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Ju Huang, Michel Nguyen, Yan Liu, Anne Robert, B. Meunier. The TDMQ Regulators of Copper Homeostasis Do Not Disturb the Activities of Cu,Zn-SOD, Tyrosinase, or the Co III Cofactor Vitamin B12. *European Journal of Inorganic Chemistry*, 2019, 2019 (10), pp.1384-1388. 10.1002/ejic.201801332 . hal-02333091

**HAL Id: hal-02333091**

**<https://hal.science/hal-02333091>**

Submitted on 10 Nov 2020

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# The TDMQ Regulators of Copper Homeostasis Do Not Disturb the Activities of Cu,Zn-SOD, Tyrosinase, or the Co(III) Cofactor Vitamin B12.

Ju Huang,<sup>[a,b]</sup> Michel Nguyen,<sup>[b]</sup> Yan Liu,<sup>[a]\*</sup> Anne Robert,<sup>[b]\*</sup> Bernard Meunier<sup>[a,b]</sup>

**Abstract:** Copper chelators based on a tetradentate monoquinoline (TDMQ) scaffold are currently developed as potential drugs against Alzheimer's disease. Since TDMQ chelators have a high affinity for Cu(II) and are expected to regulate copper homeostasis in the brain, the question whether these chelators might be able to demetallate, and therefore inhibit, essential copper proteins is obviously important, and should be answered before clinical development could be considered. We therefore examined the possible interaction of these chelators with physiological copper proteins, and related metal biomolecules. Our present work evidenced that TDMQ chelators (up to millimolar concentrations) do not affect *in vitro* the catalytic activities of Cu,Zn-superoxide dismutase and tyrosinase, two copper enzymes expressed in the brain and involved in the regulation of redox processes. In addition, TDMQ were found unable to demetallate vitamin B12, a Co(III) cofactor essential for neurotransmitter synthesis and normal brain function. The validation of the compatibility between TDMQs and several physiological metal complexes is an important positive issue supporting the development of TDMQ chelators as drug-candidates.

## Introduction

Disruption of copper homeostasis in the brain is considered as a causal factor of the oxidative stress characteristic of Alzheimer's disease (AD).<sup>[1]</sup> TDMQs are specific copper(II) chelators which are currently developed as regulators of copper homeostasis in the brain and, consequently, as drugs able to stop the cognitive decline linked to neuronal death (Figure 1).<sup>[2]</sup> Since the affinity of TDMQ chelators for Cu(II) is high (log  $K_{app}$  values up to 15–16 at pH 7.4), it was obviously necessary to check if they will interfere or not with the structure and/or the activity of important copper proteins like Cu,Zn-superoxide dismutase (SOD) or tyrosinase (TYR). SOD, which is ubiquitous in aerobic organisms, is able to protect living systems from potentially detrimental effects of the

superoxide radical, generated by the 1-electron reduction of dioxygen.<sup>[3]</sup> In addition, the involvement of oxidative damage to Cu,Zn-SOD in the pathogenesis of sporadic AD and Parkinson's disease has been proposed.<sup>[4]</sup> Present in the central nervous system (CNS), TYR has a di-copper active site which catalyzes the hydroxylation of monophenols to *o*-diphenols (monophenolase activity), and the subsequent oxidation of the *o*-diphenol to *o*-quinone.<sup>[5]</sup> In CNS, the TYR-catalyzed hydroxylation of L-tyrosine produces L-dopa involved in the biosynthesis of melanine, but also of catecholamines neurotransmitters, and thus serves as an alternative pathway to supply catecholamines.<sup>[6]</sup>

Although cobalt is less frequently encountered in metalloenzymes than copper, the cobalt corrin complex vitamin B12 is an important cofactor of several enzymes.<sup>[7]</sup> Any modification of the stability of vitamin B12 by drug-candidates would have a significant impact on metabolism. Therefore, we addressed this important issue in the presence of strong chelators such as TDMQs. In fact, the frequent and deleterious effect of B12 deficiency has been reported in the elderly and in neuropathological conditions including Alzheimer's disease.<sup>[8]</sup>

Our results evidenced that TDMQ20 and TDMQ22 were unable to retrieve cobalt ion from B12 and are, therefore, unlikely to disturb B12 function. The concentrations of TDMQ able to significantly inhibit tyrosinase or Cu,Zn-SOD were found in the range 0.5 - 2 mM, far above that ones expected for the pharmacological use of these drug-candidates.

