

Clioquinol promotes the degradation of metal-dependent amyloid- β ($A\beta$) oligomers to restore endocytosis and ameliorate $A\beta$ toxicity

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Alzheimer's disease (AD) is a common, progressive neurodegenerative disorder without effective disease-modifying therapies. The accumulation of amyloid- β peptide ($A\beta$) is associated with AD. However, identifying new compounds that antagonize the underlying cellular pathologies caused by $A\beta$ has been hindered by a lack of cellular models amenable to high-throughput chemical screening. To address this gap, we use a robust and scalable yeast model of $A\beta$ toxicity where the $A\beta$ peptide transits through the secretory and endocytic compartments as it does in neurons. The pathogenic $A\beta$ 1–42 peptide forms more oligomers and is more toxic than $A\beta$ 1–40 and genome-wide genetic screens identified genes that are known risk factors for AD. Here, we report an unbiased screen of ~140,000 compounds for rescue of $A\beta$ toxicity. Of ~30 hits, several were 8-hydroxyquinolines (8-OHQs). Clioquinol (CQ), an 8-OHQ previously reported to reduce $A\beta$ burden, restore metal homeostasis, and improve cognition in mouse AD models, was also effective and rescued the toxicity of $A\beta$ secreted from glutamatergic neurons in *Caenorhabditis elegans*. In yeast, CQ dramatically reduced $A\beta$ peptide levels in a copper-dependent manner by increasing degradation, ultimately restoring endocytic function. This mirrored its effects on copper-dependent oligomer formation in vitro, which was also reversed by CQ. This unbiased screen indicates that copper-dependent $A\beta$ oligomer formation contributes to $A\beta$ toxicity within the secretory/endosomal pathways where it can be targeted with selective metal binding compounds. Establishing the ability of the $A\beta$ yeast model to identify disease-relevant compounds supports its further exploitation as a validated early discovery platform.

phenotypic small-molecule screen | metal chelation

Alzheimer's disease (AD) is a common and devastating neurodegenerative disorder that is projected to increase in frequency as our population ages. The lack of disease-modifying therapies requires new approaches to address the underlying mechanisms of cellular dysfunction and identify potential therapeutics.

The amyloid- β peptide ($A\beta$) plays a major role in AD and ultimately leads to neuronal death and cognitive impairment (1). $A\beta$ peptides of ~40 aa are generated by the successive cleavage of the amyloid precursor protein (APP) by β - and γ -secretases. Mutations in APP or γ -secretase cause familial AD and bias APP cleavage toward a 42-aa $A\beta$ peptide that predominates in $A\beta$ plaques, is more aggregation prone, and is toxic to neurons (2, 3). Although $A\beta$ plaques are a common, conspicuous feature of pathology in diseased brains, increasing evidence suggests that small oligomers of $A\beta$ are the most toxic species (4, 5).

The conservation of protein homeostasis mechanisms—such as protein trafficking and chaperone networks—among all eukaryotes makes yeast a powerful discovery platform for modeling the cellular toxicities caused by neurodegenerative disease proteins (6). In neurons, the cleavage of APP to generate the $A\beta$ peptide occurs within the secretory and endosomal pathways (7). APP is trafficked through the secretory pathway to the plasma membrane and subsequently internalized and recycled through endosomal vesicles

and the trans-Golgi network back to the plasma membrane (7). During this recycling, the $A\beta$ peptide is liberated from APP by β/γ -secretases, thus enabling the peptide to interact with multiple membranous compartments within the cell.

We have taken advantage of the great conservation of the secretory and endocytic pathways between yeast and neurons to study $A\beta$ in a simple, highly tractable model organism—budding yeast. By expressing $A\beta$ as a fusion to an endoplasmic reticulum (ER) targeting signal, we have mimicked in yeast the multi-compartmental distribution of $A\beta$ (8). This approach bypasses the need to recapitulate the entire APP processing pathway and generates an $A\beta$ peptide with exactly the same sequence as is found in the human brain. The ER targeting signal directs cotranslational transport of the primary translation product into the ER, where the signal sequence is removed by signal peptidase. The peptide then transits through the secretory pathway and is secreted from the cell. In yeast, the cell wall prevents secreted $A\beta$ from diffusing away, allowing it to interact with the plasma membrane and undergo endocytosis. As in the human nervous system (2), the aggregation-prone 42-aa peptide is more prone to forming oligomeric species than the 40-aa peptide (9) and is more toxic (8).

This model allowed us to take advantage of yeast genetics to perform a completely unbiased screen of the yeast genome for suppressors or enhancers of $A\beta$ toxicity. Of the ~6,000 genes we tested, we recovered only a handful of modifiers. There are

Significance

Identifying disease-modifying therapies for Alzheimer's disease (AD) has been an insurmountable challenge. To provide a new discovery tool for high-throughput compound screening, we used a simple yeast model that makes toxic amounts of β -amyloid ($A\beta$), a peptide central to AD pathology. Previous genetic analysis established that $A\beta$ compromises yeast biology in a manner relevant to human AD. We screened 140,000 compounds for reversal of toxicity and identified a class of protective metal-binding compounds related to clioquinol (CQ), a compound that alleviates $A\beta$ toxicity in mouse AD models. Treating yeast with CQ promoted rapid degradation of $A\beta$ oligomers, rescuing cellular processes perturbed by this insidious peptide and restoring viability. Our approach provides a method for identifying compounds that may eventually help treat AD.

