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Proteomics evidence of the role of TDMQ20 in the cholinergic system and synaptic transmission in a mouse model of Alzheimer's disease

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Running Title

Proteomics pattern of TDMQ20, an anti-AD drug-candidate, on 5xFAD mice

Abstract

The interaction between copper ions and amyloid peptide Aβ has been reported to be involved in Alzheimer's disease (AD) pathology. Based on copper coordination biochemistry, we designed specific copper chelators (TDMQs) in order to regulate copper homeostasis in AD brain, and to inhibit the deleterious oxidative stress catalyzed by copper-Aβ complexes. We previously reported that TDMQ20, a highly selective copper chelator selected as drug-candidate, was able to extract copper from Cu-Aβ_{1-16} complex and to restore cognitive and behavioral deficits in AD mouse models. For a better understanding of the mechanism of action of TDMQ20, we decided to investigate the change of profile of proteins expressed in 5xFAD mice after an oral treatment of TDMQ20 (dose = 10 mg/kg, once every two days during 3 months, in total 45 times). Clioquinol (CQ), a non-specific chelator, has been used as comparator.

Here, we report the proteomic alterations in the cortex of 5xFAD mice using iTRAQ (isobaric tags for relative and absolute quantification) proteomics technology. The results indicated that 178 differentially expressed proteins (DEPs) have been identified in AD mouse group with respect to wild type (WT) animals (AD/WT). After treatment by TDMQ20, 35 DEPs were common in AD/WT and TDMQ20/AD groups in an opposite up- or down-regulated manner, respectively. In addition, among the 35 DEPs mentioned above, 10 common target proteins have been identified in AD/WT, TDMQ20/AD and CQ/AD groups, among which three target proteins were successfully validated by Western blot analysis. In particular, the expression level of ChAT and CHRM4 is significantly increased upon TDMQ20 treatment with respect to 5xFAD mice, while CQ did not significantly change the expression of these proteins. Our study suggests the involvement of this copper chelator TDMQ20 on the cholinergic system, a feature which may explain the improved cognitive and behavioral performance in AD mice upon oral treatment by TDMQ20.

Keywords

Alzheimer's disease, copper chelator, TDMQ20, iTRAQ proteomics, cholinergic neurotransmission, oxidative stress

Highlights

- TDMQ20 reverses proteomic profiles through multiple pathways in 5×FAD AD mouse model.
- TDMQ20 exerts a significant inhibition effect on PI3K-Akt pathway in the 5×FAD AD mouse model.
- TDMQ20 modulates the expression level of ChAT and CHRM4 in cholinergic system of 5×FAD AD mouse model.
- TDMQ20 dramatically reduces Aβ plaque load in the cortex of 5×FAD AD mouse model.
- TDMQ20 can be considered a promising candidate in treating AD via the regulation of cholinergic and synaptic functions.

In brief

Sun *et al*. describe the effect of a new copper chelator TDMQ20 on the profile of protein expression in AD mice. TDMQ20 inhibits the PI3K-Akt pathway in AD mouse cortex at the proteomic level. It has great potential for AD treatment by modulating ChAT and CHRM4 along the cholinergic system and decreasing cortical Aβ plaques.

INTRODUCTION

Among the 55 million of persons currently living with dementia worldwide, 60-70% of them are due to Alzheimer's disease (AD), the most common type of dementia (1). At the moment, AD is the largest unmet medical need in neurology. In AD brains, the main pathology is featured by protein deposits of aggregated amyloid-β (Aβ) and hyperphosphorylated form of tau protein (p-tau), appearing as amyloid plaques and neurofibrillary tangles, respectively, distributed in several brain regions like cortex, hippocampus, olfactory bulb, amygdala and basal forebrain (2,3). At the moment, there is still a lack of efficient drugs able to stop the development AD in the early stages and/or prevent the cognitive decline and behavioral changes induced by the disease. Since the approval of memantine in 2003, and despite huge research efforts made by universities or pharmaceuticals companies, the level of failures in clinical trials of drug-candidates is close to 100% (4). GV-971, a sodium oligomannate, and aducanumab, an antibody targeting β-amyloids, have been approved by China National Medical Products Administration (NMPA) in 2019 and 2021, respectively (5,6). However, this biopharmaceutical has been rejected by the European Agency in December 2021 (7).

Therefore, it is still urgent to explore other potential targets to enlarge the drug spectrum with different mechanisms, due to the complexity of the etiology of AD. Consequently, several research groups, including our own, decided to develop different metal chelators as drug candidates for the treatment of AD (8-11). The driving force of this research domain is the rupture of the homeostasis of redox active metal ions such as copper and iron in AD brains and their accumulation in amyloid plaques (12,13). In the presence of endogenous reductants, copper-amyloid complexes are able to catalyze the reduction of dioxygen and to produce reduced, reactive oxygen species (ROS) responsible for neuron death (14). In addition, β-amyloid oligomers induce mitochondrial damage in a copper catalyzed nitric oxide mediated process (15).

Based on our previous studies on copper chelators (10) and our understanding of the failure of non-specific copper chelators (16), we designed a new series of tetradentate ligands based on a 8-aminoquinoline specific for copper chelation named TDMQ (17-20). One of these chelators, TDMQ20, has been selected as drug-candidate and evaluated on three different mouse AD-models, two models based on non-transgenic mice, and one being the classical transgenic 5xFAD mice. TDMQ20 was able to reduce memory impairments in all three AD-models and was also able to reduce the brain level of malondialdehyde, a marker of oxidative stress (21).

