Chapter 24

Using Molecular Tweezers to Remodel Abnormal Protein Self-Assembly and Inhibit the Toxicity of Amyloidogenic Proteins

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Abstract

Molecular tweezers (MTs) are broad-spectrum inhibitors of abnormal protein self-assembly, which act by binding selectively to lysine and arginine residues. Through this unique mechanism of action, MTs inhibit formation of toxic oligomers and aggregates. Their efficacy and safety have been demonstrated in vitro, in cell culture, and in animal models. Here, we discuss the application of MTs in diverse in vitro and in vivo systems, the experimental details, the scope of their use, and the limitations of the approach. We also consider methods for administration of MTs in animal models to measure efficacy, pharmacokinetic, and pharmacodynamic parameters in proteinopathies.

Key words Amyloidosis, Proteinopathy, Amyloid, Protein aggregation, Synaptic toxicity, Blood–brain barrier, Alzheimer's disease, Parkinson's disease

1 Introduction

Proteinopathies are characterized by abnormal folding and self-assembly of proteins into cytotoxic oligomers and aggregates. There are over 30 proteinopathies [1, 2], of which prominent examples are Alzheimer's disease (AD) and Parkinson's disease (PD). In addition, abnormal protein self-assembly contributes to the pathologic process in diverse conditions, such as human immunodeficiency virus (HIV) infection [3], traumatic brain injury [4], cancer [5], and preeclampsia [6]. The aberrant oligomerization and aggregate formation may occur intra- or extracellularly, and the resulting protein assemblies disrupt various processes in susceptible cells and organs of affected individuals [7]. The reasons for the preferential targeting of particular cells or tissues by each amyloidogenic protein are not known and the exact mechanisms by which the abnormal protein assemblies cause disease are still untangling.

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To date, there are no therapeutic strategies available to cure proteinopathies [1]. In most cases, no therapy is available at all. For one rare disease, familial amyloidotic polyneuropathy, the drugs tafamidis and diflunisal slow the disease progression [8]. For other diseases, such as AD, PD, and amyotrophic lateral sclerosis, drugs are available that offer moderate and temporary symptomatic relief, but not disease-modifying therapy. Thus, there is a pressing need to obtain a deeper understanding of disease mechanisms and develop strategies for effective prevention and treatment of proteinopathies.

Common structural and pathogenic features of aggregating proteins in various diseases can be targeted by therapeutic strategies such as antibodies, chaperones, and small molecules [9–11]. Our chapter discusses one class of small molecules—molecular tweezers (MTs), which are unique compounds with promising activity against multiple amyloidogenic proteins [12, 13]. MTs bind to amyloidogenic proteins regardless of assembly state and remodel the assembly process into formation of nontoxic and non-amyloidogenic structures that can be degraded efficiently by the natural clearance mechanisms. The current lead MT derivative, CLR01 (Fig. 1), has been used successfully to inhibit abnormal protein aggregation and dissociate pre-formed aggregates in vitro, protect cultured cells from the toxicity of various amyloidogenic proteins, and provide therapeutic effects in multiple animal models [12, 13]. Importantly, CLR01 has been shown to have a high safety margin [14], supporting the use of MTs as attractive therapeutic drug candidates for proteinopathies.

1.1 Molecular Tweezers and Their Mechanism of Action

Lysine-specific molecular tweezers were first reported by Fokkens et al. in 2005 [15] and their ability to remodel abnormal protein aggregation and prevent the toxicity of the resulting oligomers and aggregates was discovered subsequently by the Bitan group [16]. Labile binding of MTs to lysine residues competes with a combination of key electrostatic and hydrophobic interactions that mediate the formation of toxic oligomers and fibrillation nuclei of amyloidogenic proteins. Because the binding is highly labile [17] and occurs with micromolar affinity, it does not interfere with the structure or function of normal proteins (unless substantially higher concentrations are used). This unique mechanism of action of MTs is not specific to one particular protein and thus they can be used as broad-spectrum nanochaperones that inhibit assembly and toxicity of many amyloidogenic proteins.

Recently, a new function was discovered for CLR01—disruption of viral membranes [18], which appears to be distinct from the effect of the compound on amyloidogenic proteins. Presumably, the specificity of the effect to viral membranes is related to the high concentration of cholesterol and sphingomyelin in these membranes relative to membranes of mammalian cells [18], but the

mechanistic details are not known and it is not yet clear whether this activity is unique to CLR01 or common to other MTs. Additional support for the membrane activity of CLR01 also was found in a study of the effect of different assembly modulators on Aβ42 in the presence of lipid membranes [19].

1.2 In Vitro Studies

CLR01 has been the main derivative explored to date for its ability to remodel abnormal protein self-assembly and inhibit the toxicity of the resulting oligomers and aggregates. Several studies have shown that CLR01 binds with high selectivity to lysine and arginine residues in amyloidogenic proteins [16, 17, 20, 21] and does not act by a nonspecific, colloidal mechanism [18]. Mass spectrometry (MS) coupled with electron-capture dissociation, and solution-state NMR studies were used to identify the main binding sites in amyloid β-protein (Aβ) [16] and α-synuclein (α-syn) [20], whose aggregation is involved in AD and PD, respectively.

