TDMQ20, a Specific Copper Chelator, Reduces Memory Impairments in Alzheimer's Disease Mouse Models

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ABSTRACT

Beside targeting amyloid or tau metabolisms, regulation of redox metal ions is a recognized therapeutic target for Alzheimer's disease (AD). Based on the bioinorganic chemistry of copper, we designed specific chelators of copper(II) (TDMQs) in sight to regulate copper homeostasis in the brain, and to inhibit the deleterious oxidative stress catalyzed by copper-amyloid complexes. An oral treatment by TDMQ20 was able to fully reverse the cognitive and behavioral impairment in three different murine models, two non-transgenic models mimicking the early stage of AD, and a transgenic representing a more advanced stage of AD. To our knowledge, such comparative study using the same molecule has never been performed.

Regular C57 BL/6J mice received a single injection of human Cu-Aβ_{1-42} in the lateral ventricles (icv-CuAβ) or in the hippocampus (hippo-CuAβ). In both cases, mice developed a cognitive impairment similar to that of transgenic 5XFAD mice. Oral administration of TDMQ20 to icv-CuAβ or hippo-CuAβ mice within a 16-days period resulted in a significant improvement of the cognitive status. The 3-months treatment of transgenic 5XFAD mice with TDMQ20 also resulted in behavioral improvements. The consistent positive pharmacological results obtained using these different AD models correlate well with previously obtained physico-chemical data of TDMQ20. The short-term novel object recognition (NOR) test was found particularly relevant to evaluate the rescue of declarative memory impairment. TDMQ20 was also able to reduce the oxidative stress in the mouse cortex. Due to its reliability and facile use, the hippo-CuAβ model can be considered as a robust non-transgenic model to evaluate the activity of potential drugs on the early stages of memory deficits.

KEYWORDS

Alzheimer's disease / copper / chelator / memory / mouse models / hippocampus

1. INTRODUCTION

Alzheimer's disease (AD) affects millions of people worldwide, and the incidence of this age-related disease is increasing due to the increase of life expectancy in many countries.[1,2] AD being mainly characterized by the formation of senile plaques of β-amyloid peptides and by neurofibrillary tangles due to the hyperphosphorylation of the tau protein, these phenomena have been considered as targets for the design of therapeutic agents.

Since memantine in 2003, no new drug has been approved, except sodium oligomannate (GV-971), recently approved by the National Medical Products Administration of China.[3] Between 2003 and 2019, 100 % of drug-candidates failed in clinical trials.[4] The quasi-exclusive use of transgenic mice to select drug-candidates for a disease which has no genetic origin for sporadic cases, might be questioned. Predictive and easy to handle mouse AD models are needed. In an attempt of diversification, we developed a non-transgenic mice model for the evaluation of the chelator PA1637.[5] In this model, an episodic memory deficit was generated by a single intracerebroventricular (icv) injection in the brain of normal mice, of low molecular weight $A\beta_{1-42}$ oligomers without copper. Despite controversies on the role of soluble amyloids,[6] they are still considered as toxic agents contributing to synaptic alteration, abnormal tau phosphorylation, glial activation and neuronal loss *in vivo*.[7] More recently, the key role of Aβ oligomers to trigger activation of the glycogen synthase kinase 3β (GSK3β), responsible for tau hyperphosphorylation, has been demonstrated.[8]

Despite the capacity of PA1637 to extract copper from copper-amyloid *in vitro*, and to inhibit the catalytic production of H_2O_2 by Cu(II)-amyloid[9,10] its development has been stopped due to its rather low bioavailability. Consequently, we designed a new series of specific chelators of Cu(II) named TDMQ, based on a 8-aminoquinoline skeleton with a chelating side-chain at C2 (Figure 1). Such tetradentate ligands have the required structure to discriminate between copper and zinc.[11-15] Two 8-hydroxyquinolines, clioquinol (CQ) and PBT2, failed in clinical trials to improve cognition and reduce amyloid plaques.[16,17] This failure might be explained by their lack of specificity for copper chelation,[18,19] and their inability to inhibit the production of reactive oxygen species (ROS) generated by the Cu-amyloids.[15,20] TDMQ ligands are able to extract copper(II) from Cu-amyloids, without disturbing the activities of Cu,Zn-SOD, tyrosinase or vitamin B_{12}.[14] TDMQ20 has been selected as lead molecule for evaluation as anti-AD drug-candidate on three different mouse models, two non-transgenic- and a transgenic one. TDMQ22,

a second chelator, differs from TDMQ20 by having a CF_3 substituent at position 6 instead of Cl at C5 and C7.[13]

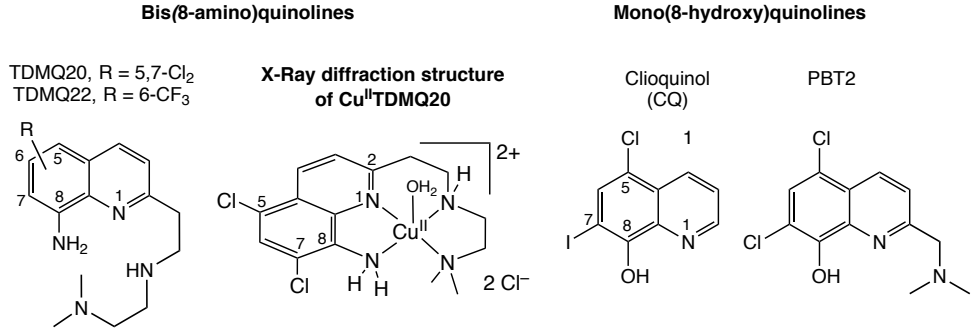

Figure 1. Structure of the chelators TDMQ20, TDMQ22, clioquinol (CQ) and PBT2, and structure of the copper(II) complex of TDMQ20, for its X-ray structure data, see Reference 11.

The deregulation of copper in AD brain and the associated memory deficits have been created by injection of the copper complex of amyloid $A\beta_{1\text{-}42}$ in the lateral ventricles (icv-CuAβ model) or hippocampus (hippo-CuAβ model) of regular mice. The third model was the transgenic mouse 5XFAD model. Data obtained with these three murine models indicated that the oral administration of TDMQ20 was able to inhibit memory deficits.