We should keep in mind that the rupture of the homeostasis of copper ions generates an excess of this metal ion in some proteins and a reduction of its availability for other ones. For example, copper is a regulator of muscarinic acetylcholine receptor in the brain of rats and its deficiency has a dramatic effect on both receptor number and binding of antagonists to this receptor (22). This fact might create a link between copper homeostasis and three of the few approved drugs (donepezil, galantamine and rivastigmine) acting as acetylcholinesterase (AChE) inhibitors (23). Cholinergic network in the central nervous system is essential for learning capacity and memory, with most of cell bodies sitting in basal forebrain region and innervations projecting to areas like cortex and hippocampus (24). Cholinergic neurons are lost in AD patients, with dramatically reduced levels of acetylcholine (ACh), a key neurotransmitter for many neurophysiological processes (25). Choline acetyltransferase (ChAT), a key enzyme for the biosynthesis of ACh, was significantly reduced in temporal lobes of patient brains (26) and in dementia induced by hypoxia (27). For all these different reasons, it is essential to understand the origin of the degradation of cholinergic neurons, and in general the neuron death that has been related to the accumulation of copper ions within amyloid proteins when these copper-amyloid complexes are activated by endogenous reductants (9,20,28).

So, the different possible modes of action of the TDMQ20 copper regulator may involve reduction of the oxidative stress created by Cu-amyloids, but also a copper-dependent regulation of several proteins involved in the redox metabolism, resulting in normalization of the activity of cholinergic neurons.

Here we report proteomics data obtained with transgenic 5xFAD mice that received by oral route 10 mg/kg of TDMQ20 once every two days during 3 months. The reported results suggest that this drug-candidate has a positive effect on the cholinergic system. Consequently, TDMQ20 should be considered as having a bi-modal mechanism of action: the first one as inhibitor of ROS production due to its capacity to extract copper from copper amyloid and the second as provider of copper to copper-deprived cholinergic neurons. This regulation of copper homeostasis, *i.e.* the capacity for a ligand to extract copper from amyloid and to release it for a copper carrier, has been suggested by "in chimico" experiments (29).

RESULTS

Relative quantification of proteins in the brain of 5xFAD mice was achieved using iTRAQ based mass spectrometry proteomics analysis. Using the statistical assay of *t*-test, DEPs between two mouse groups were screened with fold change higher than 1.2 or lower than 0.8 as the threshold, and p value < 0.05 as significance criterion. A total of 3243 DEPs were identified and classified in four categories: proteins identified in (i) 5xFAD mice compared to wild type mice (AD/WT), (ii) 5xFAD mice treated with TDMQ20 compared to untreated 5xFAD mice (TDMQ20/AD), (iii) 5xFAD mice treated with clioquinol compared to untreated 5xFAD (CQ/AD) and (iv) common DEPs among the above three categories.

DEPs between 5xFAD mice compared to WT

Among total identified proteins in AD/WT comparison, 178 DEPs were identified, 101 being up-regulated (57%) and 77 down-regulated (43%) (Figure 1A and 1B) indicating that there is no significant protein loss in AD mouse brain tissue (raw data: Supplementary 1.xlsx, sheet AD *vs* WT). Gene Ontology analysis (GO) of

biological process (BP), cell component (CC) and molecular function (MF) of these proteins is shown in Figure 1C-E. As shown in Figure 1D, fifteen enriched BP found in GO analysis linked DEPs of AD/WT category mainly included localization, cellular component organization or biogenesis, transport, establishment of localization, single-organism localization, single-organism transport and regulation of biological quality. While establishment of localization in cell, cell-cell signaling, single-organism intracellular transport, chemical synaptic transmission, anterograde trans-synaptic signaling, trans-synaptic signaling, synaptic signaling, and modulation of synaptic transmission were affected to a lesser extent. Regarding to structural stability, fifteen enriched CC processes in GO analysis displayed in Figure 1E mainly linked to DEPs related to intracellular part, cytoplasm, cytoplasmic part and, to a lesser extent, to vesicle, cytosol, extracellular region part, membrane-bounded vesicle, extracellular exosome, extracellular vesicle, extracellular organelle, extracellular membrane-bounded organelle, cell projection, cell body and neuronal cell body. MF DEPs included protein binding, enzyme binding, identical protein binding, binding, kinase binding, anion binding and purine ribonucleoside binding, indicating that these DEPs related to molecular processes were disrupted under AD pathological conditions.

AD related pathways associated with these differentially expressed proteins were identified using KEGG (Kyoto Encyclopedia of Genes and Genomes) bioinformatics tool (Figure 2A). The majority of DEPs were involved in organismal systems (Figure 2A, red bars), human diseases (violet), environmental information processing (green) and, in a minor way, in metabolism (blue) and genetic information processing (orange). Among all the enriched pathways, the synaptic vesicle cycle pathway had the lowest p value (p=2.69e-04), with 5 enriched proteins: Ap2a1, Atp6v1b2, Syt1, Cacna1a and Slc18a2 (Supplementary Figure 1). Besides, important pathways also included choline metabolism in cancer, endocrine and other factor-related calcium, dopaminergic synapse, cysteine and methionine metabolism, serotonergic synapse, thyroid hormone synthesis, ErbB

signaling pathway, hepatitis B, Wnt signaling, protein processing in endoplasmic reticulum, axon guidance, pancreatic secretion, insulin resistance (Figure 2A). Protein-protein interaction (PPI) network analysis (Figure 2B) confirms that these AD affected proteins are associated with synaptic vesicle cycle and also with metabolism, protein processing, choline metabolism, neurotransmitters and signaling, these pathways are closely related with each other, and expressional disturbance are expected to influence many functions.

DEPs between TDMQ20-treated 5xFAD mice compared to untreated animals

Compared to untreated 5xFAD AD group, 183 proteins were altered after TDMQ20 treatment, 72 proteins being up-regulated (39%) and 111 proteins being down-regulated (61%) (Figure 3A and 3B) (raw data: Supplementary 1.xlsx, sheet TDMQ20 vs AD). Gene Ontology analysis of BP, CC and MF of these proteins is shown in Figure 3C-E.

