein, S. (2008) Membrane  
837 Phosphatidylserine Regulates Surface Charge and Protein Localization. *Science* 319, 210–212.

838  
839 (41) Brown, J. W. P., Buell, A. K., Michaels, T. C. T., Meisl, G., Carozza, J., Flagmeier, P.,  
840 Vendruscolo, M., Knowles, T. P. J., Dobson, C. M., and Galvagnion, C. (2016)  $\beta$ -Synuclein  
841 suppresses both the initiation and amplification steps of  $\alpha$ -synuclein aggregation via competitive  
842 binding to surfaces. *Sci Rep* 6, 36010.

843  
844 (42) Gaspar, R., Meisl, G., Buell, A. K., Young, L., Kaminski, C. F., Knowles, T. P. J., Sparr, E.,  
845 and Linse, S. (2017) Acceleration of  $\alpha$ -synuclein aggregation. *Amyloid* 24, 20–21.

846

847 (43) Fusco, G., Pape, T., Stephens, A. D., Mahou, P., Costa, A. R., Kaminski, C. F., Kaminski  
848 Schierle, G. S., Vendruscolo, M., Veglia, G., Dobson, C. M., and De Simone, A. (2016)  
849 Structural basis of synaptic vesicle assembly promoted by  $\alpha$ -synuclein. *Nat Commun* 7, 12563.  
850

851 (44) Cohen, S. I. A., Arosio, P., Presto, J., Kurudenkandy, F. R., Biverstal, H., Dolfe, L.,  
852 Dunning, C., Yang, X., Frohm, B., Vendruscolo, M., Johansson, J., Dobson, C. M., Fisahn, A.,  
853 Knowles, T. P. J., and Linse, S. (2015) The molecular chaperone Brichos breaks the catalytic  
854 cycle that generates toxic A $\beta$  oligomers. *Nat Struct Mol Biol* 22, 207–213.  
855

856 (45) Seidler, J., McGovern, S. L., Doman, T. N., and Shoichet, B. K. (2003) Identification and  
857 Prediction of Promiscuous Aggregating Inhibitors among Known Drugs. *J Med Chem* 46, 4477–  
858 4486.  
859

860 (46) Cremades, N., Cohen, S. I. A., Deas, E., Abramov, A. Y., Chen, A. Y., Orte, A., Sandal, M.,  
861 Clarke, R. W., Dunne, P., Aprile, F. A., Bertocini, C. W., Wood, N. W., Knowles, T. P. J.,  
862 Dobson, C. M., and Klenerman, D. (2012) Direct observation of the interconversion of normal  
863 and toxic forms of  $\alpha$ -synuclein. *Cell* 149, 1048–1059.  
864

865 (47) Chen, S. W., Drakulic, S., Deas, E., Ouberai, M., Aprile, F. A., Arranz, R., Ness, S.,  
866 Roodveldt, C., Guilliams, T., De-Genst, E. J., Klenerman, D., Wood, N. W., Knowles, T. P. J.,  
867 Alfonso, C., Rivas, G., Abramov, A. Y., Valpuesta, J. M., Dobson, C. M., and Cremades, N.  
868 (2015) Structural characterization of toxic oligomers that are kinetically trapped during  $\alpha$ -  
869 synuclein fibril formation. *Proc Natl Acad Sci U S A* 112, E1994–2003.  
870

871 (48) Chen, S. W., Drakulic, S., Deas, E., Ouberai, M., Aprile, F. A., Arranz, R., Ness, S.,  
872 Roodveldt, C., Guilliams, T., De-Genst, E. J., Klenerman, D., Wood, N. W., Knowles, T. P. J.,  
873 Alfonso, C., Rivas, G., Abramov, A. Y., Valpuesta, J. M., Dobson, C. M., and Cremades, N.  
874 (2015) Structural characterization of toxic oligomers that are kinetically trapped during  $\alpha$ -  
875 synuclein fibril formation. *Proc Natl Acad Sci U.S.A.* 112, E1994–E2003.  
876