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~25,000 genes in the human genome and less than 20 (10) have been shown to confer risk for AD. However, several of the yeast genes that alter A β toxicity are either direct homologs or interacting partners of human risk factors (8, 10). For example, *YAPI802*, is the yeast homolog of PICALM, one of the most highly validated risk factors for AD (10, 11); *INP52*, is homologous to SYNJ1, which interacts with the risk factor BIN1 (12, 13); and *SLA1* is homologous to SH3KBP1, which interacts with the risk factor CD2AP (14–16). All of these proteins are involved in clathrin-mediated endocytosis in yeast and humans. In addition to ameliorating the toxicity of A β in yeast, these proteins reduced A β toxicity in both *Caenorhabditis elegans* and rat cortical neuronal models (8). The recovery of genes that promote clathrin-mediated endocytosis in unbiased genome-wide screens suggested that A β poisons this process (8). Indeed, the peptide compromised endocytosis of a transmembrane receptor (Ste3), an activity crucial for neuronal function. Importantly, the mechanism of action of these risk factors had not previously been linked to A β . Thus, the yeast model has already provided key insights on the nature of A β 's cellular toxicity in the human brain.

In this study, we used our yeast A β model to identify small molecules that ameliorate toxicity. In an extremely stringent and unbiased screen of 140,000 compounds, we identified a small number of cytoprotective compounds, including 8-hydroxyquinolines (8-OHQs). Members of this family bind metals and are among the few compounds that have been shown to alleviate A β toxicity in mouse models of AD (17, 18), and to show early potential as an AD therapeutic (19). Here, we investigate the mechanism of action for the most efficacious member of this family, clioquinol (CQ).

Results

Screen for Compounds That Rescue A β Toxicity in Yeast. To screen for compounds that ameliorate A β toxicity in yeast, we used a strain with a compromised drug efflux system that expresses A β under the control of a galactose-inducible promoter (8). This strain exhibited a level of A β toxicity that provided sufficient dynamic range and high signal-to-background signal to confidently identify rescuing compounds. Approximately 140,000 compounds were tested in duplicate in 384-well plates using increased growth (optical density) as an end point. The high reproducibility enabled robust detection of “hits” (Fig. 1A). Only ~30 compounds passed our rigorous scoring criteria (Z score > 3), establishing the high stringency of the screen. Strikingly, one-half of all recovered hits were 8-OHQs (Fig. 1B and Fig. S1).

Notably, the effectiveness of this class of compounds against A β toxicity has been established in neurons and mouse models. Two closely related 8-OHQs—CQ and PBT2—decrease A β accumulation and alleviate cognitive and behavioral symptoms in mouse models of AD (17, 18). 8-OHQs are moderate-strength metal chelators that can extract metals weakly bound to proteins and both redistribute metals within the cell and act as ionophores (17, 18). PBT2 is one of only a few compounds effective in phase II clinical trials with human AD patients (19). CQ rescued our yeast A β model in a highly dose-dependent manner (Fig. 1C and D), but at higher concentrations it inhibited the growth of both A β -expressing cells and control strains (Fig. 1C). The increase in optical density of A β -expressing cells with CQ was a direct consequence of increased viable colony-forming units and not an artifact of increased cell size (Fig. S2).

Due to our previous findings that different 8-OHQs have distinct activities in different models of proteotoxicity (20), we compared the efficacy of CQ in all of our yeast proteotoxicity models. The A β , α -synuclein (i.e., Parkinson disease), TDP-43 (i.e., frontotemporal dementia and ALS), and htt72Q (i.e., Huntington disease) yeast models have comparable levels of toxicity, but in each case the nature of the cellular toxicity is distinct as are the genetic hits obtained from unbiased genome-wide modifier screens (8, 21–23). CQ had significant, yet modest efficacy against α -syn and htt72Q and no effect on TDP-43 (Fig. 1E). However, it rescued the A β model more potently than it did any other model, indicating a considerable degree of selectivity.