As shown in Figure 3D, BP linked to DEPs in the TDMQ20/AD category mainly included cellular component organization or biogenesis, cellular component organization, and nervous system development. Then follows in order of diminishing impact: protein complex subunit organization, nitrogen compound transport, generation of neurons, neuron development, neuron projection development, response to organonitrogen compound, chemical synaptic transmission, anterograde trans-synaptic signaling, trans-synaptic signaling, synaptic signaling and modulation of synaptic transmission.

Cellular components linked to DEPs of TDMQ20/AD comparison included cytoplasm and cytoplasmic part and, to a lesser extent, vesicle, cytosol, extracellular region part, extracellular exosome, extracellular vesicle, extracellular organelle, extracellular membrane-bounded organelle, cell projection, neuron part, somadendrites, cell body and neuronal cell body (Figure 3E). Molecular function included protein binding, binding and, to a much lesser extent, nucleoside-, ribonucleoside- purine ribonucleoside-binding, identical protein binding (Figure 3 C).

KEGG pathway analysis (Figure 4A) showed that the majorities of DEPs were involved in the human diseases (brown bars), then in cellular processes (Figure 4A, red bars) and environmental information processing (green bars), organismal systems (violet bars), genetic information processing (orange bars) and metabolism (blue bars). Among all the enriched pathways, the PI3K-Akt signaling pathway had the highest significantly (lowest *p* value, $p = 3.88e\text{-}04$), with 11 enriched proteins: Ywhae, Hsp90ab1, Ywhaz, Ywhag, Hras, Cdc37, Rac1, Gnb4, Tsc1, Tnc and Ppp2r5b (Supplementary Figure 2).

PPI network of TDMQ20/AD is depicted in Figure 4B. Putting together with the above analysis, these DEPs were found to be associated with the following pathways which were closely related with each other: PI3K-Akt signaling pathway and synaptic vesicle cycle, as well as protein processing in endoplasmic reticulum, regulation of actin cytoskeleton, focal adhesion, hippo signaling pathway, and inositol phosphate metabolism.

DEPs between CQ-treated 5xFAD mice compared to untreated animals

When compared to the untreated AD group, 226 proteins were altered after CQ treatment of AD mice (Figure 5). Among them, 73 proteins (32%) were up-regulated and 153 proteins (68%) were down-regulated (Figure 5A and B) (raw data: Supplementary 1.xlsx, sheet CQ vs AD). As shown in Figure 5C-D, biological process of GO analysis linked to DEPs of CQ/AD groups mainly included establishment of localization, transport, single-organism localization, single-organism transport, cellular localization and regulation of localization. Protein localization, regulation transport, establishment of protein localization, protein complex subunit organization, amide transport, peptide transport, protein complex assembly protein complex biogenesis and nucleobase containing small molecule metabolic process were also altered to a lesser extent (Figure 5D). Cellular components significantly linked to DEPs of CQ/AD groups included cytoplasm, cytoplasmic part, vesicle, extracellular region, extracellular region part, membrane-bounded vesicle, extracellular exosome, extracellular vesicle,

extracellular organelle, extracellular membrane-bounded organelle, neuron part, cell projection, neuron projection and cell body (Figure 5E). Molecular function mainly included protein binding and binding, and also enzyme binding, small molecule binding, purine ribonucleoside binding, purine nucleoside binding and several other binding functions. KEGG pathway analysis (Figure 6A) showed that the majorities of DEPs were involved in metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases. Among all the enriched pathways, the endocrine and other factor-regulated calcium reabsorption pathway had the lowest *p* value, and accounted for 8 proteins as Ap2b1, Plcb1, Atp2b1, Atp1b1, Atp1b2, Calb1, Prkca and Adcy6. PPI network is shown in Figure 6B. Putting together with the above analysis, these proteins were found to be associated with fatty acid degradation, cysteine and methionine metabolism, carbon metabolism, thyroid hormone synthesis, phospholipase D signaling pathway, endocrine and other factor-regulated calcium reabsorption, synaptic vesicle cycle and GABAergic synapse, and cholinergic synapse.

Common DEPs in AD/WT, TDMQ20/AD and CQ/AD categories

We further analyzed the commonly altered DEPs in the AD/WT, TDMQ20/AD and CQ/AD series. Among the 35 common DEPs (Supplementary Figure 4) identified in AD/WT and TDMQ20/AD categories, and 30 common DEPs (Supplementary Figure 5) identified in AD/WT and CQ/AD categories, 10 proteins were found common in all three AD/WT, TDMQ20/AD and CQ/AD categories, with DEPs in AD/WT that were reversed in TDMQ20/AD and CQ/AD (Figure 7, panels A and B). Five proteins which were down-regulated in AD/WT, namely Chrm4, Chat, Rin1, Lgalsl, and Mrps9 displayed a consistent up-regulated pattern both in TDMQ20/AD and CQ/AD categories. Conversely, proteins Plp1, Vps29, Rac1, Ptprz1 and Cmpk1, which were overexpressed in AD/WT were down-regulated in both TDMQ20/AD and CQ/AD (Figure 7B). According to PPI network analysis, these 10 common DEPs are mainly involved in pathways related

to cognition, behavior, learning and memory, anterograde trans-synaptic signaling as well as neuron projection development (Figure 7C). Most significantly regulated proteins with gene names Chat, Rin1, Chrm4, Ptprz1, Rac1 and Plp, were associated with these pathways.