In most of the in vitro and cell culture experiments, a control derivative named CLR03, which lacks the hydrophobic side arms of the tweezer structure (Fig. 1), was used as a negative control. For example, oligomerization and aggregation of Aβ42 were disrupted effectively by CLR01, whereas CLR03 was inactive, as expected [16]. The aggregation kinetics were followed using thioflavin T (ThT) fluorescence [22] and the morphology of the aggregates characterized by electron microscopy. Formation of oligomers was studied by dot-blots using the anti-oligomer antibody, A11 [23]. More recently, ion-mobility spectroscopy-coupled mass-spectrometry experiments showed that CLR01 disrupted specifically formation of Aβ42 hexamers and dodecamers [24]. Importantly, these oligomers previously have been shown to be key toxic structures linked to Aβ42 toxicity [25–28].

CLR01, but not CLR03, inhibited the aggregation of α-syn and disaggregated pre-formed α-syn fibrils when added either in the middle of the growth phase or after mature fibrils formed

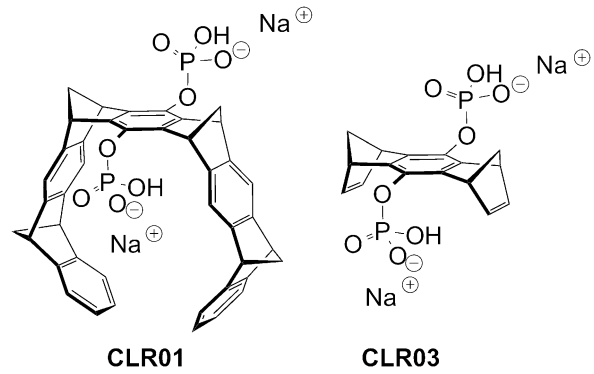

Fig. 1 Chemical structure of CLR01 and CLR03. These compounds are slightly basic and are partially protonated at physiological pH

[20]. Fibril dissociation was a slow reaction completed over ~2 months and required a tenfold excess of CLR01, consistent with the mechanism of action of MTs. Similar results were observed with Aβ40 or Aβ42 [16]. In the case of islet amyloid polypeptide, a highly amyloidogenic peptide associated with pancreatic β-cell death in type-2 diabetes, CLR01 halted fibril growth but did not dissociate preexisting fibrils [21]. In contrast, fibrils of amyloidogenic peptides derived from semen proteins, which greatly enhance HIV infection, were dissociated by CLR01dose-dependently within ~2 h [18]. These results suggest that CLR01's ability to dissociate pre-formed fibrils of amyloidogenic proteins depends strongly on the thermodynamic and kinetic stability of the fibrils.

To date, CLR01 has been found to inhibit the aggregation of 16 different amyloidogenic proteins, including tau, transthyretin (TTR), β_2-microglobulin, insulin, calcitonin [16, 29], and several others that have not yet been published. Recently, aggregation of amyloidogenic p53 mutants has been shown to be disrupted by CLR01, suggesting the potential application of MTs as anticancer agents [30].

1.3 Cell Culture Studies

Thanks to their ability to modulate the self-assembly process of amyloidogenic proteins, MTs would be expected to inhibit the toxicity of oligomers and aggregates of these proteins. Indeed, in all the cases tested to date, CLR01 showed dose-dependent inhibition of the toxicity of diverse proteins in cell viability assays using different cell lines [16, 20, 21, 29, 30].

CLR01 also was found to inhibit synaptic toxicity caused by Aβ42. When added to primary cortical or hippocampal neurons at nanomolar or low micromolar concentrations, Aβ42 caused retraction of dendritic spines and inhibition of both basal synaptic activity and long-term potentiation (LTP), a cellular correlate of learning and memory [31]. CLR01 prevented these toxic effects effectively when added at tenfold excess to the neuronal culture. Dendritic spine retraction and varicosities were alleviated (Fig. 2a) and spine density was rescued to ~80% of the baseline level in the presence of CLR01 (Fig. 2b). Similarly, basal synaptic activity was rescued and LTP was restored to ~80% of baseline [31].

A surprising activity of CLR01 was observed when it was tested with seminal amyloid proteins. CLR01 inhibited the formation of infectivity-enhancing seminal amyloids and dissociated pre-formed fibrils. Interestingly, it also inhibited viral infection directly when tested in TZM-bl cells [18]. CLR01 prevented the formation of virion-amyloid complexes and directly disrupted the membrane integrity of the viruses. Following these surprising findings, the antiviral activity of CLR01 was tested further in other viruses. Infection by the enveloped viruses herpes simplex virus type 2 and hepatitis C virus was inhibited, whereas infection by the nonenveloped human cytomegalovirus was not, supporting the direct effect