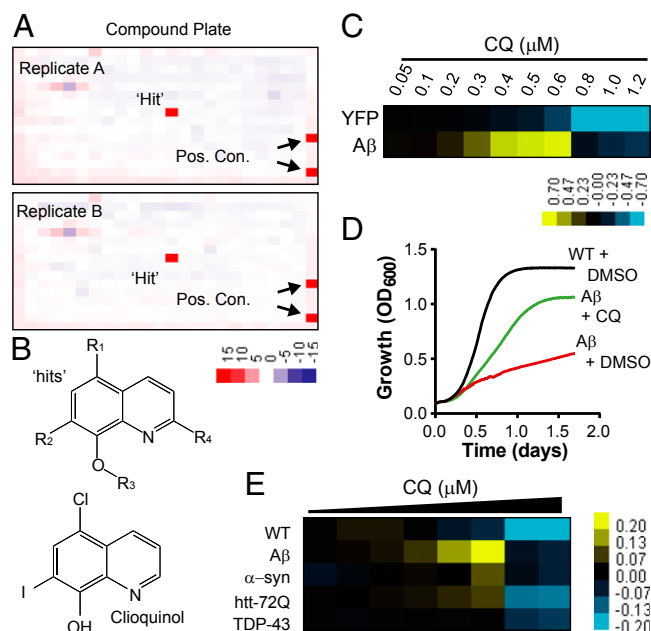


Fig. 1. Small-molecule screen identifies 8-OHQ that protect against A β toxicity. (A) Representative duplicate 384-well screening plates containing a single “hit” and positive controls. Values are Z scores, where “red” indicates rescue and “blue” indicates toxicity. (B) General structures of 8-OHQ hits and CQ. (C) Dose–response heat map of CQ treatment of both YFP- and A β -expressing strains. Data indicate the difference in OD₆₀₀ between CQ- and DMSO-treated samples. Yellow, rescue. Blue, toxicity. Concentrations are in micromolar. (D) Growth curves of WT yeast and the A β strain treated with the maximal effective dose of CQ or DMSO alone. The y axis is OD₆₀₀, and the x axis is time in days. (E) Dose–response curves of WT and multiple proteotoxicity yeast models treated with CQ in 384-well plates. Data are reported as the difference in OD₆₀₀ between the CQ-treated and DMSO-treated conditions. Yellow indicates rescue, and blue reflects toxicity. The doses of CQ range from 5 μ M down to 0.3 μ M CQ with a 30% dilution at each step.

Although CQ showed some protection in other models, we previously showed that CQ does not rescue the toxicity caused by diverse yeast genes when overexpressed from the same galactose-regulated promoter (20). In addition, CQ was unable to rescue growth defects caused by five different gene deletion mutations and seven toxic compounds with diverse modes of action (Table S1). Given the selectivity of CQ, we focused on investigating its activity in the yeast A β toxicity model.

CQ Restores Endosomal Trafficking. Our recent unbiased screen for suppressors of A β toxicity uncovered several proteins involved in clathrin-mediated endocytosis whose homologs are AD risk factors in humans (8). We directly tested the effect of A β on clathrin-mediated endocytosis using Ste3, a G-protein–coupled receptor for the mating pheromone. Indeed, steady-state Ste3 endocytosis was severely perturbed by A β and rescued by the genetic suppressors involved in this process (8). Here, we extended the analysis with another widely used reporter of endocytic function, a putative 13-pass transmembrane transporter for methionine, Mup1. This reporter provides a facile means of investigating stimulus-dependent trafficking: the endocytosis of Mup1 is rapidly induced by the addition of methionine.

As expected, Mup1-GFP localized primarily to the plasma membrane of WT cells in the absence of methionine (Fig. 2, Upper Left). After 30 min of methionine treatment, Mup1-GFP was endocytosed and exclusively detected in endosomes and the vacuole (Fig. 2, Upper Right). In the absence of methionine, A β expression perturbed steady-state trafficking of Mup1, detected by its localization to both the plasma membrane and to intracellular foci (Fig. 2, Lower Left). The addition of methionine promoted

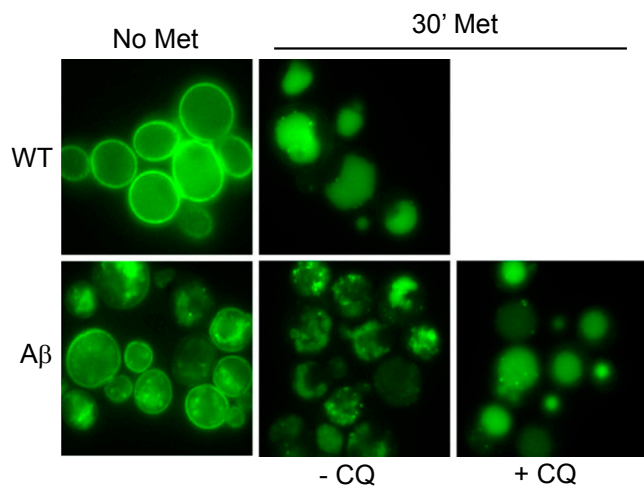


Fig. 2. CQ restores endosomal trafficking of Mup1-GFP. Mup1-GFP endocytosis was monitored in WT and A β strains (CQ, 0.8 μ M). Cultures without methionine (Left) or with 30-min methionine treatment (Right) enabled visualization of stimulated endocytosis of Mup1-GFP from the plasma membrane to the vacuole.