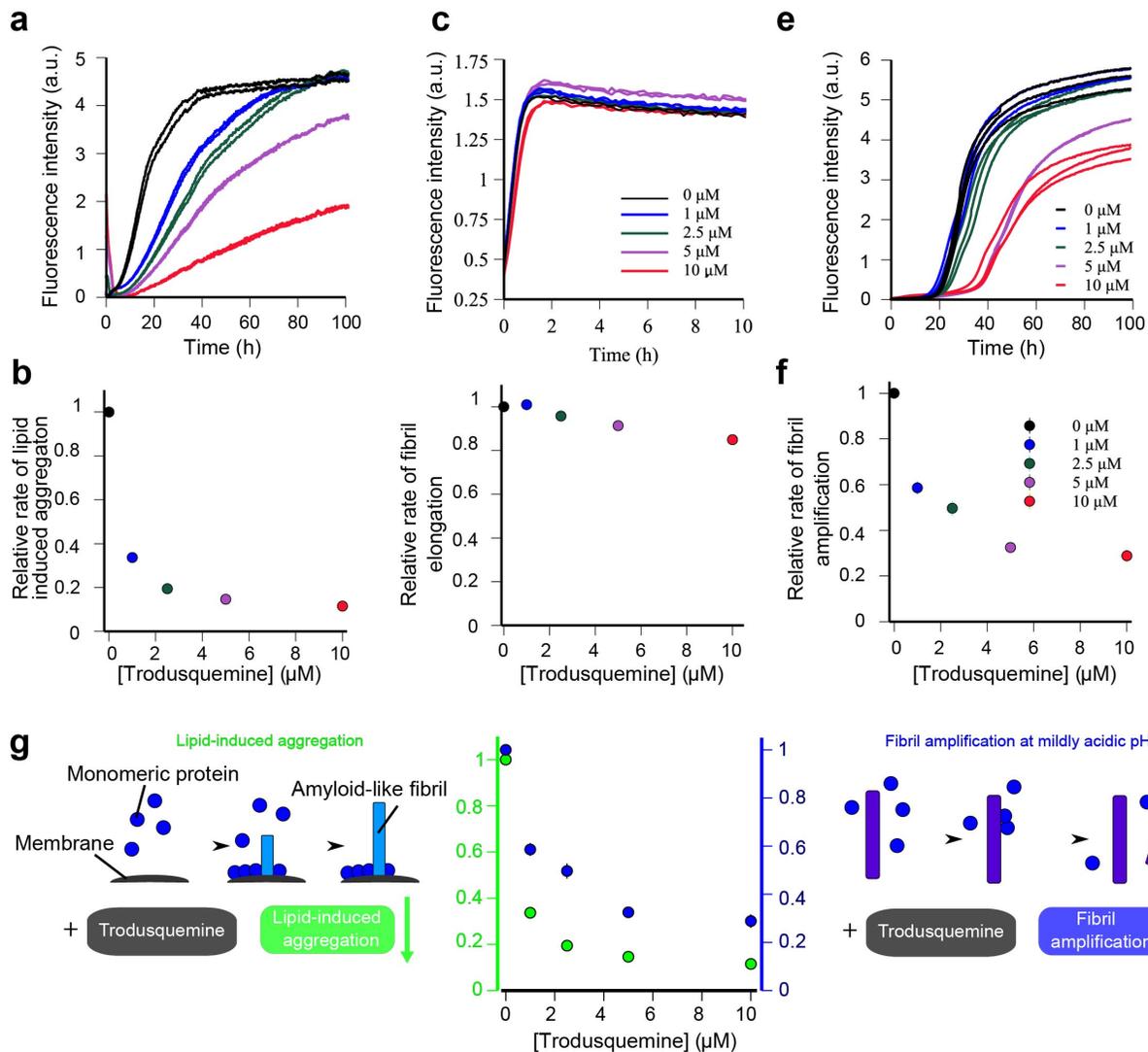
877 (49) Flagmeier, P., De, S., Wirthensohn, D. C., Lee, S. F., Vincke, C., Muyldermans, S.,  
878 Knowles, T. P. J., Gandhi, S., Dobson, C. M., and Klenerman, D. (2017) Ultrasensitive  
879 Measurement of Ca (2+) Influx into Lipid Vesicles Induced by Protein Aggregates. *Angew Chem*  
880 *Int Ed Engl* 56, 7750–7754.  
881