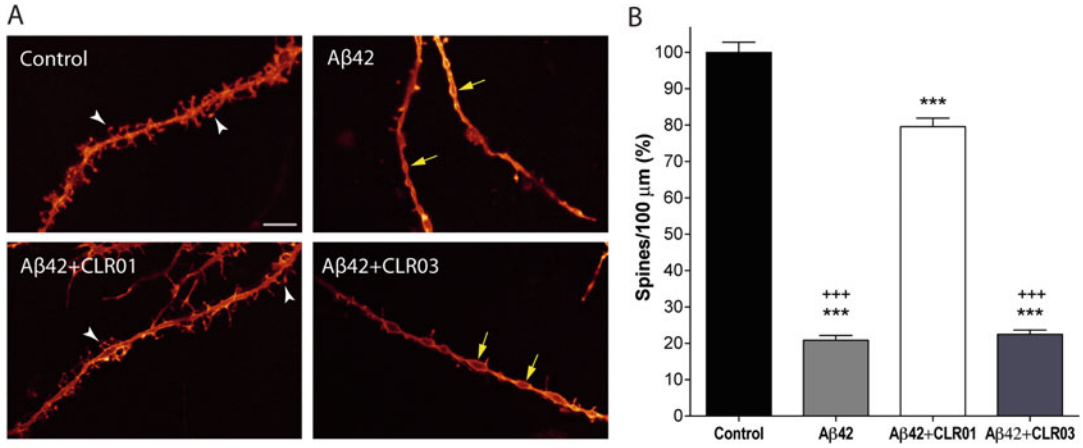

Fig. 2 CLR01 protects neurons from Aβ42-induced changes in dendritic spine number and morphology. (**a**) Rat primary hippocampal neurons incubated for 72 h with media alone or with Aβ42 in the absence or presence of CLR01 or CLR03. Arrows point to Aβ42-induced varicosities. Scale bar = 5 μm. (**b**) The number of dendritic spines per 100 μm was quantified. $^{***}p < 0.001$ compared with control; $^{+++}p < 0.001$ compared with Aβ42 + CLR01. Published originally by Oxford University Press: Brain 135(12): 3735–3748, *Protection of primary neurons and mouse brain from Alzheimer's pathology by molecular tweezers*

of CLR01 on viral membranes and suggesting that the compound could be used as a broad-spectrum antiviral microbicide [18].

1.4 In Vivo Studies

The in vivo efficacy of CLR01 was examined in animal models of several proteinopathies. The compound was evaluated in a triple-transgenic (3 × Tg) mouse model of AD, which overexpresses mutant human presenilin 1 (PS1(M146 V)), amyloid β-protein precursor (APP(KM670/671NL)), and tau(P301L). The mice develop amyloid plaques and neurofibrillary tangles in AD-relevant brain regions (hippocampus, cortex, amygdala) and deficits in synaptic plasticity and memory [32]. 15-month-old 3 × Tg mice were treated for 28 days with 0.04 mg/kg per day CLR01 in saline (vehicle), or with vehicle alone, applied subcutaneously (s.c.), continuously using osmotic minipumps [31]. Immunohistochemical (IHC) analysis of brain sections of mice showed a significant, ~33% decrease in Aβ burden in the hippocampus and cortex of CLR01-treated mice compared to vehicle-treated mice (Fig. 3a–c). Hyperphosphorylated tau (p-tau), but not total tau, also decreased substantially following CLR01 treatment (Fig. 3d–h). Presumably downstream of CLR01's effect on Aβ and p-tau burden, levels of microgliosis also decreased with CLR01 treatment, with no effect found on levels of microglia in CLR01- or vehicle-treated wild-type animals (Fig. 3i–k).

Recently, in a small study in a Tg rat model of AD, which expresses familial AD-linked mutant forms of human APP (K670N/M671L/V717I) and PS1 (M146V) [33], CLR01 was