2. RESULTS AND DISCUSSION

2.1. Mouse AD models, and drug treatment.

In two non-transgenic mouse models, the cognitive deficit was created by injection of human Cu^{II}-$A\beta_{1\text{-}42}$ complex either in the lateral ventricles (icv-CuAβ model) or in the hippocampus (hippo-CuAβ model) of regular young C57BL/6J mice (two months of age), at D0. The icv-CuAβ model of AD is similar to the icv injection of $A\beta_{1\text{–}42}$ oligomers used to evaluate the tetradentate PA1637 chelator.[5] In the second non-transgenic model, hippo-CuAβ, the Cu-$A\beta_{1\text{–}42}$ solution was injected in the hippocampus. This region of the brain is involved in declarative memory and learning as well as in spatial memory, and is particularly vulnerable in the early stages of AD.[21,22] Atrophy of hippocampus is strongly correlated with cognitive decline.[23] This non-transgenic hippo-CuAβ model can therefore contribute to mimic the early stages of AD.

In these non-transgenic AD mice, the drug treatment consisted of oral gavage with TDMQ20, TDMQ22 or CQ used as comparator, 8 times between D4 and D20 (each dose was 10 mg kg^{-1}).

We also evaluated TDMQ20 on the transgenic 5XFAD transgenic mouse model (C57BL6 genetic background), that expressed human APP and PSEN1 transgenes with a total of five AD-

linked mutations: the Swedish (K670N/M671L), Florida (I716V) and London (V717I) mutations in APP (the precursor of amyloids) and the two mutations in PSEN1 (M146L and L286V) (PSEN1 is the gene coding for presenilin-1, a constituent of γ-secretase). This model recapitulates several AD-related aspects of the disease,[24,25] with early formation of amyloid plaques and gliosis at two months. Cognitive and motor deficits start at about 6 months, and develop with age in parallel with neuron degeneration in multiple brain regions.[26,27]

For 5XFAD mice, the drug treatment was carried out once every two days, from 2 to 5 months of age, and consisted of oral gavage with TDMQ20, or CQ used as comparator, (45 times, each drug dose: 10 mg kg^{-1}). The mouse cognitive status was evaluated using the standardized behavioral tests: novel object recognition (NOR), elevated plus maze test (EPM), open field test (OFT), and Y-maze test (YMT), at the end of the treatment (between D22 and D26 for the icv-CuAβ model and the hippo-CuAβ model), and between 5.2 and 5.6 months of age for the 5XFAD mice.

2.2. TDMQ20 inhibits of the cognitive impairment of AD mice.

2.2.1. Novel object recognition (NOR)

The NOR test, based on the spontaneous propensity of rodents to explore new objects, allows the evaluation of short-term- or long-term memory,[28] assessing cognitive deficits in a wide range of pathological conditions, in transgenic and non-transgenic murine models of AD.[29] This assay is particularly relevant to AD, and consequently for the evaluation of the efficacy of potential drugs since it is dependent on the integrity of the temporal regions affected in the early stages of AD.

The recognition index is calculated as the percentage of explorations of a novel object with respect to the total number of explorations for short-term memory or long-term memory {$[(n_Y/n_{X+Y})\times100]$ or $[(n_Z/n_{X+Z})\times100]$, respectively, where n_X, n_Y, n_Z stand for the number of explorations of familiar object X, novel object Y in short-term memory evaluation, or novel object Z in long-term memory evaluation, respectively}. The recognition index is reported in Figure 2 for the icv-CuAβ (panel A), the hippo-CuAβ (panel B), and the 5XFAD (panel C) mouse models.

First of all, for short-term memory evaluation, the recognition index was significantly lower for the icv-CuAβ mice or hippo-CuAβ mice compared to the healthy controls (Figure 2, panel A or B, respectively), indicating that this test was relevant to discriminate between healthy and non-transgenic AD mice, a necessary condition to evaluate the activity of potential drugs on these models. Contrary, the difference of the recognition index was not statistically significant between

5XFAD mice and their wild type littermates (panel C), consistent with bibliographic data indicating that, in transgenic models of AD, the performance of mice in the NOR is age-dependent and deficits appear only after 6 months of age,[29] or even later.[30]

This result suggests that icv-CuAβ- and hippo-CuAβ mice are mimicking early cognitive impairment of AD, whereas such damages are probably undetectable in young 5XFAD mice.