Mup1-GFP internalization, but delivery to the vacuole was greatly impeded (Fig. 2, Lower Center). CQ completely restored endosomal transport of Mup1-GFP to the vacuole in the strain expressing A β (Fig. 2, Lower Right). Thus, A β causes defects in endosomal transport that are manifested downstream of initial transporter internalization and these defects are rescued with CQ treatment.

CQ Rescues a *C. elegans* Model of A β Toxicity. To establish that the ability of CQ to rescue the toxicity of A β in the secretory compartment was conserved in neurons, we tested a nematode model of A β toxicity. In this model, A β is expressed in the secretory pathway of glutamatergic neurons (a neuronal subtype particularly relevant to AD) using the *eat-4* promoter and a *C. elegans* ER signal sequence (8). An advantage of *C. elegans* is that the transparency of the cuticle allows surviving neurons to be visualized in living animals expressing GFP in the same neurons. A β expression produced an age-dependent loss of glutamatergic neurons. Importantly, the endocytosis-related genes we originally identified in our yeast screen, which are also known AD risk factors in humans, suppress A β toxicity in this nematode model (8). Expressing A β in glutamatergic neurons enables a quantitative measure of neurodegeneration and differs from previous approaches in the nematode where A β expressed within the body wall muscle cells caused a motor phenotype (24, 25).

As previously described, the percentage of worms with the WT number of neurons decreased from 3 to 7 d post larval hatching [Fig. 3A] (8)]. Because the cuticle of the worm is relatively impenetrable, we used an established protocol to administer CQ acutely at the earliest larval stage (26). After 24 h, the worms were transferred to normal growth media and allowed to develop and age in the absence of CQ. Even with this single early dosing, CQ increased the percentage of worms with the WT number of neurons. Rescue was apparent at both 3 and 7 d post CQ treatment (Fig. 3A and B). Thus, as in yeast, the toxicity of secreted forms of A β is rescued by CQ in glutamatergic neurons. This encouraged us to take advantage of the yeast model to further investigate CQ's mechanism of action.

CQ Rescue of A β Toxicity Requires Metal Binding. Because 8-OHQs are known metal binding compounds, we first asked whether this functionality was required for the rescue of A β toxicity. We tested CQ analogs lacking either the hydroxyl group or the aromatic nitrogen. Together, these groups coordinate the bidentate binding of transition metals ions. Because direct analogs of CQ lacking

these groups were not available, we tested analogs of chloroxine (CQ_{Cl}) (Fig. 4A). This 8-OHQ is identical to CQ except that it has a chlorine atom in place of the iodine atom. It was as effective at alleviating A β toxicity in yeast as was CQ (Fig. 4B, "Max"). We then tested CQ_{Cl} analogs lacking either the hydroxyl group (CQ_{Cl}-OH) or the nitrogen atom (CQ_{Cl}-N). Both were inactive against A β , indicating CQ efficacy requires bidentate metal binding (Fig. 4B).

Next, we tested the ability of exogenous metals to alter CQ activity. We used copper, iron, and zinc, all of which are known to bind CQ (27). On their own, none of these metals affected A β toxicity (Fig. 4C). When coadministered at a concentration that was equimolar with CQ, each metal had completely different effects (Fig. 4C). Copper abolished rescue of A β by low concentrations of CQ and also abolished the growth inhibition caused by higher CQ concentrations. Iron shifted the dose-response, increasing the required dose for rescue and reducing the growth inhibitory effects of CQ. Zinc had no effect on CQ efficacy or growth inhibition at higher concentrations. These data suggest that CQ binds copper, and to a lesser extent iron, to alleviate A β toxicity.

Effects of Metals and CQ on in Vitro A β Assembly. Metal ions, including copper, iron, and zinc, bind to A β both in vitro and within A β plaques from the brains of AD patients (28, 29). In in vitro assembly reactions with A β , the addition of copper favors the formation of nonfibrillar, amorphous aggregates (29) and oligomers (30, 31) that are more toxic to cells than amyloid fibers of A β (18, 30–32). Metal binding compounds, such as CQ and PBT2, can remove metals from A β in vitro (17, 18, 33, 34). These compounds also increase the amount of soluble A β in the brains of AD mouse models in vivo and stimulate the extraction of A β from plaques in brain samples from AD patients (17). Because the predominant form of A β 1–42 in yeast is small oligomers (8) and copper appears central to rescue of A β toxicity (Fig. 4C), we