To further validate the proteomic results, the expression of seven proteins with the gene names Chrm4, Chat, Rin1, Lgalsl, Mrps9, Ptprzl and Cmpkl, were evaluated by Western blot analysis in WT, AD, TDMQ20 and CQ mice. Results are reported in Figure 8.

Among these seven proteins, three proteins, which are significantly downregulated in AD mice with respect to control animals (ChAT, CHRM4 and Lgalsl) were found up-regulated after treatment of AD mice with TDMQ20 with respect to untreated AD mice (Figure 8A, C-E). Specially, the significantly increased level of ChAT and CHRM4 (Figure 8C and 8D, respectively) clearly suggests a role of TDMQ20 in the cholinergic system. This result is consistent with improved memory and behavioral performance of TDMQ20 treated 5xFAD mice that was previously reported (21). Conversely, treatment by CQ failed to efficiently reverse the low level of ChAT and CHRM4 in AD mice. The level of Lgalsl, downregulated in AD mice, was efficiently reversed by treatment with TDMQ or CQ (Figure 8E). Ptprz1 level was somewhat lower in AD mice than in control WT, and its level trends to be reversed by TDMQ20 and CQ. However, these differences indicate a tendency but are not statistically significant (Figure 8G). RIN1, Mrps9 and Cmpk1 levels were found similar in WT control mice and in 5xFAD mice. So, these proteins cannot be considered as relevant of AD targets in this animal model (Figure 8F, 8H, 8I).

Immunofluorescence staining of Aβ plaques in the cortex

As one of the important pathological hallmarks in AD brains, we additionally quantified amyloid plaques in the mouse cortex of each group. The amyloid plaque load of each mouse group was quantified as the percentage of area covered by 6E10-immunoreactivity (Figure 9) in each image sample, acquired using a

florescent microscope. According to the result, the cortex of mice treated with TDMQ20 exhibited a drastically reduced plaque load compared to untreated AD mice (roughly –70% compared to AD mice), suggesting a lower amount of uncontrolled copper ions promoting plaque formation in the brain of treated mice. This result is consistent with a lower concentration of malondialdehyde, a marker of oxidative stress, in the cortex of AD mice treated with TDMQ20 we reported in a previous study (30). The reduction of plaque load upon treatment with CQ was also significant (–57%), which is also consistent with previous report (31).

DISCUSSION

TDMQ20 is a highly specific copper chelator able to extract Cu from Cu-Aβ complex, thus inhibiting Cu-Aβ induced activation of dioxygen and ROS production, and to release Cu from Cu-TDMQ20 into normal physiological circulation (19). Furthermore, oral treatment of TDMQ20 on 5xFAD mice had a clear beneficial effect the short-term memory and cognitive function of this transgenic model of Alzheimer's disease (21). In order to investigate further the underlying mechanism of action of TDMQ20, and specially the possible interaction with the copper dependent cholinergic system, the current study was designed as an exploration based on proteomic and bioinformatic analysis. By using iTRAQ, proteomic alterations in the cortex of 5xFAD mice with respect to WT animals were investigated, leading to identification of DEPs in AD with respect to healthy animals. The fate of these DEPs proteins in AD mice treated by TDMQ20 (or CQ used as comparator) was then investigated in order to evaluate the relationship between (i) the pattern of protein modifications induced by TDMQ20 treatment, (ii) copper regulation in brain and (iii) ability of this drug to reverse AD impairment.

Proteomics of AD 5xFAD mice compared to healthy WT mice (AD/WT)

Amyloid precursor protein (APP) was highly upregulated with a fold change of 6.8 in our 5xFAD mice with respect to WT (raw data: Supplementary 1.xlsx, sheet AD

vs WT), result consistent with plaque accumulation in AD condition (Figure 9). More importantly, Rin1 (Ras and Rab interactor1), known to take part in the long-term potentiation (32) and neuronal plasticity (33), is downregulated with a fold change of 0.3 in 5xFAD mice. Several other DEPs identified in AD/WT were involved in synaptic vesicle cycle: proteins Slc18a and Cacna1a were upregulated, and Ap2a1, Syt1 and Atp6v1b2 were down-regulated, indicating alterations of synaptic signaling and plasticity that are likely linked to impairment of learning, memory and cognitive functions. Another important set of DEPs are related to choline metabolism pathway: ChAT, Chrm4, Slc44a1 and Plpp3 were downregulated, and Prkcg was upregulated. Reduced expression of ChAT (fold change = 0.2), already reported (34-36), is consistent with low Ach level and degeneration of cholinergic neurons in AD brain (37). The significant lower level of Chrm4 (fold change = 0.2) in our 5xFAD mice with respect to healthy animals confirms impairment of cholinergic system at this stage of AD.

Proteomics of AD mice treated with TDMQ20 compared to untreated AD mice (TDMQ20/AD)

After treatment of AD mice by TDMQ20, 183 DEPs, with 72 up-regulated and 111 down-regulated with respect to untreated animals (39% and 61%, respectively). Among these 183 DEPs, 35 DEPs were found common in AD/WT and TDMQ20/AD comparisons and are analyzed below. Most of these 35 DEPs were involved in PI3K-Akt signaling pathway involved in cellular survival and proliferation. In addition, several DEPs link to PI3K-Akt signaling pathway, with the gene names Hsp90ab1, Ywhaz, Ywhag, and Rac1 also present properties in synaptic plasticity, such as axon extension (38), synapse formation (39), dendritic spine enlargement and morphogenesis (40,41). The downregulation in AD of another protein involved in PI3K-Akt pathway, galectin-related protein Lgalsl (= HSPC159) (42) was also reversed upon treatment by TDMQ20.