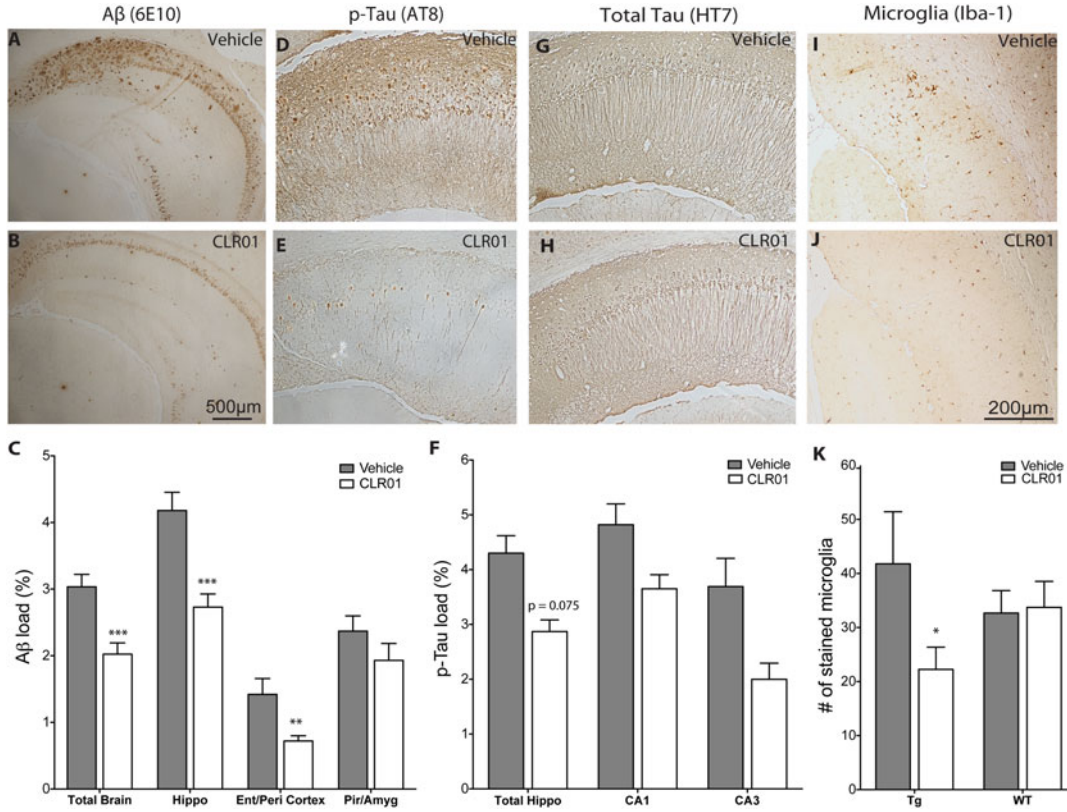

Fig. 3 CLR01 decreases abnormal Aβ and tau deposition and ameliorates microgliosis in Tg mouse brain. 3 × Tg mice were treated with 0.04 mg/kg per day CLR01 or vehicle. (**a**) Vehicle-treated Tg brain stained with monoclonal antibody (mAb) 6E10 showing amyloid plaques and intraneuronal Aβ deposition predominantly in the hippocampus. (**b**) 6E10-stained CLR01-treated Tg brain. (**c**) % Aβ burden quantified by calculating the total 6E10-stained area divided by the total area measured (Hippo—hippocampus, Ent—entorhinal, Peri—perirhinal, Pir—piriform cortex, Amyg—amygdala). (**d**) Vehicle-treated brain showing mAb AT8-positive neurofibrillary tangles in the CA1 region. (**e**) AT8-stained CLR01-treated brain. (**f**) % Aggregated p-tau load quantified by calculating the total AT8-stained area divided by the total area. (**g**) Vehicle-treated brain stained with mAb HT7 for total tau. (**h**) HT7-stained CLR01-treated brain. (**i**) Vehicle-treated brain showing Iba1-positive activated microglia in the subiculum and CA1 region. (**j**) Anti-Iba1-stained CLR01-treated brain. (**k**) Activated microglia load (# of stained microglia in a 1.14 mm^2 area) per treatment condition. The scale bar in panel B is applicable also to A and in J also to panels D–I. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to vehicle-treated mice. Published originally by Oxford University Press: Brain 135(12): 3735–3748, *Protection of primary neurons and mouse brain from Alzheimer's pathology by molecular tweezers*

administered in a similar manner to the 3 × Tg mouse experiment described above, at 0.1 or 0.3 mg/kg/day. The animals were mixed-gender and were treated at 9-months of age, an age at which they are expected to have moderate plaque pathology [34]. Due to the relatively young age of the animals, the plaque pathology found by IHC using the Aβ-specific mAb MOAB-2, which recognizes Aβ but not APP [35], was highly variable (Fig. 4d). Nonetheless, the treatment led to 45% and 52%