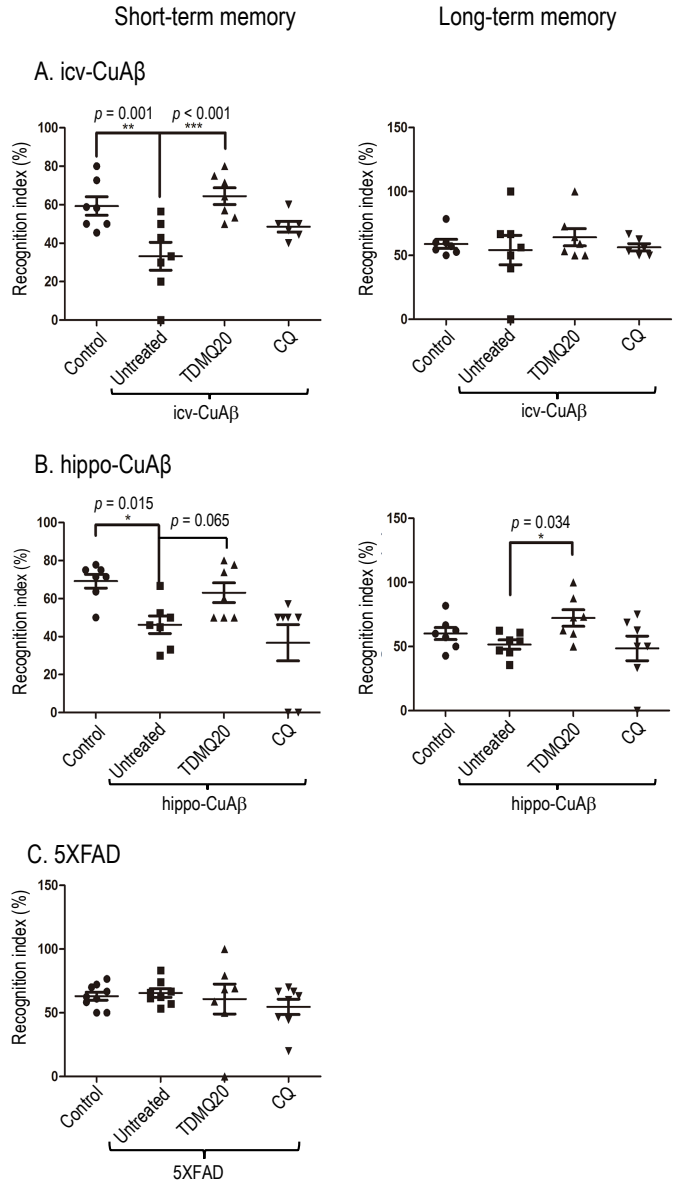

Figure 2. Declarative memory evaluated by the recognition index [(n_Y/n_{X+Y})×100] for short term memory or [(n_Z/n_{X+Z})×100] for long-term memory in the NOR task in the icv-CuAβ (panel A) and the hippo-CuAβ (panel B) non-transgenic mouse models, and in the transgenic 5XFAD (panel C) model. n_X, n_Y, n_Z stand for the number of explorations of familiar object X, novel object Y in short-term memory evaluation, or novel object Z in long-term memory evaluation, respectively. In each case, the recognition index of AD mice which received no drug-treatment (untreated group) was compared to that of healthy mice (control group), and to the recognition index of AD mice treated with TDMQ20 or clioquinol [8 × 10 mg kg^{-1} in 16 days (panels A and B), or 10 mg kg^{-1}, one every two days during 3 months (panel C)]. In panels A or B, the control/untreated groups were brain-injected with CuAβ/PBS. In panel C, the untreated and control groups were the transgenic 5XFAD mice and their non-transgenic littermates (C57BL6), respectively. All control and untreated mice received oral gavage with aqueous NaCl 0.9 w/v instead of drug. For details, see SI. Each mark represents the result obtained with a single mouse; horizontal lines represent

mean values ± SEM [panels A and B: n = 7 (controls) or 6-7 (untreated- or treated AD mice); panel C: n = 9 (controls) or 7-8 (untreated- or treated 5XFAD mice)]. $^{**}p < 0.01$, $^{*}p < 0.05$ vs. the untreated AD group (ANOVA).

After a short oral treatment by TDMQ20 icv-CuAβ mice spent significantly more time exploring the novel object in the short-term memory test, compared to untreated AD mice. A similar trend was evidenced for hippo-CuAβ mice. In fact, the recognition index of treated mice was similar to the score of control mice. Then, TMDQ20 significantly reduced the cognitive injuries created by the intracerebral injection of the Cu-$A\beta_{1-42}$ complex. By comparison, CQ did not significantly improved the short-term memory of icv-CuAβ mice and hippo-CuAβ mice. Duplication of these experiments using icv-CuAβ- and hippo-CuAβ mice provided fully consistent results provided as Supporting Information (Figure S1). The ability of TDMQ22 to restore the short-term memory of icv-CuAβ mice was close to that of TDMQ20 (SI, Figure S1, panel A).

Contrary to the short-term memory, the long-term recognition index was not significantly impaired in icv-CuAβ- and hippo-CuAβ mice (panels A and B, right). This result suggests that these models efficiently mimic the early stage of AD, in which the short-term memory is the first to be impaired, while the long-term memory, including semantic and procedural memories are not yet significantly impacted. We should note that the development of efficient drugs requires the use of mouse models of the early stages of the disease, and not a model for the late stages, where cognitive and anatomic damages are irreversible.

2.2.2. Y-maze test

The Y-maze test is a measure of spatial working memory driven by the innate curiosity of rodents to explore previously unvisited areas.[31] The spontaneous alternation of mice in the Y-maze has been validated as a hippocampal relevant spatial task.[32,33] The spontaneous alternation ratios of hippo-CuAβ- and 5XFAD mice in the Y-maze are reported in Figure 3, panels A and B, respectively. The 5XFAD mice exhibit a tendency to reduced spontaneous alternation ratio compared with wild-type mice (Figure 3), consistent with previous reports indicating that alternation ratio was impaired after the age of 6 months.[24,25] The oral treatment by TDMQ20 restored the alternation ratio of 5XFAD mice (panel B) up to the level of control mice, with nearly the same efficacy than CQ. A similar trend was obtained with hippo-CuAβ mice. Due to the result lack of statistical significance, we suggest that the spatial memory impairment is not yet established in these non-transgenic early AD mice. Therefore this test is poorly suitable to evaluate the cognitive impairment and the efficacy of drugs in 2-3 month old hippo-CuAβ mice.

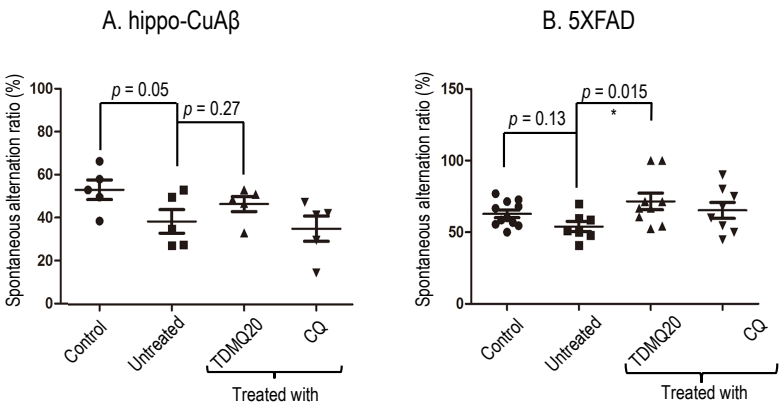

Figure 3. Spatial memory evaluated by the spontaneous alternation ratio in the in the Y-maze task for the hippo-CuAβ (panel A) non-transgenic mouse model, and the transgenic 5XFAD (panel B) model. In each case, the spontaneous alternation rate of AD mice which received no drug-treatment (untreated group) was compared to that of healthy mice (control group), and to the alternation ratio of AD mice which received TDMQ20 or clioquinol (8 × 10 mg kg^{-1} in 16 days, panel A, or 10 mg kg^{-1}, one every two days during 3 months, panel B). In panel A, the control/untreated groups were brain-injected with CuAβ in PBS. In panel B, the untreated and control groups were the transgenic 5XFAD mice and their non-transgenic littermates, respectively. All control and untreated mice received oral gavage with aqueous NaCl 0.9 w/v instead of drug. For details, see SI. Each mark represents the result obtained with a single mouse; horizontal lines represent mean values ± SEM [(A): n = 5; (B): n = 9 (controls) or 7-8 (untreated- or treated 5XFAD mice]. *p* values in the ANOVA are indicated.

2.2.3. Open field test

In the open-field assay, the unsupported rearings and travel distances of the hippo-CuAβ mice in the open-field were found significantly lower than that of age-matched controls, indicating that the locomotor deficit of hippo-CuAβ mice was well established (Figure 4A). Here also, the treatment by TDMQ20 for a short period of 16 days resulted in full rescue of the mouse abilities, with both rearings and travel distances similar to that of controls.