882 (50) Evangelisti, E., Cascella, R., Becatti, M., Marrazza, G., Dobson, C. M., Chiti, F., Stefani,  
883 M., and Cecchi, C. (2016) Binding affinity of amyloid oligomers to cellular membranes is a  
884 generic indicator of cellular dysfunction in protein misfolding diseases. *Sci rep* 6, 32721.  
885

886 (51) Eisenberg, T., Knauer, H., Schauer, A., Büttner, S., Ruckstuhl, C., Carmona-Gutierrez,  
887 D., Ring, J., Schroeder, S., Magnes, C., Antonacci, L., Fussi, H., Deszcz, L., Hartl, R., Schraml,  
888 E., Criollo, A., Megalou, E., Weiskopf, D., Laun, P., Heeren, G., Breitenbach, M., Grubeck-  
889 Loebenstein, B., Herker, E., Fahrenkrog, B., Fröhlich, K.-U., Sinner, F., Tavernarakis, N.,  
890 Minois, N., Kroemer, G., and Madeo, F. (2009) Induction of autophagy by spermidine promotes  
891 longevity. *Nat Cell Biol* 11, 1305–1314.  
892

- 893 (52) Bauer, J. H., Morris, S. N. S., Chang, C., Flatt, T., Wood, J. G., and Helfand, S. L. (2009)  
894 dSir2 and Dmp53 interact to mediate aspects of CR-dependent life span extension in *D.*  
895 *melanogaster*. *Aging-Us 1*, 38–48.
- 896  
897 (53) Hamilton, B., Dong, Y., Shindo, M., Liu, W., Odell, I., Ruvkun, G., and Lee, S. S. (2005) A  
898 systematic RNAi screen for longevity genes in *C. elegans*. *Genes Dev 19*, 1544–1555.
- 899  
900 (54) Sarin, S., Prabhu, S., O'Meara, M. M., Pe'er, I., and Hobert, O. (2008) *Caenorhabditis*  
901 *elegans* mutant allele identification by whole-genome sequencing. *Nat Methods 5*, 865–867.
- 902  
903 (55) Kim, Y., and Sun, H. (2007) Functional genomic approach to identify novel genes involved  
904 in the regulation of oxidative stress resistance and animal lifespan. *Aging Cell 6*, 489–503.
- 905  
906 (56) Dillin, A., Hsu, A.-L., Arantes-Oliveira, N., Lehrer-Graiwer, J., Hsin, H., Fraser, A. G.,  
907 Kamath, R. S., Ahringer, J., and Kenyon, C. (2002) Rates of behavior and aging specified by  
908 mitochondrial function during development. *Science 298*, 2398–2401.
- 909  
910 (57) Lee, S. S., Kennedy, S., Tolonen, A. C., and Ruvkun, G. (2003) DAF-16 target genes that  
911 control *C. elegans* life-span and metabolism. *Science 300*, 644–647.
- 912  
913 (58) Jorgensen, E. M., and Mango, S. E. (2002) The art and design of genetic screens:  
914 *caenorhabditis elegans*. *Nat Rev Genet 3*, 356–369.
- 915  
916 (59) Ayyadevara, S., Balasubramaniam, M., Johnson, J., Alla, R., Mackintosh, S. G., and  
917 Shmookler Reis, R. J. (2016) PIP(3)-binding proteins promote age-dependent protein  
918 aggregation and limit survival in *C. elegans*. *Oncotarget 7*, 48870–48886.
- 919  
920 (60) Johnson, C. S. (1999) Diffusion ordered nuclear magnetic resonance spectroscopy:  
921 Principles and applications. *Prog Nucl Magn Reson Spectrosc 34*, 203–256.
- 922  
923 (61) Wu, D. H., Chen, A. D., and Johnson, C. S. (1995) An Improved Diffusion-Ordered  
924 Spectroscopy Experiment Incorporating Bipolar-Gradient Pulses. *J Mag Res, Series A 115*, 260–  
925 264.
- 926 (62) Stejskal, E. O., and Tanner, J. E. (1965) Spin Diffusion Measurements: Spin Echoes in the  
927 Presence of a Time-Dependent Field Gradient. *J ChemPhys 42*, 288–292.
- 928  
929 (63) Capitini, C., Conti, S., Perni, M., Guidi, F., Cascella, R., De Poli, A., Penco, A., Relini, A.,  
930 Cecchi, C., and Chiti, F. (2014) TDP-43 inclusion bodies formed in bacteria are structurally  
931 amorphous, non-amyloid and inherently toxic to neuroblastoma cells. *PLoS ONE 9*, e86720–.
- 932  
933 (64) Di Natale, C., Scognamiglio, P. L., Cascella, R., Cecchi, C., Russo, A., Leone, M., Penco,  
934 A., Relini, A., Federici, L., Di Matteo, A., Chiti, F., Vitagliano, L., and Marasco, D. (2015)  
935 Nucleophosmin contains amyloidogenic regions that are able to form toxic aggregates under  
936 physiological conditions. *FASEB J 29*, 3689–3701.
- 937  
938 (65) Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics 77*, 71–94.