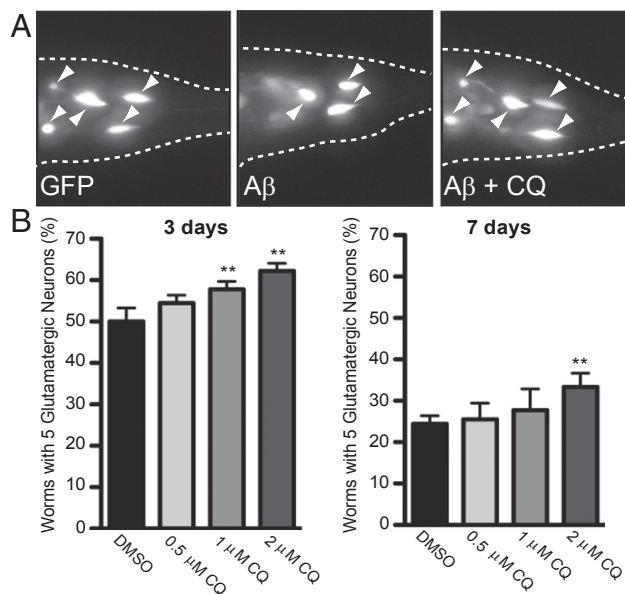


Fig. 3. CQ rescues worms expressing A β in glutamatergic neurons. (A) Fluorescent microscopy images of representative WT or A β -expressing worms. The rescue of *C. elegans* glutamatergic neurons by CQ was monitored with GFP coexpressed in the same cells as A β under control of the *eat-4* promoter. (B) Quantification of CQ rescue is reported as the percentage of worms with the WT number (5) of anatomically distinct glutamatergic neurons in the hermaphrodite tail at 3 and 7 d after drug treatment. The arrowheads indicate surviving glutamatergic neurons. Data are the average of three independent experiments with 30 worms in each set. Error indicates SD, and the asterisk (*) indicates a value of $P < 0.05$ according to Student's *t* test.

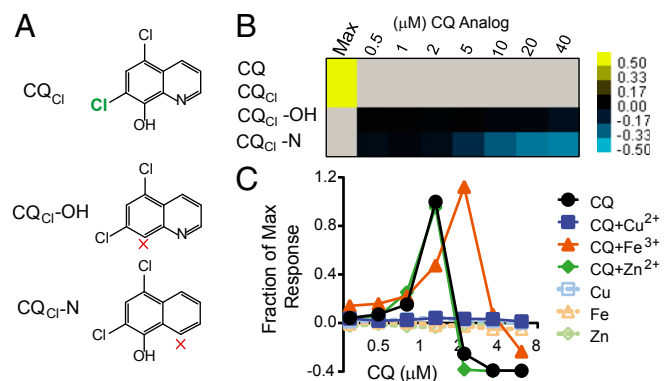


Fig. 4. CQ rescues A β through a metal-dependent mechanism. (A) Structures of CQ derivative, chloroxine (CQ_{Cl}), and analogs lacking either the hydroxyl group (CQ_{Cl}-OH) or aromatic nitrogen (CQ_{Cl}-N). (B) Dose-response curves of CQ_{Cl} analogs. Data are expressed as the difference in OD₆₀₀ between drug-treated and untreated cultures. Values above graph indicate concentration in micromolar. Yellow indicates rescue; blue indicates toxicity. Only the maximum effective concentration (1 μ M, "Max") is shown for CQ and CQ_{Cl}. (C) Dose-response curves of A β cultures treated with CQ and equimolar copper, iron, or zinc indicate interactions between CQ and copper and iron. Data reflect differences in OD₆₀₀ between treated and untreated samples normalized to maximal rescue by CQ (micromolar) in the absence of supplemented metals.

tested the effects of copper on A β oligomerization in vitro, and asked how CQ affects this process.

We first tested the effects of copper on A β 1–42 fiber formation using ThioflavinT (ThT), which binds β -sheet-rich proteins (i.e., amyloid fibrils). Copper strongly inhibited amyloid fiber formation when present in A β assembly reactions (Fig. 5A) (29). To characterize the species of A β that accumulate in the presence of copper, we used an antibody known as A11. This antibody, which was raised against a form of A β that mimics toxic oligomers, recognizes specific prefibrillar conformers of A β that are highly toxic to neurons (35). A β assembly reactions were carried out with both equimolar (1:1) and excess (5:1) copper relative to A β for 30 min and then subject to dot blot analysis with A11 and another antibody that detects total A β (6E10). Copper substantially increased the amount of A11-reactive A β (Fig. 5B). CQ had no effect on the formation of A11-reactive A β species on its own, but it strongly antagonized the oligomer-potentiating effects of copper (Fig. 5C).

CQ Dramatically Reduced A β Peptide Levels by Increasing Degradation.

To determine whether CQ has a similar ability to modulate A β within the context of the secretory compartment of a living cell, we returned to yeast. First, we examined the effects of CQ on the accumulation of A β using denaturing SDS gels. CQ dramatically reduced the accumulation of A β , with a sharp dose dependence that mirrored the sharp dose dependence of CQ's ability to rescue A β toxicity (Fig. 6A, Upper). CQ had no effect on total protein levels (Fig. 6A, Lower).

We next tested whether exogenous metals antagonized the ability of CQ to reduce A β levels. Indeed, they did so in a manner similar to their effects on the rescue of A β toxicity (Fig. 4C). Copper completely eliminated CQ's effects on A β accumulation; iron affected it modestly; and zinc had no effect (Fig. 6B). We next asked what species of A β were most affected by CQ. Nondenaturing gels, which preserved oligomer structure, demonstrated a decrease in most forms of A β when cultures were treated with CQ (Fig. 6C, Right). The levels of a control protein (YFP) expressed from the same promoter did not change (Fig. 6C, Left). Thus, the ability of CQ to rescue A β toxicity directly correlated with its ability to prevent the accumulation of oligomeric A β in a manner dependent on metal binding.