Conversely, the locomotor activity of 5XFAD mice was found rather close to that of the corresponding healthy mice, with no significant difference in the rearings between WT and 5XFAD mice (Figure 4B). This is consistent with the fact that the exploratory behavior and locomotor activity of 5XFAD mice were reported as being normal at least until 6 months,[26,27] or 9-12 months old.[25,30] However, in our hands, the grid crossings by 5XFAD animals was significantly higher than that of their WT littermates (Figure 4B), suggesting that, at 5.4 months, brain damage were already installed. In these conditions, the treatment by TDMQ20 significantly normalized the grid crossing by transgenic AD mice.

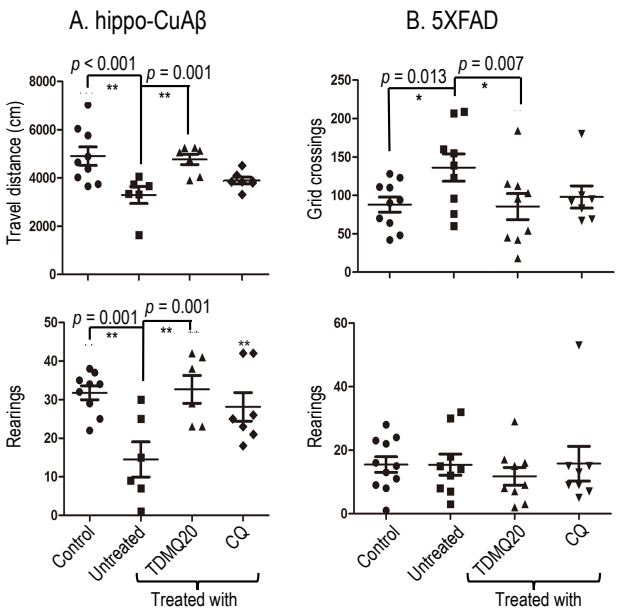

Figure 4. Locomotion ability evaluated by the crossings, travel distances, and rearings, in the open field test for the hippo-CuAβ (panel A) non-transgenic mouse model, and the transgenic 5XFAD (panel B) model. In each case, the scores of AD mice which received no drug-treatment (untreated group) was compared to that of healthy mice (control group), and to the scores of AD mice which received TDMQ20 or clioquinol (8×10 mg kg^{-1} in 16 days, panel A, or 10 mg kg^{-1}, one every two days during 3 months, panel B). In panel A, the control/untreated groups were brain-injected with CuAβ in PBS. In panel B, the untreated and control groups were the transgenic 5XFAD mice and their non-transgenic littermates, respectively. All control and untreated mice received oral gavage with aqueous NaCl 0.9 w/v instead of drug. For details, see SI. Each mark represents the result obtained with a single mouse; horizontal lines represent mean values ± SEM. [(A): n = 9 (control), or 6-7 (untreated or treated hippo-CuAβ mice; (B): n = 10 (control), or 7-9 (untreated or treated 5XFAD mice]. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs. untreated AD mice (ANOVA).

2.2.4. Elevated plus maze

The elevated plus maze assay has been described for assessing anxiety response of rodents, and screening of drugs for treatment of anxiety-related disorders.[34,35] The score of 5XFAD mice in the elevated plus maze is reported in Figure 5. 5XFAD mice exhibited an increased number of closed arm entries, and a decreased proportion of time spent in closed arms, compared to their WT littermates. Normally, mice tend to avoid the open arms, seeking protection in the closed arms. A decreased proportion of time spent in closed arms indicate a decreased anxiety of 5XFAD mice (Figure 5), that may correspond to dis-inhibitory tendencies seen in AD patients.[25,26] Oral treatment of these mice with TDMQ20 fully reversed their behavior to to the score of WT, more efficiently than the CQ treatment did.

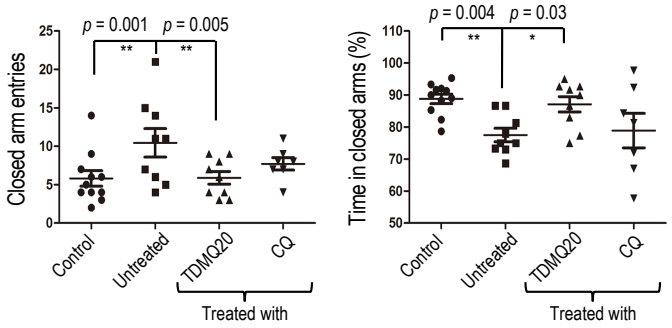

Figure 5. Anxiety evaluated by the closed arm entries and time spent in closed arms, in the elevated plus maze test for the transgenic 5XFAD model. The scores of AD mice which received no drug-treatment (untreated group) was compared to that of wild-type mice (control group), and to that of AD mice which received TDMQ20 or clioquinol (10 mg kg^{-1}, one dose every two days during 3 months). The untreated and control groups were the transgenic 5XFAD mice and their non-transgenic littermates, respectively. All control and untreated mice received oral gavage with aqueous NaCl 0.9 w/v instead of drug. For details, see SI. Each mark represents the result obtained with a single mouse; horizontal lines represent mean values ± SEM. (controls: n = 9; untrated- or treated 5XFAD mice: n = 7-8). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs. the untreated 5XFAD mice (ANOVA).

As a summary of behavioral tests (Table 1), both 2.5-2.7 month hippo-CuAβ or icv-CuAβ mice exhibited significant deficits of short-term memory in the NOR assay. The score of the hippo-CuAβ mice was also impaired in the open-field assay, validating these non-transgenic models to evaluate the activity of potential drugs on the early stages of the memory deficits. The short oral treatment with TDMQ20 was efficient to fully restore the abilities of mice in these reference tests. The score of hippo-CuAβ mice in the Y-maze was less impaired than in transgenic 5XFAD mice, but nevertheless confirmed that TDMQ20 was able to reverse the ongoing impairment of the spatial memory on young mice affected by intracerebral injection of copper-amyloids.

Conversely, the deficits of 5XFAD mice observed in the open field, Y-maze or elevated plus maze assays, are rather weak below 6 months of age. Cognitive deficits of these transgenic mice are reported to correlate well with an increasing burden of amyloid plaques in the cortex and hippocampus, axonal degeneration, spinal cord pathology and neuron loss in specific cortical layer, mimicking an advanced AD pathology.[25] Similarly, in APP/PS-1 mouse model, the cognitive deficits in both recognition memory and spatial memory become apparent only at 11-24 months of age, when the disease damages are definitively installed.[36] In these conditions, such transgenic models should be considered more relevant to describe advanced stages of AD, but are probably not really adapted to evaluate drug-candidates that should be efficient on the early stages of the disease.