- 939  
940 (66) Van Ham, T. J., Thijssen, K. L., Breitling, R., Hofstra, R. M. W., Plasterk, R. H. A., and  
941 Nollen, E. A. A. (2008) *C. elegans* model identifies genetic modifiers of  $\alpha$ -synuclein inclusion  
942 formation during aging. *PLoS Genetics* 4, e1000027–11.  
943  
944 (67) Perni, M., Challa, P. K., Kirkegaard, J. B., Limbocker, R., Koopman, M., Hardenberg, M.  
945 C., Sormanni, P., Müller, T., Saar, K. L., Roode, L. W. Y., Habchi, J., Vecchi, G., Fernando, N.  
946 W., Casford, S., Nollen, E. A. A., Vendruscolo, M., Dobson, C. M., and Knowles, T. P. J. (2018)  
947 Massively parallel *C. elegans* tracking provides multi-dimensional fingerprints for phenotypic  
948 discovery. *J Neurosci Methods*.  
949  
950 (68) Van der Goot, A. T., Zhu, W., Vazquez-Manrique, R. P., Seinstra, R. I., Dettmer, K.,  
951 Michels, H., Farina, F., Krijnen, J., Melki, R., Buijsman, R. C., Ruiz Silva, M., Thijssen, K. L.,  
952 Kema, I. P., Neri, C., Oefner, P. J., and Nollen, E. A. A. (2012) Delaying aging and the aging-  
953 associated decline in protein homeostasis by inhibition of tryptophan degradation. *Proc Natl*  
954 *Acad Sci U S A* 109, 14912–14917.  
955  
956



957

958

959 **Figure 1. Trodusquemine inhibits both the lipid-induced initiation and the fibril-induced**

960 **amplification steps in  $\alpha$ -synuclein aggregation *in vitro*.** (a) Change in ThT fluorescence

961 intensity when 100  $\mu\text{M}$  monomeric  $\alpha$ -synuclein was incubated in the presence of 100  $\mu\text{M}$  DMPS

962 vesicles and 50  $\mu\text{M}$  ThT in 20 mM phosphate buffer (pH 6.5) under quiescent conditions at 30

963  $^{\circ}\text{C}$ . Trodusquemine was added to the solutions at increasing concentrations (black: 0  $\mu\text{M}$ , dark

964 blue: 1  $\mu\text{M}$ , green: 2.5  $\mu\text{M}$ , purple: 5  $\mu\text{M}$ , red: 10  $\mu\text{M}$ ); two independent traces are shown for