Regarding nervous system, TDMQ20/AD DEPs also showed a significant connection with the synaptic vesicle cycle. Cplx1 and Cplx3, which regulate

synaptic vesicle exocytosis and neurotransmitter secretion (43,44), were significantly up-regulated in TDMQ20 treated AD mice. The above-mentioned downregulation of Rin1, involved in synaptic plasticity in AD, was significantly reversed upon treatment by TDMQ20. Specially, the muscarinic receptor Chrm4 was up-regulated with a high fold change value of 6.6, with respect to untreated AD mice. Chrm4 is localized at the pre-synapse of axon terminals and on dendritic membranes at the post-synapse (45), with high density in striatum, cortex and hippocampus (31), and alterations of Charm4 have been reported in AD as early as 1982. This confirms the very likely role of TDMQ20 in regulation of cholinergic signaling in the brain, possibly through modulation of synaptic neurotransmission as indicated by biological process from GO analysis.

Proteomics of AD mice treated with CQ compared to untreated AD mice (CQ/AD)

Despite clioquinol (CQ) was reported to exhibit beneficial effects on cognition in a transgenic mouse model of AD (46), it was discarded due to severe neurotoxicity due to its lack of metal selectivity (46). As historical prototype of copper chelator, it was used as comparator in the present study. AD mice treated with CQ exhibited 73 (32%) upregulated and 153 (68%) downregulated DEPs with respect to untreated AD mice. DEPs were mostly related to establishment of localization, transport and single-organism localization in biological process and mostly link to cytoplasmic (Cell Components). Unlike TDMQ20 treated mice, KEGG pathway analysis showed a very high percent of DEPs involved in metabolic pathways which might be related to detrimental side-effects of CQ in clinics. Other pathways like endocytosis, adrenergic signaling in cardiomyocytes, pancreatic secretion, carbon metabolism, purine metabolism, are also involved. Among all the analyzed pathway, the endocrine and other factor-regulated calcium reabsorption pathway showed the most significance, with eight proteins including Ap2b1, Plcb1, Atp2b1, Atp1b1, Atp1b2, Calb1, Prkca and Adcy6. In the nervous system, the effect of CQ on several types of synapse pathways. Rin1 displayed significant higher level in CQ treated mice than in untreated AD animals. In cholinergic synapse pathway,

similarly to TDMQ20, Chrm4 was highly upregulated with respect to AD. Other proteins related to cholinergic system: Map2k1, Gng3 and Kcnq2 were downregulated.

Differentially expressed proteins in common to AD/WT, TDMQ20/AD and CQ/AD categories

Ten target proteins were found common to these three groups, with similar expression pattern either upregulated or downregulated from one to another. Compared to AD/WT category, five proteins Chrm4, Chat, Rin1, Lgalsl, Mrps9 displayed a consistent up-regulated pattern, while five proteins Plp1, Vps29, Rac1, Ptprz1 and Cmpk1 were downregulated in both TDMQ20/AD and CQ/AD categories. Based on the KEGG pathway analysis, these common DEPs were mainly involved in cognition, learning and memory, behavior, anterograde trans-synaptic signaling and neuron projection development. According to the commercial availability of the antibodies of these common DEPs, seven proteins with the gene names Chrm4, Chat, Rin1, Lgalsl, Mrps9, Ptprzl and Cmpkl were selected and validated by Western blot analysis. Results showed that three proteins, with the gene names Chrm4, Chat and Lgalsl, were found significantly up-regulated after the treatment of TDMQ20, and the decreased level of Lgalsl in AD mice was also reversed by CQ treatment.

Among the ten proteins reversed to normal levels by both TDMQ20 and CQ, three of them were successfully validated by Western blot analysis for TDMQ20 treatment. Specially, the relative abundance levels of ChAT and CHRM4 (impaired in 5xFAD mice) were significantly increased upon TDMQ20 treatment (with fold changes of 4.3 and 6.6, respectively), while CQ did not induce significant changes of these protein levels (Figure 8), suggesting a specific action of TDMQ20 on these two masterpiece proteins of the cholinergic system.

Table 3. List of top 10 common DEPs reversed by treatment by TDMQ20 or CQ

No.	UniProt ID	Gene names	Protein names	TDMQ:AD Fold change	AD:WT Fold change	CQ:AD Fold change
1	P32211	Chrm4	Muscarinic acetylcholine receptor M4 (Mm4 mAChR)	6.6	0.2	5.6
2	Q03059	Chat	Choline O-acetyltransferase (CHOACTase) (ChAT) (Choline acetylase) (EC 2.3.1.6)	4.3	0.2	4.8
3	Q921Q7	Rin1	Ras and Rab interactor 1 (Ras interaction/interference protein 1)	3.1	0.3	4.0
4	Q8VED9	Lgalsl	Galectin-related protein (Galectin-related protein A) (Lectin galactoside-binding-like protein A)	2.3	0.5	1.7
5	Q9D7N3	Mrps9	28S ribosomal protein S9, mitochondrial (MRP-S9) (S9mt)	1.9	0.6	1.5
6	D3Z645	Vps29	Vacuolar protein sorting-associated protein 29 (Vesicle protein sorting 29)	0.7	1.4	0.6
7	Q3TLP8	Rac1	Ras-related C3 botulinum toxin substrate 1	0.7	1.3	0.5
8	B9EKR1	Ptprz1	Receptor-type tyrosine-protein phosphatase zeta (R-PTP-zeta) (EC 3.1.3.48)	0.8	1.3	0.8
9	A0A0R4J093	Cmpk1	UMP-CMP kinase (EC 2.7.4.14) (Deoxycytidylate kinase) (CK) (dCMP kinase) (Nucleoside-diphosphate kinase) (EC 2.7.4.6) (Uridine monophosphate/cytidine monophosphate kinase)	0.8	1.2	0.8

			(UMP/CMP kinase) (UMP/CMPK)			
10	P60202	Plp1	Myelin proteolipid protein (PLP) (Lipophilin)	0.5	0.7	0.5

Plaque load in the mouse cortex

Amyloid plaque, consisting of misfolded and aggregated amyloid β peptides is one of the well-known hallmarks of AD pathological states. In our present study, we calculated the plaque load in mouse cortex as the ratio of area covered by the anti-Aβ antibody 6E10 to the entire imaged area acquired by a fluorescent microscope. Treatment by TDMQ20 remarkably reduced plaque load in the cortex by about 70%, suggesting a beneficial role of TDMQ20 in clearing pathological Aβ deposits in AD mice. CQ treatment also contributed to an approximately 57% reduction of plaque load in mouse cortex, though not as much as TDMQ20 did. These results further consolidate TDMQ20 as a good candidate for AD treatment.