Table 1. Improvement of the scores of AD mice treated by TDMQ20, with respect to untreated AD controls.

		AD mouse model		
		icv-CuAβ	hippo-CuAβ	5XFAD
Behavioral test	NOR (short term)	++ [a]	++	nr [d]
	Y-maze	nd [c]	+ [b]	+ [b]
	Open field	nd	++	+ [b,e]
	Elevated plus maze	nd	nd	++
Cortex oxidative status	MDA level	+ [b]	nd	+ [b]

[a] ++ Means: Statistically significant with p-value ≤ 0.05 (ANOVA); [b] + Means: tendency, but with p-value > 0.05; [c] Not determined; [d] Not relevant (no significant difference between WT controls and 5-month old 5XFAD mice; [e] Only for grid crossing.

2.3. TDMQ20 inhibits cortex oxidative injuries

The ability of TDMQ20 to rescue cognitive deficits is consistent with the capacity of this copper homeostasis regulator to reduce *in vitro* the formation of ROS.[13,20] In fact, neuro-inflammation is considered a key factor of the loss of neurons and synapses in AD.[30] The molecular origin of brain inflammation might be linked to the oxidative stress initiated by the reductive activation of dioxygen catalyzed by copper-amyloids. It is therefore relevant to evaluate the oxidative status of the mouse brains. For this purpose, the malondialdehyde (MDA) level is significant of the potential occurrence of peroxide induced lipid injuries.[37]

The level of MDA, measured by the thiobarbituric reactive species (TBARS) assay, in the cortex of icv-CuAβ- and 5XFAD mice is reported in Figure 6A and 6B, respectively.

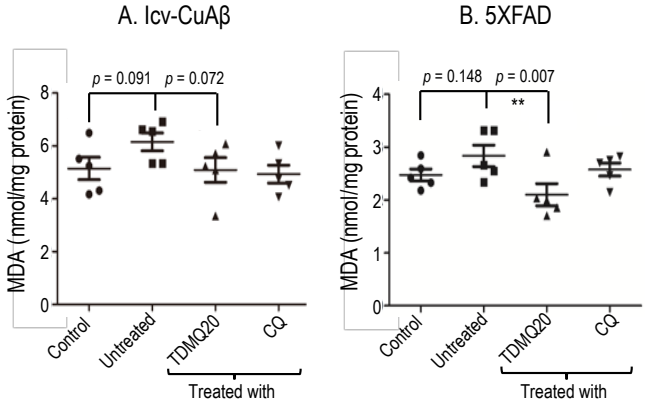

Figure 6. Effect of TMDQ20 on the level of malondialdehyde in the cortex of icv-CuAβ AD mice (A) and 5XFAD mice (B). (A): The control mice were brain-injected with PBS 1X/DMSO, 80/20, v/v; untreated mice were brain injected with CuAβ in 1X/DMSO, 80/20, v/v. (B): The controls were WT C57BL/6J mice. All control and untreated

mice received oral gavage with aqueous NaCl 0.9 w/v instead of drug. Each mark represents the result obtained with a single mouse; horizontal lines represent mean values ± SEM of n = 5 experiments. Relevant *p* values are indicated (ANOVA).

The concentration of MDA in the cortex of AD mice, transgenic (5XFAD) or not (icv-CuAβ), was slightly higher than that of the controls (Figure 6), supporting the involvement of an oxidative stress in these mice. In both cases, the treatment with TMDQ20 reduced the MDA level in the cortex until the level of control mice, indicating a normalization of the oxidative status in the cortex. This encouraging data is consistent with the ability of TDMQ20 to inhibit *in vitro* the production of ROS induced by Cu-Aβ.[13,15]

2.4. Preliminary safety and druggability studies of TDMQ20

2.4.1. Single dose

In an acute toxicity assay in WT C57BL/6J mice, TDMQ20 was found completely safe after administration of an oral dose 150 or 200 mg kg^{-1}, corresponding to 15-20 times the dose used to treat AD mice, with all mice surviving without any detectable deficit (Table 2). The drug exhibited some delayed toxicity by ip route at 150 mg kg^{-1}: two mice over 11 died at D3 and D15, respectively, indicating an LD_{50} value higher than 150 mg kg^{-1}. By comparison, clioquinol was found significantly more toxic, with LD_{50} values close to 200 mg kg^{-1} and 150 mg kg^{-1}, by oral and ip route, respectively (2/5 and 7/11 mice died on the day of drug administration).

Table 2. Preliminary toxicological evaluation of TMDQ20 compared to CQ on C57BL/6J mice, after a single oral or intraperitoneal (ip) dose.

		Survival of mice treated with a single dose of TDMQ20 or CQ		
Drug	Dose (mg kg^{-1})	Administration route	Survival	Date of death
TDMQ20	150	oral	6/6	-
	200	oral	5/5	-
	150	ip	9/11	D3, D15
CQ	150	oral	6/6	-
	200	oral	3/5	D1
	150	ip	4/11	D1

2.4.2. Multiple doses

The toxicity of TDMQ20 was then evaluated after on oral administration of 100 mg kg^{-1} every day to WT C57BL/6J mice. Two mice died at D8 and D9 (survival 8/10), *ie.* after administration of 10-11 times the total drug dose used in the AD treatment (80 mg kg^{-1} in total). The assay was

388 then discontinued at D9. Mice (n = 9) were then treated with TDMQ20 by oral route at 80 mg kg-
389 $^{-1}$, 3 times a week during 2 weeks, corresponding to a total dose of 480 mg kg^{-1}. In these conditions,
390 with a schedule similar to that used for the evaluation of the pharmacological activity of TDMQ20
391 (80 mg kg^{-1} in total), all mice survived without any detectable sign of toxicity during the treatment,
392 suggesting a safety index ≥ 8. In addition, the treatment of 5XFAD mice clearly indicated that
393 this drug-candidate was well tolerated upon long-term oral treatment (45 doses, 450 mg kg^{-1} in
394 total), even in animals weakened by the disease.