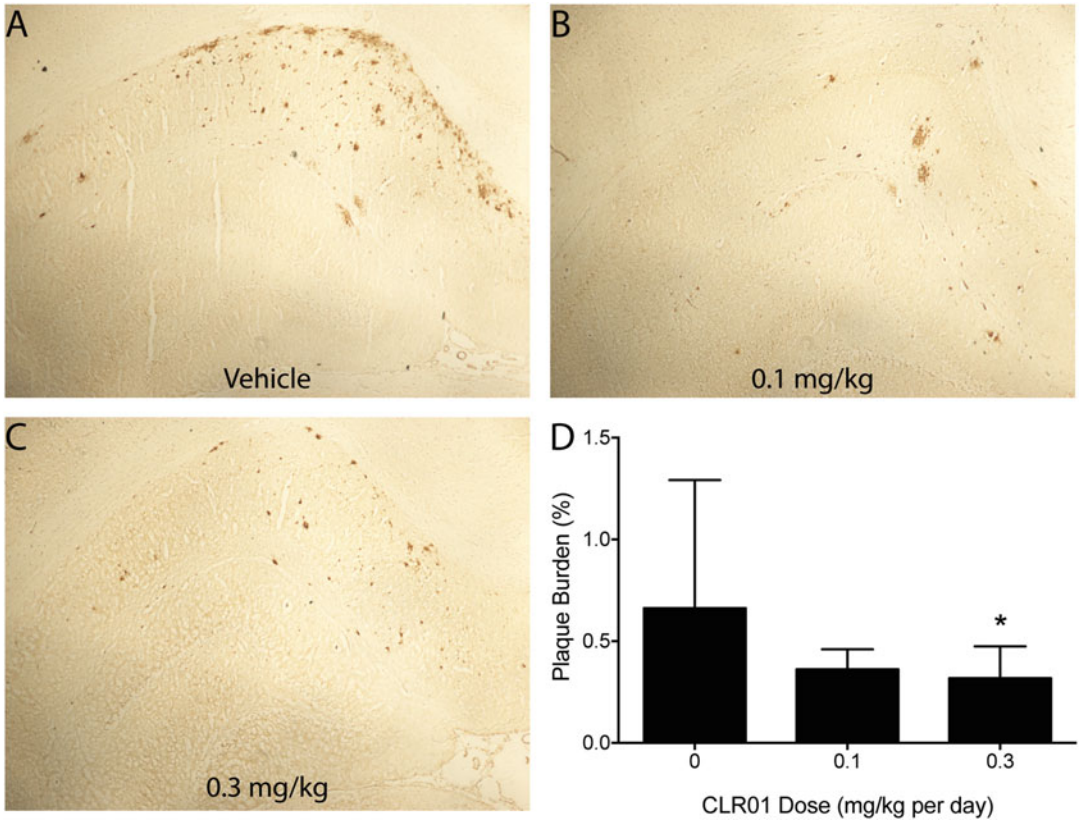

Fig. 4 CLR01 reduces amyloid plaque burden in Tg AD rats. (**a**–**c**) Representative brain sections. (**d**) % Plaque burden quantified by calculating the total MOAB-2-stained area divided by the total area measured. *$p < 0.05$

reduction in plaque burden in the 0.1- and 0.3-mg/kg/day treatment groups, respectively (Fig. 4, whole brain analysis).

In the context of Parkinson's disease, CLR01 was tested in a zebrafish (ZF, *Brachydanio rerio*) embryo model. In this model, neuronal expression of human, wild-type α-syn led to severe deformation and death within 48–72 hours post fertilization (hpf). Addition of 1 or 10 μM CLR01 to the water in which the embryos developed at 8 hpf caused a dramatic improvement in phenotype and survival [20]. IHC analysis showed that in untreated ZF, α-syn formed abundant cytoplasmic aggregates, whereas in CLR01-treated fish α-syn was completely soluble in the cytoplasm. Interestingly, the treatment led to ~80% reduction in total α-syn concentration levels in the ZF neurons. Additional experiments using proteasome inhibitors or a GFP-coupled degron system showed that by keeping α-syn from aggregating, CLR01 enabled its rapid clearance, predominantly by the 26S ubiquitin-proteasome system (UPS) [20]. A recent subsequent study showed that the pesticide Ziram, which increases significantly the risk of developing PD [36], caused selective aminergic neuronal death in ZF embryos, which

was linked to aggregation of the endogenous ZF synuclein. CLR01 was found to significantly rescue the survival and phenotype of Ziram-treated embryos [37], similarly to its effect in the ZF model expressing human α-syn.

To determine whether CLR01 was effective against TTR amyloidosis in vivo, the compound was tested in Tg mice expressing human mutant TTR(V30M) on a mouse TTR-null background and heterozygous for deletion of the heat shock transcription factor 1 (HSF1)—a model of familial amyloidotic polyneuropathy [38]. The mice develop progressive amyloidosis in the gastrointestinal (GI) tract and peripheral nervous system. Treatment with 1.2-mg/kg/day CLR01 via s.c. osmotic minipumps for 35 days led to a significant decrease in TTR deposition in the stomach, colon, and dorsal-root ganglia, and in associated markers of disease, including apoptosis, endoplasmic reticulum stress, and protein oxidation [29].

The safety of CLR01 was evaluated in both acute and chronic administration experiments in wild-type mice [14]. Acute administration of 100 mg/kg CLR01 caused obvious signs of distress, primarily hunching and freezing, which was alleviated completely by 2 h following administration. 10 mg/kg did not appear to cause any distress. Histological and serological analysis showed expected liver injury, but not damage to other organs. No mortality was recorded in either of the groups. In follow-up chronic administration experiments, 10 mg/kg CLR01 for 30 days yielded no signs of discomfort. There were no histological findings and the only significant serum change was ~40% decrease in cholesterol [14]. These findings indicate that CLR01 has a high safety margin in mice.

2 Materials

2.1 In Vitro Studies

1. Active MTs, e.g., CLR01, in a powder form [39].
2. CLR03 in a powder form (as a negative control).
3. Appropriate buffer for dissolving MTs depending on the desired study. For details of different buffers used previously *see* **Note 1** in Subheading 4.1. For a discussion of solubility, refer to **item 1** in Subheading 3.1.
4. Protein sample under study. *See* **Note 2** in Subheading 4.1.
5. Appropriate assay for monitoring the effect of MTs (*see* **item 5** in Subheading 3.1).

2.2 Cell Culture Studies

1. Active MTs in a powder form.
2. CLR03 in a powder form (as a negative control).
3. Sterile water for making MT stocks.
4. Cell culture system for studying the effects of MTs.

5. Growth medium, growth supplements, growth factors, antibiotics, differentiation medium as appropriate for the cell line under study.
6. Protein under study, if the protein is added exogenously (*see* **Note 2** in Subheading 4.2).
7. An appropriate assay for monitoring the effect of MTs.