EXPERIMENTAL PROCEDURES

Experimental design and statistical rationale

5xFAD mice (genetic background: C57BL/6 x SJL) were used as AD model. These mice express 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, and the M146L and L286V mutations in PSEN1. Starting when they were two-month-old, these AD mice were orally treated either with TDMQ20 or CQ and compared with untreated mice. The expression of proteins in the cortex of these three AD mouse groups and in the healthy control WT C57BL/6 x SJL mice was compared using iTRAQ labeling and bioinformatics analysis described below. GraphPad Prism 8.0.2 software was used for statistical analysis. Data were expressed as mean ± SEM unless otherwise indicated, with $P < 0.05$ defining differences as statistically significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Differences among means were determined using the two-tailed *t* test.

Materials and animals

TDMQ20 was synthesized according to Reference 18. Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline, abbreviated as CQ) was purchased from Sigma (Sigma-Aldrich Co., St. Louis, USA). All other reagents were supplied by Sigma-Aldrich, AB Sciex or Promega, except otherwise stated.

Thirty specific pathogen-free (SPF) 5xFAD mice and 10 non-transgenic C57BL/6 x SJL control mice (male/female ratio = 1/1, weighing 20-25 g, 8 weeks) have been used. Animals were housed by groups in standard laboratory conditions: air-conditioned room (20-25ºC), 12 hours light/dark illumination cycle, free access to food and water. Food was provided by Guangdong Medical Experimental Animal Center. All protocols were implemented in accordance with NIH guidelines (NIH publication No. 85-23, revised 1985) for animal care and welfare. All animal care and experiments were approved by the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences in China and approved by the Regional Ethical Committee for Animal Experimentation at Shenzhen University (Permit Number: AEWC-20140615-002) and performed according to the animal protection law.

Animal grouping and treatment

Two-month-old mice were separated into four groups and drug-treated as indicated in Table 1. Drugs were delivered to stomach of mice once every two days (oral administration by gavage, volume of each gavage = 100 μL, of a dose = 10 mg/kg) for three months (in total 45 times), and followed with behavioral tests as conducted previously (21).

Table 1 Mice grouping and drug administration

Group	**Drug**	**Drug dose (mg/kg)**
WT (C56BL/6J control)	0.9% NaCl	0
AD (5xFAD mice)	0.9% NaCl	0

TDMQ20 (5xFAD treated with-)	TDMQ20 in 0.9% NaCl	10
CQ (5xFAD treated with-)	Clioquinol in DMSO	10

Sample preparation

Fourteen days after the end of treatment (5.5 months old mice), mice were killed by cervical dislocation. Brains were rapidly excised and placed on an ice-cold plate for dissection of cortices. Six cortex tissue samples were taken from each group of mice for proteomics analysis. Tissue samples were lysed in lysis buffer containing 7 M urea, 2 M thiourea, 4 w/v% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 40 mM dithiothreitol (DTT), and 40 mM Tris base), sonicated 10 times for 5 s with 10 s pause in an iced water bath, and centrifuged for 10 min at 12 000 rpm, 4 °C. The supernatant was withdrawn and stored at −80 °C until use.

Trypsin digestion and iTRAQ labeling

For proteomics analysis, equal amounts of above protein samples from 6 mice cortex (random selection) in each group were pooled separately to yield two distinct pools in each group. Protein digestion and iTRAQ labeling were performed as previously reported (30,47). In brief, approximately 100 μg of each processed tissue protein sample was reduced with 10 mM DTT (Sigma Aldrich, Missouri, USA) for 1 h at 37 °C, followed by alkylation using 50 mM iodoacetamide (IAA, Sigma Aldrich, Missouri, USA) for 15 min at room temperature in the dark. The samples were then desalted and buffer-changed three times with 100 μL of 0.5 M triethylammonium bicarbonate (TEAB) (B Sciex, Foster City, CA, USA) by Amicon® ultra centrifugal filters (10 kDa cutoff; Millipore, Billerica, MA). The proteins were digested with trypsin (Promega, Madison, WI, USA) at a ratio of 1:30 at 37 °C overnight. The digested peptides were then labeled with iTRAQ reagents as follows (Table 2): the WT group was labeled with iTRAQ 117 and 118, while the AD group with iTRAQ 119 and 121, the TDMQ20 treated AD group with iTRAQ 113 and 114, and the CQ treated AD group with iTRAQ 115 and 116.

The labeled samples were mixed together and lyophilized. After labeling, the peptides were subjected to reverse-phase chromatography at pH 10, to remove the excess of labeling reagents and salts by HPLC system (Agilent Technologies, Santa Clara, CA, USA). Peptides were eluted and combined into 16 batches. They were then lyophilized and stored at -80 °C.