395 The aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and urea
396 were measured in the serum of 5XFAD mice, treated or not by TDMQ20 (45 × 10 mg kg^{-1} in 3
397 months, total dose = 450 mg kg^{-1}), at the end of the treatment period and compared to the levels
398 found in the WT C57BL/6J controls. There was no significant difference between the levels
399 obtained for the WT controls, the 5XFAD mice and the 5XFAD mice treated by TDMQ20,
400 indicating the lack of any detectable damage on liver and kidneys upon long-term oral treatment
401 with TDMQ20 in these conditions (45 doses, 450 mg kg^{-1} in total).

402

403 ***2.4.3. Druggability***

404 *In vitro* preliminary metabolic studies indicate that the TDMQ20 significantly inhibits CYP 2D6,
405 but not CYP 3A (< 20 %) and 2C9 (< 5 %) (SI). We have to remember that the drug prozac is
406 also an inhibitor of CYP2D6. The measured pKa values indicate that, at physiological pH,
407 TDMQ20 should exist as a mixture of species, due to mono- and diprotonation of the side chain,[15]
408 like many CNS drugs containing protonable nitrogen sites.[38] The calculated log P value for
409 TDMQ20 is 2.5,[39] and topological polar surface area (tPSA) is 53 Å^2,[39] in the range consistent for
410 passive transport across the blood-brain barrier.[38] The membrane crossing capacity has been
411 confirmed by the Caco-2 data. In standard conditions at 10-5 M, A-B permeability was 8.4 with
412 a recovery of 35% and B-A permeability was 10.1 with a recovery of 74%. In addition, TDMQ20
413 was Ames negative on TA98, TA100, TA1535 and TA1537, even after microsomal activation
414 (SI), indicating that this ligand was not mutagenic in bacteria.

415

416 In conclusion, TDMQ20 efficiently reversed the cognitive or behavioral impairment using two
417 non-transgenic mouse models of the early stage of AD and in a transgenic model. With the icv-
418 CuAβ and hippo-CuAβ models, the short-term NOR test is specially robust to evaluate the

impairment of the declarative memory in mice, making such models easy to select molecules that should be active on the early stages of AD.

The efficacy of TDMQ20 by oral administration of low doses on three different mouse AD models is an encouragement for further pharmacological evaluations of this specific copper chelator.

3. METHODS

3.1. Materials.

Human $A\beta_{1\text{-}42}$ (CF_3COO^- as counter-ions) was obtained from Aladdin, China. According to a protocol adapted from Reference 40, $A\beta_{1\text{-}42}$ (1 mg) was dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP, 1 mL) and sonicated for 15 minutes. The solution was centrifugated at 12,000 rpm for 15 min. The concentration of the $A\beta_{1\text{-}42}$ solution was determined using Micro BCA Protein Assay kit from ThermoFisher Scientific™ (code 23235), working with BSA (bovine serum albumin) as a calibration protein. HFIP was then removed by lyophilization, and the solid $A\beta_{1\text{-}42}$ was then stored at -20 °C until used. The chelators TDMQ20 and TDMQ22 were synthesized as previously reported.[12,13] Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline, CQ), $CuCl_2$, DMSO and usual chemicals were purchased from Sigma. Phosphate buffered saline 10X (PBS 10X) was prepared as follows: NaCl 137 mmol, KCl 2.7 mmol, Na_2HPO_4 10.0 mmol, KH_2PO_4 1.76 mmol, sterile water qs. 100 mL. PBS 1X was prepared by dilution of PBS 10X in sterile water. Isoflurane was supplied by Shenzhen RWD life Science Co.

3.2. Animals.

Specific pathogen free (SPF) C57 BL/6J mice, weighting at 20–25 g (8 weeks) were purchased from the Guangdong Medical Experimental Animal Center. 5XFAD mice (strain name: B6.Cg-Tg(APPSwFlLon,PSEN1*M146L*L286V)6799Vas/Mmjax), with genetic background C57BL/6 x SJL, were purchased from the Jackson Laboratory (Stock# 34848-JAX|5XFAD, Bar Harbor, ME, USA) and propagated in the College of Life Sciences and Oceanography in Shenzhen University. Their offsprings were assessed by PCR detection (Mei5 Biotechnology, China) of the AD pathogenic genes. Their non-transgenic littermates were screened out as healthy controls. Animals were separated in groups containing males and females in a 50/50 ratio. They were housed in standard laboratory conditions: air-conditioned room, temperature 20-25ºC, 12 h light/dark illumination cycle, free access to food and water. Drinking water was distilled water

obtained from a Millipore Direct-Q 5 UV water purification system (Millipore SAS, France) and food was purchased from the Guangdong Medical Experimental Animal Center. Before icv and intra-hippocampal injections of Cu-Aβ, mice were anesthetized with isoflurane, using a R500 Series Compact Small Animal Anesthesia Machine (Shenzhen RWD Life Science Co.) according to the supplier instructions. Mice were killed by cervical dislocation after anesthesia with isoflurane. All animal experiments were conducted in accordance with ethical animal research standards of the Chinese Council on Animal Care, and approved by the Animal Ethical and Welfare Committee of Shenzhen University (permit number: AEWC-20140615-002). All efforts were made to minimize animal suffering.

3.3. Experimental methods for the icv-CuAβ and hippo-CuAβ models.

3.3.1. Preparation of the Cu-Aβ$_{1–42}$ solution

Preparation of the Aβ$_{1–42}$ solution. Human Aβ$_{1–42}$ monomer powder obtained after HFIP treatment (0.1 μmole measured by BCA titration) was dissolved in DMSO (50 μL). This mother solution was then diluted by addition of 200 μL of deionized water. The final solution (400 μM measured by BCA assay, H_2O/DMSO = 80/20, v/v) was stirred using a vortex (1800 rpm, 1 minute) and sonicated for 1 minute. This limpid solution was aliquoted in seven parts of 30 μL each, stored at –20 ºC away from light, covered with an aluminum foil (Solution A).

Preparation of the $CuCl_2$ solution. A 40 mM stock solution of copper(II) chloride was prepared by dissolution of $CuCl_2$ (2.70 mg) in DMSO (500 μL). This stock solution was diluted 20 times in DMSO, to provide a 2 mM solution (Solution B).

Preparation of the Cu-Aβ$_{1–42}$ solution. The copper-Aβ$_{1–42}$ complex was prepared in a 1/1 metal/peptide ratio, as follows. An aliquot of the human Aβ$_{1–42}$ (above Solution A, 30 μL) was kept at room temperature for 30 min. One mole equivalent of $CuCl_2$ in DMSO (Solution B, 6 μL), was then added in the flask containing the solution A, the mixture was vortexed for 30 sec, and then allowed to stand at 37 °C for 15 min. Then, H_2O (18 μL) was added and vortexed, followed by addition of PBS 10X (6 μL), then vortexed for 30 sec. The solution was kept at 37 °C for 15 min. It was again vortexed for 30 sec before each injection in the mouse brains. The absence of precipitate was carefully checked before each injection. The final ratio H_2O/DMSO was 80/20, v/v. This solution (Solution C) was freshly prepared each day, before the intracerebroventricular (icv-CuAβ model) or intrahippocampal (hippo-CuAβ model) injections.