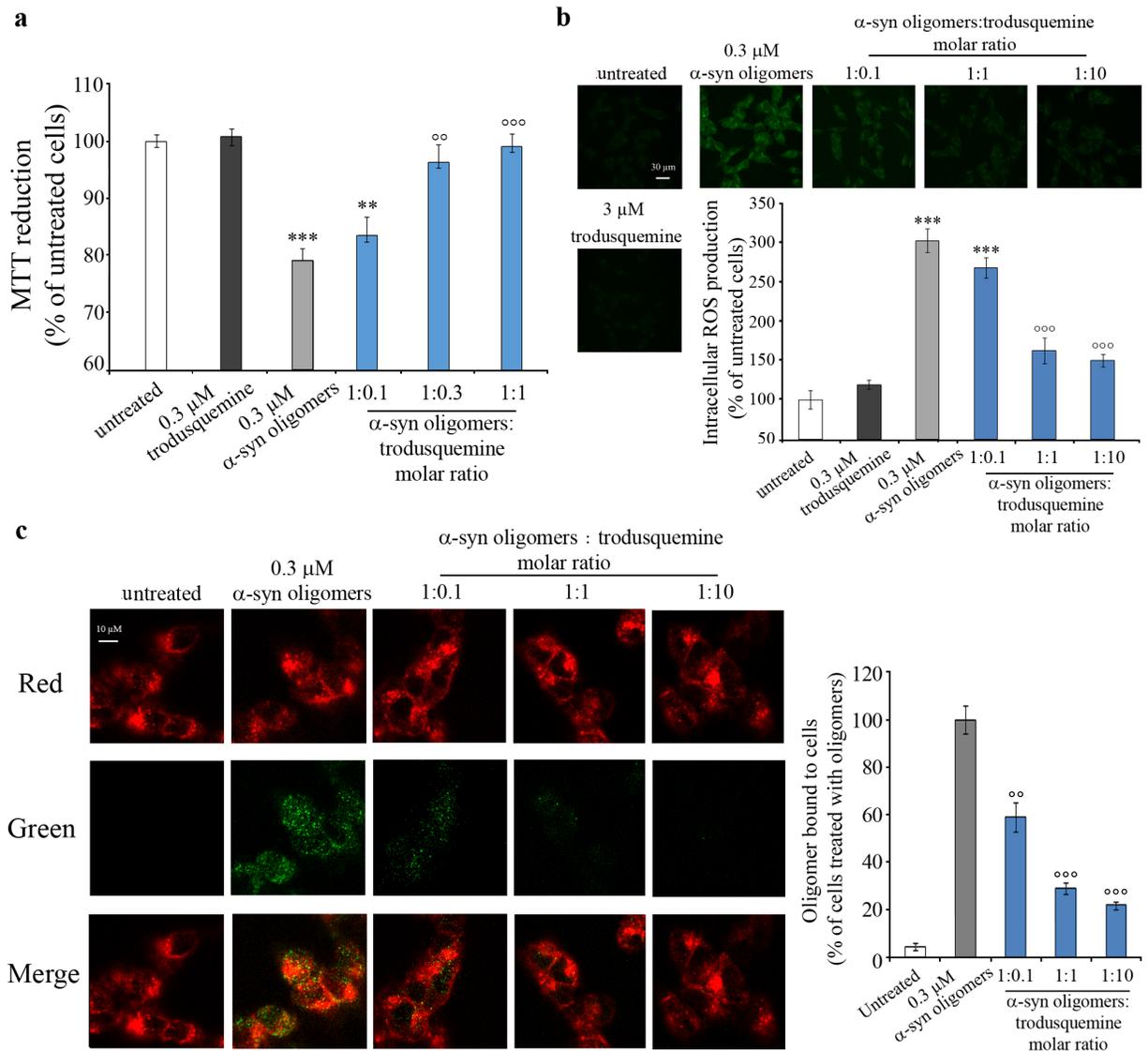
965 each concentration. (b) Relative rates of lipid-induced aggregation. The data were analyzed by

966 globally fitting the early times of the kinetic traces using a single-step nucleation model<sup>15,41</sup> (see

967 **Materials and Methods** for details). (c) Change in ThT fluorescence intensity when 100  $\mu\text{M}$

968 monomeric  $\alpha$ -synuclein was incubated in the presence of 5  $\mu$ M pre-formed fibrils (monomer  
969 equivalents) and 50  $\mu$ M ThT in 20 mM phosphate buffer (pH 6.5) under quiescent conditions at  
970 37 °C. Increasing concentrations of trodusquemine were added to the solution (colours as in  
971 panel **a**) and two independent traces are shown for each concentration. **(d)** Effects of  
972 trodusquemine on the relative rates of fibril elongation. The rates of elongation were extracted  
973 through linear fits to the early time points of the kinetic traces shown in **(c)**<sup>13,15,41</sup>. **(e)** Change in  
974 ThT fluorescence when 100  $\mu$ M monomeric  $\alpha$ -synuclein was incubated in the presence of 50 nM  
975 pre-formed fibrils and 50  $\mu$ M ThT in 20 mM phosphate buffer (pH 4.8) at 37 °C under quiescent  
976 conditions. Trodusquemine was added at different concentrations (colours as in panel **a**); two  
977 independent traces are shown for each concentration. **(f)** Effects of trodusquemine on the relative  
978 rates of fibril amplification. The rates were analyzed by determining the change in fibril number  
979 concentration at the half time of the aggregation reaction<sup>15,41</sup>. **(g)** Schematic representation of the  
980 effects of trodusquemine on lipid-induced aggregation and fibril amplification.