Table 2. iTRAQ labeling of the samples

Group	TDMQ20	CQ	WT	AD
iTRAQ label	113/114	115/116	117/118	119/121

Nano LC-MS/MS (Mass Spectrometry) analysis and iTRAQ quantification

Nano LC-MS/MS analysis was conducted as previously described (30,47). Briefly, the 16 pooled batches were reconstituted in 30 μL of 0.1% formic acid and 2% acetonitrile, among which 8 μL of each batch was added to a Triple TOF 6600 system fitted with a NanosprayIII source (AB Sciex). Data were obtained with a 2.4 kV ion spray voltage, 30 psi curtain gas, 5 psi nebulizer gas, and an interface heater temperature of 150 °C. TOF-MS was conducted with a scan scope of 350-1500, and for MS/MS was 400-1250. Smart information-dependent acquisition was activated with automatic collision energy and automatic MS/MS accumulation. The protein was identified and quantified by ProteinPilot v5.0 (AB Sciex) with the Paragon algorithm against the Uniprot "complete proteome" mouse proteins database. Based on a 95% confidence level, at least one unique peptide per protein group was required for identifying proteins, and two quantified peptides were required for quantifying proteins. The cutoff values of 1.2-fold for up-regulated and 0.8-fold for down-regulated proteins, p value <0.05, were considered as significant results. This significant change was further determined by conversion of $\log_2$ ratios, followed by data normalization and performance of a statistical t test by using OMICSBEAN online tools (http://www.omicsbean.cn)(48).

Bioinformatics analysis

Bioinformatics analysis was performed by using OMICSBEAN online tools and STRING (Search Tool for the Retrieval of Interacting Genes/Proteins, version 9.1, http://string-db.org/) database. Differentially expressed proteins (DEPs) between the different mouse groups were analyzed using Gene Ontology annotation (GO), Kyoto Encyclopedia of Genes and Genomes pathways (KEGG) and protein-protein interaction (PPI) networks.

Western Blot Analysis

The validation of targeted proteins was performed by Western blot analysis. Proteins were extracted from the cortex tissue after homogenizing and their concentrations were measured by Pierce™ BCA Protein Assay Kit (ThermoFisher, MA, USA). After optimizing the protein concentration, samples were boiled for 10 min at 100 °C in water bath together with a 4x loading buffer (BOSTER, California, USA). Proteins were separated on denaturing gel electrophoresis (10% SDS-PAGE (Epizyme, Shanghai, China) for 50 minutes. Separated proteins were further transferred into a nitrocellulose membrane (0.45 μm, Millipore, Darmstadt, Germany). Then, membranes were gently shaken in a blocking solution (Beyotime, Shanghai, China) for about two hours. Primary antibodies (anti-CHRM4, anti-CMPK1, anti-LGALSL, anti-MRPS9, rabbit polyclonal, ABClonal, Wuhan, China; anti-PTPRZ, mouse monoclonal, BD Biosciences, NJ, USA; anti-ChAT, rabbit polyclonal, Cell signaling Technology, Massachusetts, USA; anti-RIN1, rabbit polyclonal, Invitrogen, MA, USA) were added and incubated with membranes overnight, at 4 ℃. Corresponding secondary antibodies (anti-rabbit/mouse horseradish peroxidase conjugated, Abmart, NJ, USA) were added after washing 3 × 10 min with 4% TBST buffer (tris-buffered saline and Tween 20, pH 7.50) and further incubated for 2 hours at room temperature. After washing 3 × 10 min with TBST buffer, blots were visualized by incubating with ECL reagent (Advansta, CA, USA) and exposed with the Tanon-5200 chemiluminescence imaging system (Tanon, Shanghai, China).

Immunofluorescence staining and image acquisition

Mouse brains were removed after cervical dislocation and were prepared as paraffin-embedded slices cut with ~4 μm thickness. Six slices from each group were selected and deparaffinized by immersing in xylene (2 × 5 min), and then rehydrated by immersing in the following solutions for 3 min each: 100% ethanol, 95% ethanol, 85% ethanol, 75% ethanol, 50% ethanol, 30% ethanol and then deionized water. Slices were then washed in PBS for 2 × 5 min. After drawing a circle around each slice with a hydrophobic barrier pen, permeabilization buffer containing 0.8% Triton X-100 in PBS was added to each slice for 30 min. Slices were further incubated in blocking solution containing 10% normal goat serum (Solarbio, Beijing, China) at RT for 2 h, and then with primary antibodies (anti-6E10, mouse polyclonal, Biolegend, San Diego, USA; anti-MAP2, chicken polyclonal, Invitrogen, MA, USA) overnight at 4 °C. Slices were washed 3 × 10 min with PBS and then incubated with the secondary antibodies (goat anti-chicken Alexa Fluor 488, goat anti-mouse Alexa Fluor 568, Life Technologies GmbH) for 2 h at RT in the dark. After 3 × 10 min washing in PBS, slices were further stained with DAPI (10 μg/mL, Beyotime, Shanghai, China) for 15 min in the dark. Then slices were washed for 3 × 10 min with PBS before mounting on glass coverslips with Dako fluorescence conserving medium (Dako, Hamburg, Germany). The dilution of antibodies used here were 1:500 in blocking solution. Images were acquired by using Olympus BX53 fluorescence microscope under a 20 × objective, with resolution of 1920 × 1900 pixels.

CONCLUSIONS

Our current study has been designed as an investigation of protein-based molecular mechanism underlying the improved cognitive and behavioral performance in a transgenic 5xFAD mouse model of Alzheimer's disease upon treatment with the copper chelator TDMQ20. Abnormally expressed proteins in the cortex of AD mice are closely involved in several key functional brain pathways, such as choline

metabolism, trans-synaptic signaling, chemical synaptic transmission, and synaptic vesicle cycle.