The decrease in oligomeric A β could be due to either its reduced accumulation or accelerated degradation. To distinguish between these two possibilities, we used two methods to assess degradation rates. First, we treated cells with the protein synthesis inhibitor, cycloheximide (CHX), and monitored A β levels by immunoblotting. After expressing A β for 24 h with either DMSO or CQ, cells were treated with CHX for 15 or 45 min. In CQ-treated cells, A β was rapidly degraded within 15 min of inhibiting translation (Fig. 6D). This increased degradation was not observed with a control protein (Pgk1). In contrast, the A β peptide that accumulated in cells treated with DMSO did not significantly degrade during this same time frame (Fig. 6D).

Inhibiting translation in this global manner might alter cellular degradation pathways and confound interpretations. We therefore used [³⁵S]methionine pulse labeling and immunoprecipitation. Cells expressing A β were transiently labeled with [³⁵S] methionine to allow incorporation of radiolabeled methionine into newly synthesized proteins. After chasing cultures with non-labeled methionine, we monitored the remaining A β by immunoprecipitating the peptide from cell lysates. In agreement with the CHX experiment, CQ specifically increased degradation of A β (Fig. 6E and Fig. S3). Together, the two experiments indicate that the stability of A β oligomers formed within the secretory pathway is dependent on metals and that, by affecting these oligomers, CQ can directly antagonize the cellular pathologies caused by A β .

Discussion

We have used an unbiased phenotypic small-molecule screen of ~140,000 compounds to identify a clinically relevant class of compounds that prevent the toxic effects of A β expressed within the secretory pathway. Although multiple metal-related A β pathologies and modes of action for CQ—and PBT2—have been described and postulated (17, 18, 28), our data suggest a mechanism of action in the yeast model that is not ionophore based (18). Rather, CQ promoted the degradation of metal-dependent A β oligomers within the secretory and endosomal compartments.

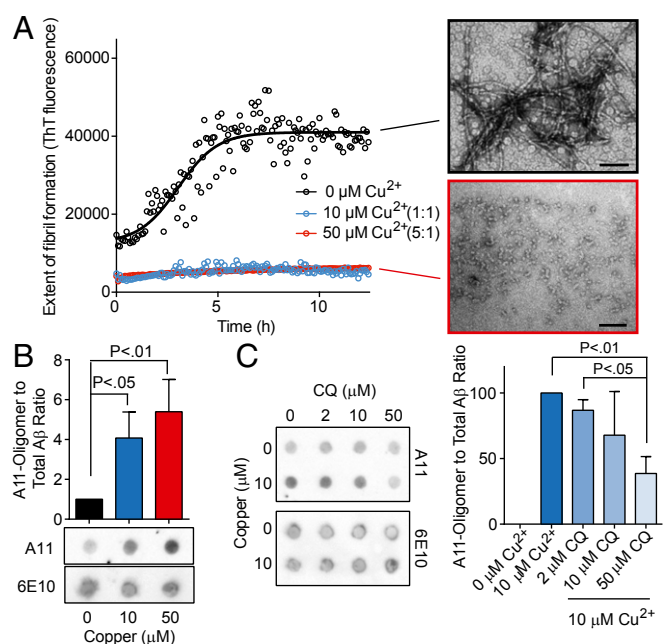


Fig. 5. Effects of copper and CQ on A β oligomers. (A) ThT fluorescence of A β assembly reactions in the presence of copper (micromolar) at indicated molar ratios compared with A β . EM shows representative fields of A β with no or 50 μ M Cu²⁺. (B) Dot blot analysis of A β assembly (10 μ M) in the presence or absence of both copper and CQ at 1:1 and 5:1 molar ratios. A11 monitors oligomeric A β conformations and 6E10 monitors total A β . (C) Dot blot (Left) and quantitation (Right) of A β assembly reactions in the presence of indicated concentrations of copper and CQ.