General structure of TDMQ chelators

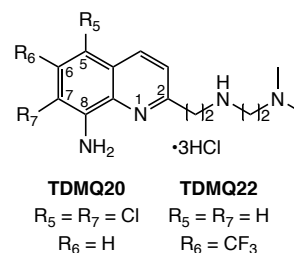


Figure 1. Structure of the copper chelators TDMQ20 and TDMQ22.

## Results and Discussion

**TDMQ20 or TDMQ22 + Vitamine B12.** Vitamine B12 is a corrinoid cobalt(III) complex involved in folate metabolism, and whose deficiency in neurodegenerative diseases has been reported.<sup>[9]</sup> The vitamin B12 deficiency in the Alzheimer's brains is mostly exerted via inactivation of protein phosphatase 2 and hyperphosphorylation of tau protein.<sup>[10]</sup> For this reason, any event possibly leading to inactivation of B12, and specially demetallation by a metal chelator must be avoided.

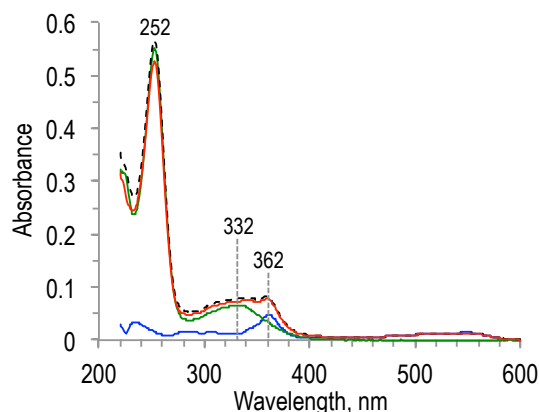
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We first evaluated the ability of TDMQ22 to extract Co(III) from B12. After incubation during 30 min, the UV-visible spectrum of a mixture of B12 and TDMQ22 (1/1 mol ratio) at pH 7.4 (Figure 2, red trace) exhibited two maxima at 332 nm, for TDMQ22, and at 362 nm, for B12 Co(III) complex. This spectrum, which is clearly the overlapping of the individual spectra of B12 and TDMQ22 (blue and green traces, respectively), indicated that TDMQ22 was unable to act as a competitive ligand for Co(III).



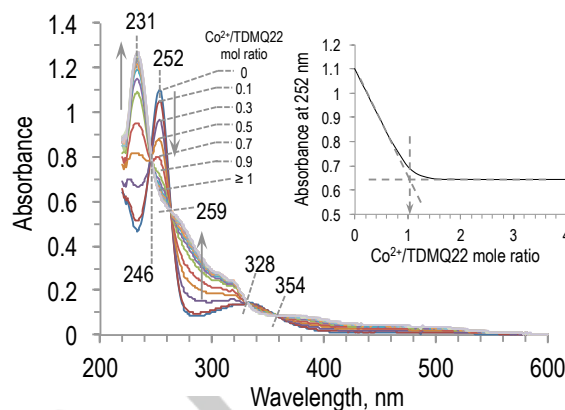
**Figure 2.** UV-visible spectrum of vitamin B12 in the presence of TDMQ22 (mole ratio = 1/1), 20  $\mu$ M in Hepes buffer 50 mM, pH 7.4 (red trace). The spectra of vitamin B12 alone (20  $\mu$ M, blue trace) and TDMQ22 alone (20  $\mu$ M, green trace) are given for comparison. The dashed black trace is the arithmetic addition of blue and green spectra.

In fact, when the titration of TDMQ22 was carried out by UV-visible spectroscopy, using  $\text{Co}^{\text{III}}(\text{NH}_3)_6\text{Cl}_3$  as source of cobalt, TDMQ22 remained unmetallated, even in the presence of 200 mole equivalents (mol equiv) of  $\text{Co}^{3+}$  salt. The spectrum of the mixture was the arithmetic addition of the spectra of TDMQ22 free ligand and of the  $\text{Co}^{3+}$  salt (Figure S1). The same result was obtained with TDMQ20 (data not shown).