981  
982  
983  
984  
985  
986  
987



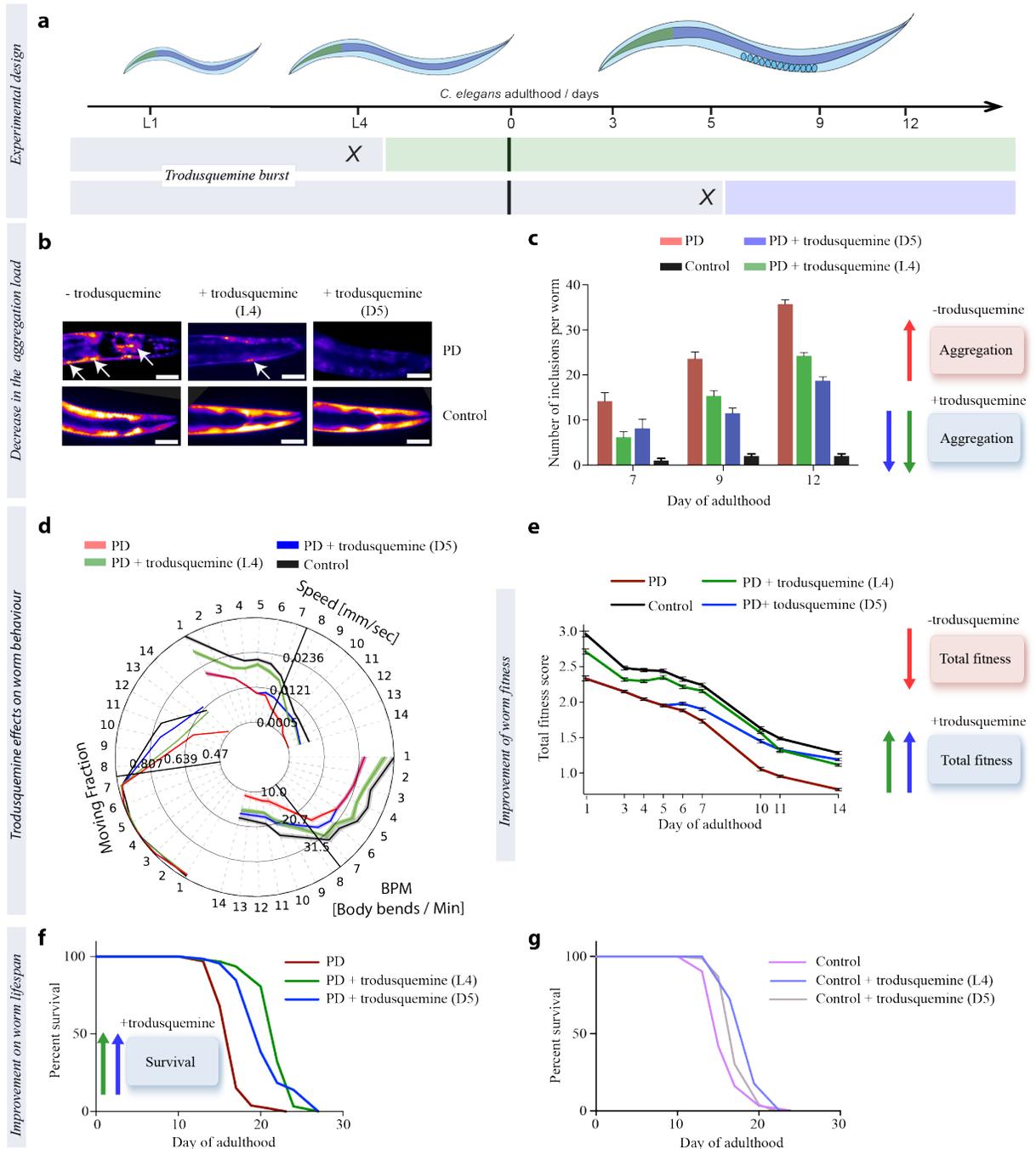
988  
 989 **Figure 2. Trodusquemine suppresses the toxicity of  $\alpha$ -synuclein oligomers in human**  
 990 **neuroblastoma SH-SY5Y cells by inhibiting their binding to cell membranes. (a)** Type B\*  
 991  $\alpha$ -synuclein oligomers were resuspended in the cell culture medium at a concentration of 0.3  $\mu$ M  
 992 (monomer equivalents), incubated with or without different concentrations (0.03, 0.1 and 0.3  
 993  $\mu$ M) of trodusquemine for 1 h at 37 °C under shaking conditions, and then added to the cell  
 994 culture medium of SH-SY5Y cells for 24 h to test the ability of the cells to reduce MTT.  
 995 Experimental errors are S.E.M. \*\* $P \leq 0.01$ , and \*\*\* $P \leq 0.001$ , respectively, relative to untreated  
 996 cells. °° $P \leq 0.01$  and °°° $P \leq 0.001$ , respectively, relative to cells treated with  $\alpha$ -synuclein  
 997 oligomers. (b) Top panel: Representative confocal scanning microscope images of SH-SY5Y