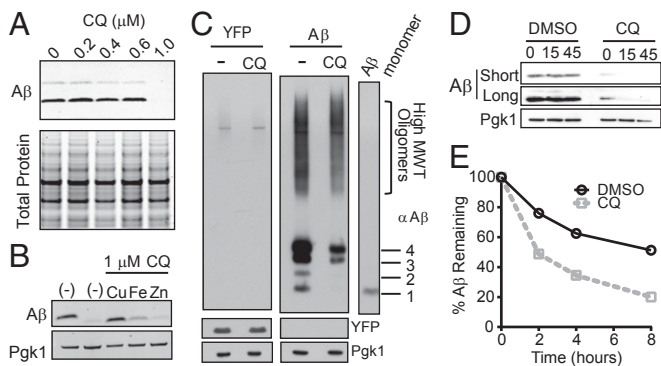


Fig. 6. CQ promotes A β degradation. (A) Denaturing SDS/PAGE and A β immunoblot show reduction of A β levels in response to CQ treatment compared with total protein (Coomassie). (B) A β immunoblots of cells treated with CQ and equimolar Cu $^{2+}$, Fe $^{3+}$, and Zn $^{2+}$. Pgk1 is a loading control. (C) A β immunoblot of nondenaturing gel shows a decrease in all forms of A β . A β oligomeric states are indicated to the *Right* of the monomer control lane. A YFP control strain is shown on the *Left*. (D) Immunoblot analysis of A β -expressing yeast with DMSO control or CQ after a short CHX time course. Short and long exposures are shown for comparison. (E) The percentage of A β remaining after [35 S]methionine pulse-labeling A β -expressing yeast in presence or absence of CQ. A β was immunoprecipitated and quantified at 2-h intervals after pulse labeling.

Thus, our yeast A β model has allowed us to focus on an early aspect of A β misfolding and the identification of CQ indicates that these early events are directly modulated by metals. Moreover, the results from this chemical screen indicate that modulating A β :metal complexes directly ameliorates the defects caused by A β oligomers within the secretory/endosomal pathways, which we previously linked to AD risk alleles through genetic screens (8). This connection further establishes the potential for this class of small molecules to mitigate both early and late aspects of A β cellular pathologies.

Endogenous transition metals play an important role in A β pathology and their modulation has potential promise in treating AD patients (19, 36, 37). We suggest that the metal-dependent enhanced degradation of A β we observe here for CQ rescue of A β toxicity plays a role in neuronal systems as well. However, it is important to note that multiple mechanisms likely operate on the longer timescales of A β accumulation in mouse models and in human disease. For example, accumulation of transition metals in extracellular A β plaques formed late in disease can limit intracellular metal availability (28). CQ can dissolve plaques that have formed in late stages of disease (in both mice and humans) (17) and liberate trapped metals. This then presumably allows the ionophoric capability of CQ and PBT2 to simultaneously restore metal homeostasis and up-regulate proteases that degrade extracellular A β (18, 28, 36). Deciphering both early and late aspects of A β pathology will be important in understanding cellular pathologies and developing new therapeutic approaches in the treatment of AD.

The nearly complete elimination of A β accumulation that is mediated by CQ in yeast far exceeds any effect on protein stability that we have observed for any other compound or genetic suppressor in any of our yeast models of protein-folding pathologies (20, 38). CQ modestly rescues α -syn and htt72Q toxicity [Fig. 1E (20)]. However, it has little effect on the accumulation of these proteins (20). Presumably, this difference in the activities and efficacy of CQ is due to some, as yet unknown, difference in the cellular context and biophysical properties of different metal-binding proteins [of which α -syn is one (39)]. We found that the degradation of A β was not affected by the ablation of any single pathway (i.e., autophagy; Fig. S4). This, together with the extreme rate of CQ-mediated A β degradation, suggests that multiple mechanisms are at play.

Empowered by significant improvements in DNA sequencing, a considerable effort is now underway to catalog polymorphisms in the human genome and identify those that contribute to pathologies related to protein homeostasis. It is now clear that the total number of human polymorphisms is much greater than previously realized (40), creating an intense need for rapid and tractable model systems to explore genotype–phenotype causality. Our yeast A β system meets this need. A previous unbiased genetic screen for modifiers of A β toxicity allowed us to link toxicity to recently discovered human AD risk alleles (8) with no previously known relationship to A β . Here, the facile yeast system enabled high-throughput compound screening that allowed us to rapidly identify relevant compounds and interrogate their mechanism of action. This ultimately validates our approach as we pursue new compounds with unknown protective mechanisms. Although this initial screen primarily revealed the central importance of metals in cellular A β pathology, we anticipate that other compounds with distinct modes of action will be identified. In general, for AD and other protein-misfolding diseases, yeast chemical genetic approaches will help elucidate new targets and “druggable” pathways and aid discovery and validation (38). It is imperative to integrate several orthogonal model systems and many other recent advances (10, 22, 38, 41, 42) to further our understanding of, and eventually our ability to treat, devastating diseases such as AD.

Methods

Constructs and Yeast Strains. The signal sequence:A β (ssA β) construct has been described (8). The chemical screening strain used here was generated by integrating either *GAL1*-regulated A β or YFP constructs into the *trp1* and *ura3* loci of a WT strain and deleting the drug efflux pump (*pdr5::KanMX*) using standard methods (Table S1). The *MUP1-GFP* A β strain was generated by recombining a PCR product of GFP and a downstream *HIS3* auxotrophic marker amplified from a chromosomal *MUP1-GFP* WT yeast strain (38).