3.3.2. Injection of Cu-Aβ$_{1–42}$ in mouse brain ventricle. (icv-CuAβ model).

Injections were performed using the protocol adapted from Reference 5. The 35 mice were randomly assigned to one of the five following groups (7 mice in each group): control mice, mice which received a single bilateral intracerebroventricular injection of Cu-Aβ_{1-42} complex (icv-CuAβ group), icv-CuAβ mice treated with TDMQ20 (TDMQ20 group), icv-CuAβ mice treated with TDMQ22 (TDMQ22 group), and icv-CuAβ mice treated with clioquinol (CQ group). The intracerebroventricular injections were made at 2 months (mo) of age, with PBS 1X containing 20 vol% of DMSO for the control group, or Cu-Aβ_{1-42} Solution C, the four other groups. The coordinates of the injection sites in each lateral ventricle were: (AP) = – 0.5 mm from bregma; (L) = +/– 1 mm from bregma; and (D) = – 2.5 mm from skull. The skull was drilled, and an injector (27 G, 35 mm) was guided successively into the two lateral ventricles, using a mouse brain localization injector (RWD, Shenzhen, China). Then, 2.5 µL of solution C or PBS 1X containing 20 % DMSO was injected directly into each of the two lateral ventricles (total volume of injection = 5 µL per mouse), using a 5 µL syringe mounted on an automated pump. In each ventricle, the solution was delivered at a rate of 0.5 µL/min. After each injection, the injector was let in place for 5 min to avoid flow back of the solution.

3.3.3. Injection of Cu-Aβ$_{1-42}$ in mouse hippocampus (hippo-CuAβ model).

The mice were randomly assigned to one of the four following groups (7 mice in each group): control mice, mice which received a single bilateral intrahippocampal injection of Cu-Aβ_{1-42} complex (hippo-CuAβ), hippo-CuAβ mice treated with TDMQ20, hippo-CuAβ mice treated with clioquinol (CQ group). The injections in the two CA3 hippocampus regions were made at 2 months of age, with PBS 1X containing 20 vol% of DMSO for the control group, or Cu-Aβ_{1-42} Solution C, for the three other groups. The coordinates of the injection sites were: (AP) = –2.18 mm from bregma; (L) = +/– 2.3 mm from bregma; and (D) = – 2.15 mm from skull. The skull was drilled, and an injector (27 G, 35 mm) was guided successively into the bilateral CA3 hippocampal regions. The delivery of the Cu-Aβ_{1-42} (solution C, 2 x 2.5 µL of the Cu-Aβ_{1-42} solution C, or the PBS 1X/DMSO, 80/20, v/v into the bilateral hippocampal CA3 region was carried out in the same way than icv injections, except that the flow rate was 0.25 µL/min (compared to 0.5 µL/min for cerebroventricular space, in order to avoid damage to the smaller hippocampus).

3.3.4. Preparation of drug solutions, drug dose, and treatment.

Mother solutions of TDMQ20, TDMQ22 were prepared by dissolving 25 mg of each drug in 10 mL of NaCl 0.9 % (w/v) solution. Clioquinol (25 mg) was dissolved in 10 mL of DMSO. 100 µL

of such drug solutions contained 0.25 mg of drug. While the injections of Cu-Aβ_{1-42} into ventricles or hippocampus were carried out on D0, the mice were treated intragastrically 8 times (at D4, D6, D9, D11, D13, D16, D18, and D20) as follows: NaCl 0.9% (w/v) in distilled water (100 μL) for the control groups, the icv-CuAβ group, and the hippo-CuAβ group, TDMQ20 in NaCl 0.9 % (100 μL) for the TDMQ20 groups, TDMQ22 in NaCl 0.9% (100 μL) for the TDMQ22 groups, and CQ in DMSO (100 μL) for the CQ group. Each drug dose was therefore 10 mg kg^{-1} for TDMQ20, TDMQ22, and CQ. Mice were individually weighted on the day of the intracerebral injection of Cu-Aβ (2 months of age) for the icv-CuAβ- and hippo-CuAβ models, and the exact volume of drug solution for each mouse was calculated accordingly. For 5XFAD mice, animals were weighted at the age of two months (beginning of treatment) and the volumes of drug solutions were calculated accordingly**.**

3.4. Experimental methods for the 5XFAD transgenic model.

At the age of 2 months, mice were weighted and randomly assigned to one of the following groups: untreated 5XFAD mice, 5XFAD mice treated with TDMQ20, 5XFAD mice treated with CQ, compared to wild type C57BL/6 x SJL mice. The drugs were dissolved in 0.9% NaCl (TDMQ20) or DMSO (CQ), and were delivered to the stomach of mice once every two days during the three following months (45 treatments, total drug dose: 450 mg kg^{-1}). Each drug dose consisted of 10 mg kg^{-1} of mouse weight contained in 100 μL of solvent. All control and untreated mice received oral gavage with aqueous NaCl 0.9% instead of drug. The behavioral tests were performed at the end of the treatment, between 5.0 and 5.5 months of age.

3.5. Behavioral tests.

3.5.1. Novel object recognition test (NOR).

The test was carried out as previously reported.[28] Mice were tested in an uncovered square box (40 cm × 40 cm × 40 cm). This open arena is divided into 25 equal squares. At D22 after the injection of Cu-Aβ in the ventricles or hippocampus (two days after the last administration of drug), each mouse was placed for 5 min in the empty arena of the box placed in the test room equipped with daylight lamp, in order to habituate it to the environment and to the apparatus (habituation phase). At D23, the home cages were left for acclimation in the test room for one hour prior to the beginning of the test session (acclimation phase). Each mouse was then placed for 5 min in the arena containing two identical objects (X and X') (acquisition phase). After a 90

min retention interval in its home cage, the mouse was placed back into the arena and exposed to one of the familiar objects (X) and to a novel object (Y), for a short-term (90 min) recognition memory test. The mouse was initially placed in the apparatus facing the wall and allowed to explore the objects for 5 min. When the mouse's nose pointed or touched an object within 1 cm, it was recorded as an exploration behavior. The number of times the mouse explored one of the two objects was recorded. At D24, each mouse was similarly placed into the arena for 5 min, in the presence of a different pair of dissimilar objects (a familiar and a novel one; X and Z, respectively), for a long-term (1 day) recognition memory test. The recognition index, defined as the ratio of the number of times the novel object was explored to the number of times both objects were explored, was used to measure the memory function. So, the recognition indexes were calculated as $n_Y/n_{(X+Y)}$ and $n_Z/n_{(X+Z)}$ for the short-term memory and long-term memory, respectively, where n_X, n_Y, n_Z stand for the number of explorations of objects X, Y or Z, respectively. Between trials, the arena was cleaned with 75% ethanol to eliminate animal clues, and then with water. For the 5XFAD mice, the schedule of the test was the same as described above, and the test begun at 5.3 ± 0.1 months of age.