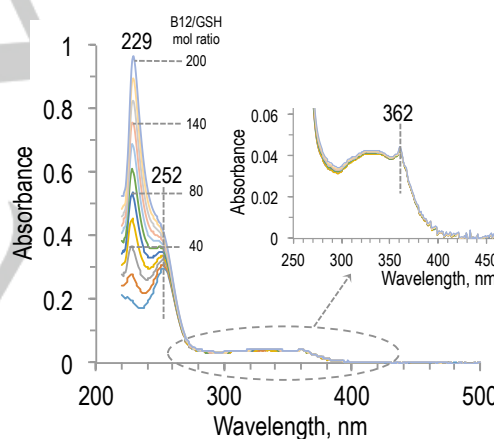
On the contrary, when TDMQ22 was titrated using  $\text{Co}^{\text{II}}\text{Cl}_2$  (Figure 3), the absorbance of TDMQ22 ( $\lambda_{\text{max}} = 252$ ) decreased upon addition of  $\text{Co}^{2+}$ . New absorbances appeared at 231 and 267<sub>sh</sub> nm, along with lower bands at ca. 301, 315 and 380 nm, which were assigned to the Co(II) complex of TDMQ22. Well-defined isosbestic points were detected at 246, 259, 328 and 354 nm. The plot of absorbance at 252 nm with respect of the  $\text{Co}^{2+}/\text{TDMQ22}$  mole ratio clearly indicate that the metal complexation was complete in these experimental conditions (no equilibrium in solution), and that the metal/ligand stoichiometry of the complex was 1/1. Results obtained with TDMQ20 instead of TDMQ22 were fully similar (Figure S2). The same result was obtained after incubation of vitamin B12 with TDMQ20 or TDMQ22 for 1 h at 37  $^{\circ}\text{C}$  (data not shown).

Since the affinity of porphyrin and corrole rings for a metal depends on its oxidation state, and because glutathione (GSH) is an efficient biological reductant, the putative transfer of cobalt from B12 to TDMQ22 was investigated in the presence of increasing concentration of GSH. In addition, reduction of Co(III) to Co(II) is required for demetallation of Co(III)-porphyrin complexes.<sup>[11]</sup> The addition of aliquots of GSH in an equimolar mixture of B12 and TDMQ22 in Hepes buffer, pH 7.4, is reported in Figure 4. Starting from the specific absorbances of TDMQ22 (252 and 332 nm) and B12 (362 nm), the only significant change

of the spectrum upon addition of GSH was the increasing absorbance at 229 nm, due to GSH itself. In these conditions, addition of up to 200 mole equivalents of GSH was therefore not able to induce the reductive demetallation of B12.



**Figure 3.** Titration of TDMQ22 (40  $\mu$ M) by  $\text{CoCl}_2$  (0-7 mole equiv) in Hepes buffer 50 mM, pH 7.4. Arrows indicate the absorbance changes upon addition of cobalt. Insert: Absorbance at 252 nm as a function of the  $\text{Co}^{2+}/\text{TDMQ22}$  mole ratio.



**Figure 4.** Titration of a mixture B12 + TDMQ22 (1/1 mole ratio, 10  $\mu$ M) by glutathione (GSH, up to 200 mole equiv) in Hepes buffer 50 mM, pH 7.4. Insert: Vertical expansion of the range 250-450 nm.

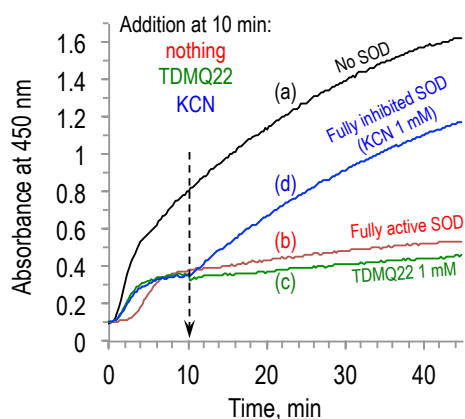
Similarly, absorbance of B12 at 362 nm remained unchanged upon titration by glutathione in the presence of 1 mole equivalent of TDMQ20, indicating that this chelator was unable to demetallate B12 (Figure S3).