Small-Molecule Screen and Compounds. Small-molecule screening was carried out as described (20). Briefly, late log phase A β cultures grown in 2% (wt/vol) raffinose-containing media were diluted ($OD_{600} = 0.03$) into galactose-containing media. Compounds (100 nL) were pinned to 384-well plates containing diluted culture and incubated for 40 h at 30 °C. OD_{600} values were then used to calculate Z scores [(OD_{600} well – OD_{600} plate average)/ OD_{600} plate average]. Compounds were purchased as follows: CQ (Sigma-Aldrich); CQ_{Cl} (Sigma-Aldrich); CQ_{Cl}-OH (Santa Cruz Biotechnology); CQ_{Cl}-N (Frontier Scientific Services).

Growth Assays. Growth assays with compounds were performed by either Bioscreen C or 384-well plates analysis. In both cases, log phase raffinose cultures were diluted ($OD_{600} \sim 0.02$) into galactose-containing media, compounds or DMSO added, and cultures grown at 30 °C with either intermittent shaking (Bioscreen C) or endpoint (384-well) OD_{600} readings. Data were typically expressed as the difference in OD_{600} between compound- and DMSO-treated cultures. For all metal experiments, metals were added at the same time as CQ. Specificity experiments were performed in conditions with at least 50% reduction in growth in response to gene deletion or toxic compounds (Table S2). Toxic compound concentrations were selected to inhibit growth of WT yeast by at least 50%.

MUP1-GFP Assay. The effects of A β expression on Mup1-GFP endocytosis were performed as described (38). Cultures induced for ~ 16 h with 5GalMet in a Bioscreen C with or without CQ (0.8 μ M), methionine (50 μ g/mL) added for 30 min, and GFP localization then imaged by fluorescence microscopy.

C. elegans CQ Treatment. The *C. elegans* strain (UA166) expressing an ssA β construct in glutamatergic neurons has been described (8). Compound treatment (20) and analysis of glutamatergic neurons (8) have been described.

In Vitro A β Oligomer Formation. Lyophilized, chemically synthesized A β (1–42) (American Peptide Company) was dissolved at ~ 1 mg/mL in pH 11.5 NaOH and flash frozen. Exact concentration was measured using amino acid analysis (Dana Farber Molecular Biology Core). For ThT assays, A β was dissolved in PBS (pH 7.2) to a final concentration of 10 μ M and ThT was added to a final concentration of 20 μ M. The solution was allowed to aggregate at 30 °C under quiescent conditions and fluorescence at 480 nm was monitored for 12 h using a Tecan Safire plate reader. For dot blots, 300 μ L of solutions

containing 10 μM A β with varying concentrations of Cu and CQ were loaded onto a 0.1- μm nitrocellulose membrane using the Bio-Rad vacuum filter trap device. The membrane was then blocked in 5% (wt/vol) nonfat dry milk in PBS for 1–2 h followed by an overnight incubation in A11 antibody [Millipore; 1:250 in 5% (wt/vol) milk/PBS] and a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Sigma). Quantification of dot blots was performed using ImageJ (NIH). Transmission electron microscopy was performed by incubating A β solutions aggregated for ~12–15 h on a Ni-Formvar grid followed by negative staining by 2% (wt/vol) uranyl acetate.

Effect of CQ on A β Levels. The ssA β and YFP strains were grown to log phase in raffinose media and then diluted to an OD₆₀₀ of 0.01 (YFP) or 0.03 (A β) in media containing galactose with either 0.6 μM CQ or DMSO and grown in a Bioscreen C plate at 30 °C to an OD₆₀₀ of ~0.6 (~16 h). Lysates were generated and blots probed as previously described (8).

Analysis of A β Degradation. For CHX experiments, A β yeast strains were cultured and induced as above with or without CQ. After A β induction, CHX was added (35 $\mu\text{g}/\text{mL}$) and aliquots removed at indicated time points. Samples were brought to 40 mM sodium azide, washed with ice-cold water, and A β levels analyzed (8).

For [³⁵S]methionine pulse-labeling experiments, cells expressing ssA β were grown as described above, harvested, washed twice in media lacking methionine, resuspended in the same media (OD₆₀₀ = 1.0), and incubated

at 30 °C for 15 min. L-[³⁵S]Methionine (Perkin-Elmer; specific activity > 1,000 Ci/mmol) was then added (50 $\mu\text{Ci}/\text{mL}$) for 20 min. Cultures were diluted into media containing excess cold methionine (450 $\mu\text{g}/\text{mL}$) to an OD₆₀₀ of ~0.5. Aliquots were removed, brought to 20 mM Na azide and 0.1 mg/mL BSA and placed on ice. A β was immunoprecipitated with the 6E10 antibody (Covance) and Protein A/G Plus Agarose (Pierce). After washing, beads were boiled in sample buffer and supernatant subjected to SDS/PAGE. For total label, A β immunoprecipitation total extract was resolved and the entire lane quantified. Imperial blue (Pierce)-stained gels were washed with water, equilibrated with 1 M sodium salicylate in 10% (wt/vol) glycerol, and dried. The gel was exposed to a FujiFilm imaging plate at –20 °C, the plate scanned with a FujiFilm BAS-2500 Bio-Imaging Analyzer, and the data quantitated with Multigage, version 2.2, software.

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