3.5.2. Elevated plus maze test (EPM).

The elevated plus maze test was carried out using the protocol adapted from References 41 and 42. The experiment was started at D26 from intracerebral injection. The home cages were left for acclimation in the test room equipped with daylight lamp, for one hour prior to the beginning of the test session. The elevated plus maze apparatus consisted of four arms of 30 cm length × 5 cm width, two of them with 30 cm high walls, elevated at 60 cm above the floor. The mouse was initially placed in the center of the maze, facing an open arm and allowed to explore the maze for 5 min. The number of times the mouse entered the open and closed arms was recorded, as well as the time spent in the open and closed arms. If the mouse fell off an open arm, the trial was stopped and excluded from the analysis. For 5XFAD mice, the test was carried out at 5.4 ± 0.1 mo. of age. Between trials, the arena was cleaned with 75% ethanol to eliminate animal clues, and then with water.

3.5.3. Open field test (OFT).

The test was carried out at D26 after intracerebral injection, using the protocol adapted from References 43 and 44. The home cages were left for acclimation in the test room equipped with daylight lamp, for one hour prior to the beginning of the test session. The test field consisted of an empty square box of 100 × 100 × 30 cm, with 25 square grids on the floor. Each mouse was

initially put at the center of the field, and allowed to explore it for 5 min. A digital camera was placed 2 m above and its field of view covered the entire experimental field. The number of rearings, crossings of the floor grid, and walking distances of mice were recorded. For 5XFAD mice, the test was carried out at 5.4 ± 0.1 months of age.

3.5.4. Y-maze test (YMT).

The Y-maze test was carried out using the protocol adapted from Reference 45. The home cages were left for acclimation in the test room equipped with daylight lamp, for one hour prior to the beginning of the test session. Spontaneous alternation behavior test has been performed in a symmetrical black plexiglas Y-maze with three arms (30 cm long × 10 cm wide × 30 cm high) at 120° angles. For 5XFAD mice, the test was carried out at 5.5 ± 0.1 months of age. The mice were initially placed in the center of the maze and allowed to explore the maze for 5 min. A successful alternation was defined as any three consecutive choices of three different arms without re-exploration of a previously visited arm. Alternation rate (%) was calculated as follows: number of triads containing entries into all three arms/maximum possible alternations (the total number of arms entered – 2)×100.

3.6. Biochemical analyses.

3.6.1. Titration of malondialdehyde (MDA).

After all behavioral experiments have been performed, all the mice had a rest for one day and then were sacrificed. The concentration of malondialdehyde (MDA) was determined in cortex of C57 BL/6J control mice, and icv-CuAβ mice ± TDMQ20 or CQ, using the thiobarbituric assay according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). The cortex (*ca.* 100 mg was homogenized in 1 mL of phosphate buffer (pH 7.4, 10 mM). After centrifugation for 10 min (3,000 g), the MDA content in the supernatant was measured by UV-visible spectrometry at 532 nm. The levels of MDA are indicated as nmol/mg protein.

3.6.2. Titration of AST, ALT, creatinine and urea.

After all behavioral experiments have been performed, all 5XFAD mice had a rest for one day and then were sacrificed (5.6 months of age). The concentration of AST, ALT, creatinine and urea in the serum were measured using the automatic biochemical analyzer iMagic-M7, the assay kits and the software of the iCubio company (Shenzhen, China).

3.7. Preliminary toxicologic experiments

3.7.1. Single dose (acute toxicity).

TDMQ20 and CQ were administered by gavage or by intraperitoneal injection, at 150 or 200 mg kg^{-1}, to C57 BL/6J mice (groups of 5-11 mice each), using 25 mg mL^{-1} solutions in 0.9% NaCl (for TDMQ20) or in DMSO (for CQ). Mice were observed for 16 days after the administration.

3.7.2. Multiple doses.

C57 BL/6J mice received TDMQ20 (25 mg mL^{-1} solution in 0.9% NaCl) by intragastric administration at 100 mg kg^{-1} per day during 9 days (10 mice), or at 60 mg kg^{-1} per day, three times per week during two weeks (12 mice). Mice were weighted each day.

3.8. Statistical analysis.

Figures 2-6 and Figure S1 are presented as univariate scatterplots of all the individual data points obtained, with mean and SEM superimposed. Data with multiple independent experiments were analyzed by one-way ANOVA, followed by multiple comparisons LSD test when the ANOVA was significant ($p < 0.05$). p-levels < 0.05, 0.01 and < 0.001 were noted *, ** and ***, respectively. For each test, each mice was evaluated only once (no repeated measures). The factor of the treatment was entered as a within-subjects (independent) factor. The F-statistic, degrees of freedom, p-value and the magnitude of the difference were calculated. All statistical calculations are provided as Supporting Information (SI).

ASSOCIATED CONTENT

The Supporting Information is available free of charge at https://pubs.acs.org/doi/...

The Supporting Information contain:

- Figure S1 (duplicate of Figure 2),
- Raw data and statistical analysis of results reported in Figures 2-6,
- ADME-toxicological data of TDMQ20.

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Author Contributions

B. M., Q. L. and A. R. designed research; H. T., Y. Li and Y. Liu performed chelator synthesis; J. Z. and Q. S. performed biological assays and animal experiments; B. M., Q. L., J. Z. and A. R. analyzed the data; B. M., A. R. and Q. L. wrote the manuscript.

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Conflict of Interest

Y. L., A. R. and B. M. are co-authors of a pending patent on use of TDMQ chelators as a therapeutic for Alzheimer's disease (WO 2017/202360 A1). None of the authors have a financial interest in pharmaceutical companies.

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821 **Scheme for Table of Contents only**

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