In fact, the affinity of Co(III) and Co(II) for porphyrins and, even more, for corrins is very high<sup>[12]</sup> (demetallated only in  $\text{H}_2\text{SO}_4$  100%, 2 h, 25  $^{\circ}\text{C}$  <sup>[11]</sup>). So, this enzyme cofactor is not expected to be demetallated under physiological conditions, even in the presence of chelators such as TDMQ20 or -22.

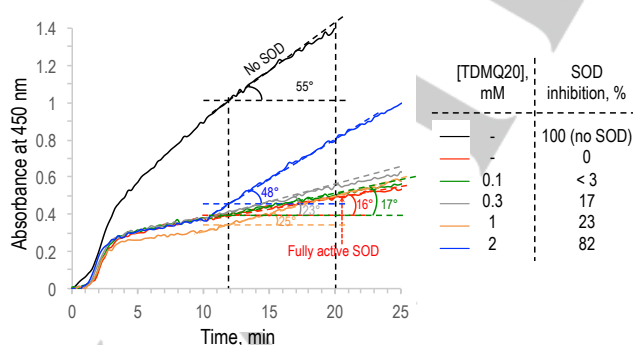
**TDMQ20 or TDMQ22 + Cu,Zn-SOD.** The copper ion of Cu,Zn-SOD being required for activity, it was worth checking that the SOD should not loose its activity in the presence of high affinity

TDMQ copper chelators. In fact, the constant for dissociation of copper from human Cu,Zn-superoxide dismutase was reported to be  $6.0 \times 10^{-18}$  ( $\log K_{\text{aff}} = 17.2$ ) at pH 7.4,<sup>[13]</sup> not far from the  $\log K_{\text{aff}}$  values of TDMQ20 and -22 for Cu(II) (16.5 and 15.1, respectively)<sup>[2]</sup>.

The principle of Cu,Zn-SOD activity measurement is detailed in Figure S4. Superoxide anion, generated by the xanthine/xanthine oxidase system (X/XO), reduces a specific tetrazolium salt, and the reduced formazan dye is detected at 450 nm (Figure 5, black trace). This reaction is fully inhibited by one unit of bovine Cu,Zn-SOD that scavenges  $\text{O}_2^{\cdot-}$  (Figure 5, red trace). To assess the absence of modification of SOD activity by TDMQ22, this chelator was added after 10 min (final concentration = 1 mM), in the mixture containing X/XO + tetrazolium salt + Cu,Zn-SOD. The production of the formazan dye remained fully inhibited (Figure 5, green trace), thus providing evidence that Cu,Zn-SOD remained fully active in the presence of 1 mM of TDMQ22.



**Figure 5.** Evaluation of the Cu,Zn-SOD activity in the presence of TDMQ22. Absorption at 450 nm of a reduced formazan dye generated by  $\text{O}_2^{\cdot-}$  produced by xanthine/xanthine oxidase (X/XO): (a, black trace) in the absence of Cu,Zn-SOD; (b, red trace) in the presence of fully active Cu,Zn-SOD: no  $\text{O}_2^{\cdot-}$  mediated formazan production; (c, green trace) in the presence of Cu,Zn-SOD, and TDMQ22 added at 10 min: no formazan production; (d, blue trace) in the presence of Cu,Zn-SOD, and KCN 1 mM, added at 10 min: formazan production due to Cu,Zn-SOD inhibition by KCN (given for comparison).<sup>[14]</sup> See Experimental Section, Cu,Zn-activity, conditions (a).