2.3 In Vivo Studies

The materials described below are two examples: (1) measuring blood–brain barrier (BBB) penetration of CLR01 by spiking the compound with a radiolabeled derivative following different routes of administration; and (2) administering CLR01 s.c. via osmotic minipumps for efficacy experiments. In addition to s.c. injection, several other routes of administration have been used to administer CLR01 and may be used for other MTs, including intravenous injection (i.v.), oral gavage, and intraperitoneal injection (i.p.). If osmotic pumps are used, they can be of different sizes, depending on the animal size, route of administration, delivery rate, and the duration of the experiment. The example below uses the Alzet model 1004 pump (http://www.alzet.com/downloads/1004specs.pdf), which delivers 0.11 μL/h and typically is used for up to 28 days. However, per the manufacturer's instructions, the pump use can be extended up to 35 days.

1. For efficacy studies—osmotic minipumps (model 1004; Alzet).
2. Hemostat (Kent Scientific).
3. Wound clips (7-mm Reflex clips, Alzet).
4. For BBB studies—^{3}H-CLR01 (*see* **Note 1** in Subheading 4.3).
5. A scintillation counter (*see* **Note 2** in Subheading 4.3).
6. Solvable™ (Perkin Elmer).
7. Ultima Gold™ liquid scintillation cocktail (Perkin Elmer).
8. DecapiCones (Braintree Scientific, Inc.).
9. 28 gauge × 0.5″ syringe needles (BD).
10. 30% H_2O_2.

3 Methods

3.1 In Vitro Studies

The method described here is for a broad range of experiments. Changes can be made for specific experimental designs. The high aqueous solubility of CLR01 allows dissolution in the same buffer as the protein. If other MTs are used, the concentrations may need to be adjusted based on the solubility of the specific compound. The protein:MT molar ratio should be examined and potentially optimized for specific experiments depending on the protein under study. The following are common steps for evaluating the effect of

MTs in vitro on different aspects of amyloid proteins' self-assembly, such as oligomerization, aggregation, and remodeling of oligomers or amyloid fibrils.

1. Prepare a stock solution of the MTs used in the experiment. We recommend preparing the stock solution in deionized water. This way, it is easier to recover any unused compound without any contamination of salts or buffers. Typically, 10 mM stock solutions can be made for CLR01. The limit of solubility has not been determined formally, yet based on our experience, it is between 10 and 15 mM. The solubility of other MTs may vary substantially and should be determined for each compound.
2. Prepare the protein in a buffer system in which it is to be studied. In most cases, this should be the last step and experiments should begin immediately because many amyloidogenic proteins aggregate fast (*see* **Note 3** in Subheading 4.1).
3. Add MT to the protein under study at different molar ratios to obtain a dose–response relationship (*see* **Note 4** in Subheading 4.1). The negative control MT, CLR03, may be added only at the highest concentration ratio if appropriate (*see* **Note 5** in Subheading 4.1). A positive control is the protein alone, with no MTs added.
4. Incubate the protein:MT mixtures under suitable aggregation conditions. Online monitoring of the samples may be done by measuring various parameters, such as absorbance (e.g., for turbidity assays) or fluorescence (e.g., for thioflavin T assays), using common plate readers. If desired, aliquots of the reaction mixtures may be withdrawn periodically and analyzed separately.
5. Typical methods for analysis of the aggregation reaction, conformational change, and morphology of the protein assemblies include thioflavin T fluorescence, dynamic light scattering, circular dichroism spectroscopy, and electron microscopy [40, 41] (*see* **Note 6** in Subheading 4.1).

3.2 Cell Culture Studies

The steps below are general instructions for testing the effect of MTs in cell lines. Some steps may need to be modified to suit the specific cell line being used. Specific details for PC-12 cells, the cell we have used most often, are mentioned in the Notes section.

3.2.1 Plating Cells for Measurement

1. Harvest the cells in media when cell confluence reaches 70–80% of flask surface (*see* **Note 2** in Subheading 4.2).
2. Plate in a 96-well plate at a cell density of 30,000 cells/well (90 μL/well, *see* **Notes 3** and **4** in Subheading 4.2).
3. Incubate cells in a cell culture incubator at 37 °C and 5% CO_2 for 24–48 h (*see* **Note 5** in Subheading 4.2).

3.2.2 Adding the Protein and MTs to Cells and Assay

1. Prepare stock solutions of MTs at 1–10 mM concentration by dissolving in sterile water (*see* **Note 6** in Subheading 4.2), and then dilute in media for a dose–response experiment (*see* **Notes 7** and **8** in Subheading 4.2).
2. Make stock solutions of the protein under study if the protein is added exogenously (*see* **Note 1** in Subheading 4.2).
3. Remove a small volume of the media (approximately 40 μL) and add the protein:MT mixture to make the final total volume in each well 100 μL (*see* **Note 9** in Subheading 4.2).
4. Incubate the cells in a cell culture incubator at 37 °C and 5% CO_2 for the time required for the specific assay to be performed (*see* **Notes 10–12** in Subheading 4.2).