Among DEPs common to AD/WT, TDMQ20/AD categories, ChAT and CHRM4 were further validated by Western blot analysis, suggesting a recovery of the cholinergic system functioning induced by TDMQ20, a feature which may account for improved cognitive, behavioral performances and spatial memory of AD mice upon treatment by TDMQ20. Especially, after treatment of AD mice by TDMQ20, ChAT level was similar to that observed in healthy WT mice. Down-regulation of Lgalsl observed in AD mice was also fully reversed upon TDMQ20 treatment. These results suggest that, beside "traditional" targeting of AChE, ChAT and Chrm4 involved in cholinergic system also deserve consideration as potential targets for AD chemotherapy. Noteworthy, cortex of mice treated with TDMQ20 do not present the panel of metabolic DEPs that might to be responsible for CQ toxicity.

Moreover, the load of amyloid plaques in mouse cortex was drastically reduced after treatment by TDMQ20, suggesting a lower amount of uncontrolled copper ions promoting plaque formation in the brain of treated mice. This result is consistent with the decreased level of malondialdehyde observed in the three different AD mouse models upon TDMQ20 treatment in our previous study. The simultaneous reversal of the deprivation of proteins involved in the copper dependent cholinergic system, suggests the capacity of TDMQ20 to act as regulator of copper homeostasis *in vivo*. The correlation of such a mechanism of action of TDMQ20 and drastic improvement of the cognitive performances of treated animals validates copper homeostasis as a priority target and TDMQ20 as a drug candidate.

Author Contributions

B.M., Q.L., and A.R. designed the research; Y.L. and colleagues performed the chelator synthesis; F.S., J.Z., and H.Z. performed biological assays, animal

experiments, and analyzed the data; F.S., Q.L., A.R., and B.M., wrote the manuscript.

Conflicts of Interest

None.

Supporting Information

Figure S1-S3, heat-map of DEPs with expression intensities in AD/WT, TDMQ20/AD, and CQ/AD categories, respectively; Figure S4, common DEPs in AD/WT and TDMQ20/AD categories; Figure S5, common DEPs in AD/WT and CQ/AD categories (PDF)

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ABBREVIATIONS

The abbreviations used are: Aβ, amyloid-β peptide; Ach, acetylcholine; AChE, acetylcholine esterase; AD, Alzheimer's disease; APP, amyloid precursor protein; ChAT, choline *O*-acetyltransferase; Chrm4, muscarinic acetylcholine receptor M4; Cmpk1, UMP-CMP kinase; CQ, clioquinol; DEPs, differentially expressed proteins; GO, gene ontology; iTRAQ, isobaric tag for relative and absolute quantitation; KEGG, Kyoto encyclopedia of genes and genomes; Lglsl, galectin related protein; Mrps9, 28S ribosomal protein S9; PPI, protein-protein interactions; Ptprz1, Receptor-type tyrosine protein phosphatase zeta; Rin1, Ras and Rab

interactor 1; SFP, specific pathogen free; TDMQ, tetradentate monoquinoline chelator.

FIGURE LEGENDS

Figure 1. Proteomic and bioinformatic analysis of DEPs in AD/WT category. (A) Volcano-diagram of DEPs. (B) Number of up-and down-regulated DEPs. (C) GO (Gene Ontology) analysis of DEPs including BP (biological process), CC (cell component), MF (molecular function). (D) Biology process. (E) Cell component.

Figure 2. Bioinformatic analysis of DEPs in AD/WT category. (A) KEGG pathway analysis. (B) PPI network of DEPs.

Figure 3. Proteomic and bioinformatic analysis of DEPs in TDMQ20/AD category. (A) Volcano-diagram of DEPs. (B) Number of up-and down-regulated DEPs. (C) GO (Gene Ontology) analysis of DEPs including BP (biological process), CC (cell component), MF (molecular function). (D) Biology process. (E) Cell component.

Figure 4. Bioinformatic analysis of DEPs in TDMQ20/AD category. (A) KEGG pathway analysis. (B) PPI network of DEPs.

Figure 5. Proteomic and bioinformatic analysis of DEPs in CQ/AD category. (A) Volcano-diagram of DEPs. (B) Number of up-and down-regulated DEPs. (C) GO (Gene Ontology) analysis of DEPs including BP (biological process), CC (cell component), MF (molecular function). (D) Biology process. (E) Cell component.

Figure 6. Bioinformatic analysis of DEPs in CQ/AD category. (A) KEGG pathway analysis. (B) PPI network of DEPs.

Figure 7. Common DEPs in AD/WT, TDMQ20/AD and CQ/AD categories. (A)

Venn diagram of common DEPs among three categories. (B) Heat-map of common DEPs among three categories with expression intensities. (C) PPI analysis of common DEPs among three categories.

Figure 8. Western blot validation of proteins. (A), (C)-(G), Choline O-acetyltransferase (ChAT), Muscarinic acetylcholine receptor M4 (Chrm4), Galectin-related protein (Lgalsl), Ras and Rab interactor 1 (Rin1), 28S ribosomal protein S9 (Mrps9). (B), (H)-(I), UMP-CMP kinase (Cmpk1), Receptor-type tyrosine-protein phosphatase zeta (Ptprz1). Quantification was conducted by ImageJ software as ratio of optic density between each band and their housekeeping proteins (α-Tubulin or β-Actin), data analysis and graph were done with Prism GraphPad 8.0.2.2, n=3.

Figure 9. Plaque load in mouse cortex of each group. (A) Representative images of immunohistochemical stainings of amyloid-β plaque (stained with 6E10, red), nuclei (stained with DAPI, blue), microtubule associated protein 2 (MAP2, green) in the cortex. (B) Plaque load in mouse cortex. Analysis was conducted by Image J, calculated as the proportion of area covered by 6E10 staining. Scale bar, 50μm.

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