998 cells showing the levels of intracellular ROS following a 15 min incubation with 0.3  $\mu\text{M}$   $\alpha$ -  
999 synuclein type B\* oligomers (monomer equivalents) in the absence or presence of 0.03, 0.3 and  
1000 3.0  $\mu\text{M}$  trodusquemine. The green fluorescence arises from the CM-H<sub>2</sub>DCFDA probe reacts with  
1001 ROS. Lower panel: quantification. \* and ° symbols as in panel A. (c) Representative confocal  
1002 scanning microscopy images of the apical sections of SH-SY5Y cells treated for 15 min with  $\alpha$ -  
1003 synuclein of type B\* oligomers (0.3  $\mu\text{M}$  monomer equivalents) and different concentrations  
1004 (0.03, 0.3 or 3.0  $\mu\text{M}$ ) of trodusquemine (left panels). Red and green fluorescence indicate the cell  
1005 membranes and the  $\alpha$ -synuclein oligomers, respectively. Right: quantification of oligomer  
1006 binding to the cells as fraction of the cells treated with oligomers (right panel). °° and °°°  
1007 symbols as in panel A.

1008

1009

1010



1011

1012

1013 **Figure 3. Treatment with trodusquemine reduces the quantity of  $\alpha$ -synuclein aggregates**

1014 **and improves fitness in *C. elegans* PD model. (a)** Trodusquemine was administered to the PD

1015 worms at either the L4 larval stage or at D5 of adulthood. **(b)** Representative images showing the

1016 effects of trodusquemine on PD worms expressing  $\alpha$ -synuclein fused to yellow fluorescent

1017 protein (YFP). Worms expressing only YFP in the large muscle cells were used as controls. For

1018 every experiment N=50. The images shown are representative of day 12 of adulthood. The scale  
1019 bar indicates 80  $\mu$ m. **(c)** Effects of trodusquemine on inclusion formation in PD worms at 7, 9  
1020 and 12 days of adulthood respectively. In all panels, the experimental errors refer to SEM. **(d)**  
1021 Behavioural map showing the effect of trodusquemine on three phenotypic readouts of worm  
1022 fitness, i.e. paralysis rate, bends per minute (BPM) and speed of swimming, at the indicated days  
1023 of adulthood. For every time-point N=500. **(e)** Total fitness score quantification<sup>29</sup>, following  
1024 treatment with trodusquemine. For every time-point N=500. **(f-g)** Effects following the treatment  
1025 with trodusquemine on PD **(f)** and wild type **(g)** worm survival. For every condition N=75.

1026

1027

1028

1029

1030

1031

1032

1033

1034

1035

1036

1037

1038