3.3 In Vivo Studies

In vivo studies may be used to determine the safety, efficacy, pharmacokinetics, and pharmacodynamics of MTs. The species, specific disease models, and route of administration vary according to the goals of the experiment. To date, most experiments have tested CLR01 in mice or fish [13]. Below are examples of protocols suitable for measuring BBB penetration of CLR01 in mice by spiking the compound with a radiolabeled derivative, and for treating mice by s.c. administration via osmotic minipumps.

3.3.1 Measuring BBB Penetration of CLR01

1. Restrain mice using DecapiCones or other mouse restrainers.
2. Measure the body weight (BW) of mice and prepare a mixture of CLR01, 1 mg/g BW spiked with ~2 μCi/g BW of ^{3}H–CLR01. The ^{3}H–CLR01 makes up 10% of the total CLR01.
3. Inject 4 μL/g BW of the CLR01:^{3}H–CLR01 mixture into the tail vein using 28-gauge × 0.5″ syringe needles (*see* **Note 3** in Subheading 4.3).
4. After the appropriate amount of time (*see* **Note 4** in Subheading 4.3), collect the blood via cardiac puncture, perfuse the mouse thoroughly through the heart with PBS, and collect the brain.
5. Dissect the brain into the two hemispheres. Weigh out one hemisphere. Draw 100–350 μL of blood. Digest the brain hemisphere and the blood, each with 1 mL Solvable for 2 h at 60 °C.
6. To the blood sample, add 0.1 mL EDTA-Na_2 solution, then add 0.3 mL 30% H_2O_2 in small aliquots, incubate for 15–30 min at ambient temperature, cap tightly, and incubate for additional 30 min at 60 °C.
7. Add 10 mL Ultima Gold, mix well, and measure the radioactivity in the sample in a liquid scintillation counter. Take three measurements for each sample, subtract the background radioactivity, and average.
8. Brain penetration percentage is calculated as radioactivity per gram of brain divided by radioactivity per milliliter of blood.

3.3.2 Administering CLR01 to Mice Via s.c. Osmotic Minipumps

1. Anesthetize mice by intraperitoneal injection of a ketamine/xylazine cocktail (87.5 mg/kg ketamine, 12.5 mg/kg xylazine) 0.1 mL/20 g mouse BW.
2. Once the animal is anesthetized, shave and disinfect the skin over the implantation site.
3. Make an incision 1.5-times the diameter of the implant adjacent to the site chosen for pump placement and perpendicular to the long axis of the implant. If the back of the animal is the site of choice, make a mid-scapular incision across the back perpendicular to the spine.
4. Insert a hemostat into the incision and by opening and closing the jaws of the hemostat, spread the subcutaneous tissue to create a pocket for the pump. The pocket should be large enough to allow some free movement of the pump (e.g., 1 cm longer than the pump). Avoid making the pocket too large, as this will allow the pump to turn around or slip down on the flank of the animal. The pump should not rest immediately beneath the incision because this may interfere with the healing of the incision.
5. Insert a filled pump into the pocket, delivery portal first. This minimizes interaction between the compound delivered and the healing of the incision. If the pocket is not large enough to hold the implant comfortably, remove the implant and enlarge the pocket as described above.
6. Close the wound with wound clips. Two clips will normally suffice.
7. The daily dose is determined by the concentration of the CLR01 in the pump and calculated by the rate of delivery of the pump (0.11 μL/h for the Alzet 1004 model).
8. Once the treatment has been completed, anesthetize mice with pentobarbital (100 mg/kg), and collect blood via a cardiac puncture.
9. Perfuse mice with cold, fixative or non-fixative saline buffer containing protease inhibitors (*see* **Note 5** in Subheading 4.3).
10. Immediately excise and fix in 10% formalin or 4% paraformaldehyde, or flash-freeze organs of interest, by placing the sample into a 1–2 mL cryovial. Cap the vial tightly and submerge in liquid nitrogen. When the content is frozen, transfer to a − 80 °C freezer.
11. IHC, western blots, ELISA, or any other techniques may be used for analysis of the tissue.

4 Notes

4.1 In Vitro Studies

1. MTs have been used in different solvents and buffer solutions, including water [18, 19]; 10 mM sodium phosphate, pH 7.4 [16, 20]; 100 mM sodium phosphate, pH 7.7 or 9.0 [31, 42]; 100 mM sodium phosphate, pH 7.4, 10 mM tris(2-carboxyethyl)phosphine (TCEP), and 0, 10, 20, or 30% sucrose (w/w) [43]; 60 mM sodium hydroxide, 20 mM phosphate buffer; 5 mM dithiothreitol (DTT), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 mM NaCl, pH 7.6; 10 mM glycine-HCl, pH 3.5; 10 mM sodium acetate pH 3.2; or 10 mM sodium acetate pH 4.4 [16, 21].
2. For best results, fresh protein solutions should be used for most experiment types. In case the protein is already present in solution in a frozen state, freeze and thaw cycles should be minimized as it may alter the state of protein and protein aggregation pattern.
3. Certain amyloidogenic proteins require removal of pre-formed aggregates, which nucleate further aggregation and reduce reproducibility substantially. For such proteins this step should be done every time to make the assay uniform and get reproducible aggregation studies. For example, we consistently treat Aβ with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) before performing aggregation or oligomerization assays [44].
4. The concentrations of MTs to be used in an assay should be wide so as to test the minimum and maximum range of effect. If the effect on a particular protein is not known, a good rule of thumb is to start at a protein:MT concentration ratio 1:10. If no effect is observed at this ratio, one should consider whether increasing the ratio would make sense in view of future applications in biological systems, given the safety limits. If an effect is observed, the next logical step is to determine the dose–response relationship of this effect.
5. In some studies, CLR03 had unexpected effects on certain proteins [24, 30]. These effects were small relative to those of CLR01, but in those cases, CLR03 could not be considered a true negative control.
6. Molecular tweezers may interfere with the analysis by some techniques. For example, high concentrations of MTs may suppress the radical reaction required for Photo-Induced Cross-linking of Unmodified Protein (PICUP). If a technique that has not been tested before is going to be used to monitor the reaction, possible interference by MTs should be tested first.

4.2 Cell Culture Studies

1. Cell toxicity assays can be performed with peptides/proteins added exogenously [16] or plasmid transfected to express the peptide or protein endogenously, as has been described previously, e.g., for α-syn [20].
2. Mix a small volume of cells with Trypan blue (we typically mix 0.5 mL of cells in media with 0.1 mL of a Trypan blue solution) and use a hemocytometer to count viable cells. We use a Bright Line Reichert 0.1-mm deep hemocytometer. Other counting methods also may be used.
3. We typically plate PC-12 cells in 96-well plates at a cell density of 30,000 cells/well. For different cell lines, cell density may need to be optimized to avoid overcrowding of cells and to ensure proper differentiation of cells that need to be differentiated into a particular phenotype.
4. In our typical experiments, PC-12 cells are harvested with differentiation media and neuronal growth factor (NGF) is added at 150 ng/mL before plating in a 96-well plate.
5. The time of incubation may vary depending upon the requirements for preparation of cells, e.g., 80–90% confluence or differentiation into mature cells.
6. To ensure sterility, we recommend using autoclaved distilled water filtered through a 0.2-μm filter and preparing the solution inside a biological safety cabinet (laminar-flow tissue-culture hood).
7. A wide concentration range of MTs should be tested to determine the minimum concentration (lower limit) below which inhibition on peptide toxicity is not significant and up to a maximum concentration (upper limit) at which MTs show cytotoxicity [16]. For example, we tested the inhibition of toxicity induced by 5–10 μM Aβ42 using concentrations of MTs increasing by half-log steps (1, 3, 10, 30, and 100 μM). In these experiments, we used NGF-differentiated PC-12 cells and prepared serial dilutions of MTs in differentiation media containing NGF.
8. The toxicity of MTs may vary depending on the cell type used and needs to be evaluated for specific systems. For example, in NGF-differentiated PC-12 cells, CLR01 caused ~25% decrease in cell viability at 400 μM [16], whereas no toxicity was observed in TZM-bl cells at 500 μM [18].
9. The protein and MTs can be mixed together before adding to the cells [16, 20] or MTs can be added separately after adding the protein. If MTs are added after adding protein, the cells should not be left exposed to protein for more than ~20 min.
10. When adding the solution to the well, tilt the plate and let the solution trickle down along the wall of the well to avoid disrupting the cells.

11. Different cytotoxicity assays can be used such as Caspase-3, TUNEL, and other similar assays. We have used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to measure cell viability [16], lactate dehydrogenase (LDH) assay to assess cell death [45], and an ELISA kit which measures cytoplasmic histone-associated nucleosomes for measurement of apoptosis [21].
12. The assembly state of the proteins is important for studying cytotoxicity. For example, to induce toxicity in NGF-differentiated PC-12 cells, α-syn added exogenously had to be incubated for 24 h to allow formation of toxic oligomers, whereas freshly dissolved α-syn was not toxic [20].

4.3 In Vivo Studies

1. In our experiments, ^{3}H–CLR01 was prepared by Moravek Biochemicals (Brea, CA) using a method that provides ^{3}H incorporation into the hydrocarbon skeleton (i.e., non-labile protons) [46].
2. We use a Triathler liquid scintillation counter (model 425–034). Any other scintillation counter suitable for measuring tritium radiation may be used.
3. I.v. injection in the tail vein may be technically difficult because the veins are hard to visualize, especially for a learner. Consulting an experienced user should be considered. Injection into the exposed jugular vein may be an optional method in anesthetized mice.
4. The plasma half-life was found to be ~ 2.5 h and approximately 5% of the initial CLR01 levels were found in the plasma 8 h following i.v. administration. The radioactivity level in the brain did not change significantly up to 72 h post injection [14].
5. The buffer composition depends on the subsequent analysis and the experimental design. Protease inhibitors are always included. Phosphatase inhibitors also may be included if analysis of phosphorylated proteins is planned. Non-fixative buffer is needed if biochemical analyses are planned, whereas fixative buffer can be used for subsequent IHC analysis. Depending on the experimental design, some animals may be perfused with fixative buffer and others with non-fixative buffer. Alternatively, all the animals may be perfused with non-fixative buffer and tissues may be post-fixed for IHC analysis.

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