**Figure 6.** Evaluation of Cu,Zn-SOD activity in the presence of TDMQ20. Absorption at 450 nm of the formazan dye generated by  $\text{O}_2^{\cdot-}$  produced by xanthine/xanthine oxidase in the absence of Cu,Zn-SOD (black trace); inhibition of  $\text{O}_2^{\cdot-}$  mediated dye production in the presence of fully active Cu,Zn-SOD (red trace); Cu,Zn-SOD activity when TDMQ20 was added at 10 min

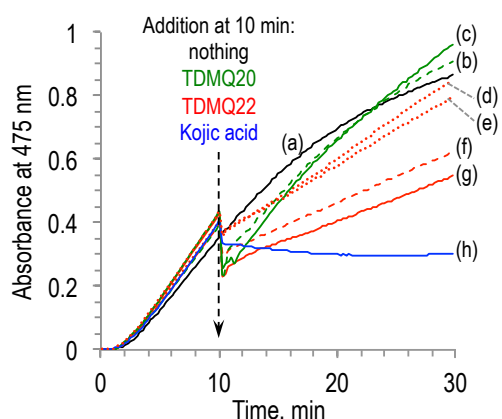
{[TDMQ20] = 0.1 mM (green), 0.3 mM (grey), 1 mM (orange), 2 mM (blue)}. Inhibition of Cu,Zn-SOD by TDMQ20 is calculated from the slope of the curve  $\text{Abs}_{450\text{nm}} = f(t)$  between 12 and 20 min. See Experimental Section, Cu,Zn-activity, conditions (a).

Similarly, no significant inhibition of Cu,Zn-SOD was detected in the presence of TDMQ20 at concentrations below or equal to 0.1 mM. Results depicted in Figure 6 indicated that, at 0.3, and 1 mM, SOD was moderately inhibited, at 17%, and 23%, respectively. A very high concentration of 2 mM, far above the pharmacological uses, was required for a nearly full inhibition of SOD (Figure 6, blue trace, 82% inhibition), giving rise to an  $\text{IC}_{50}$  value of 1.5 mM.

In order to check the activity of Cu,Zn-SOD in the presence of TDMQ chelators with longer incubation time, TDMQ20 or TDMQ22 was incubated with Cu,Zn-SOD during 1 h at 37 °C prior to the addition of xanthine that triggered the superoxide production. The activity of Cu,Zn-SOD was then monitored by formation of formazan during 45 min. Results are reported in Figure S5. In these conditions, TDMQ22 up to the huge concentration of 8.3 mM did not significantly inhibited the SOD activity (Figure S5b). Incubation of TDMQ20 at 4.2 mM resulted in the loss of ca. 15% of the SOD activity (Figure S5a, blue trace), while the activity of Cu,Zn-SOD was fully retained after being incubated with TDMQ20 at 0.83 mM. This concentration is still much higher than the chelator concentration that is expected to occur *in vivo*. In fact, the plasma and brain concentrations of clioquinol and PBT2 reported in wild type or transgenic AD mice were lower by three orders of magnitude (in the 0.3–4  $\mu\text{M}$  range, 2 hours after a 30 mg/kg single oral treatment).<sup>[15]</sup> Consequently, both TDMQ20 and TDMQ22 should probably be unable to interfere with Cu,Zn-SOD activity *in vivo*.

**TDMQ20 or TDMQ22 + Tyrosinase.** Impairment of catecholamine metabolism has been proposed to be involved in Alzheimer's disease pathology.<sup>[16]</sup> So, the lack of interference between tyrosinase, a key enzyme of L-tyrosine oxidation, and a putative drug, is requested for the development of these TDMQ ligands.

For this purpose, the tyrosinase catalyzed oxidation of L-tyrosine to L-dopachrome<sup>[17]</sup> (Figure S6) was monitored by UV-visible ( $\lambda_{\text{max}}$  of L-dopachrome = 475 nm) in the absence and in the presence of TDMQ20 and TDMQ22. Results are depicted in Figure 7. After 10 min of tyrosine oxidation, TDMQ20 [1 or 2 mM, Figure 6, green traces (b) and (c), respectively] was added to the reaction mixture. This addition did not significantly modify the absorbance at 475 nm, compared to a reaction mixture devoid of copper chelator [black trace (a)]. The same result was obtained after the addition of TDMQ22 at concentrations below or equal to 0.5 mM [red dotted traces (d) and (e), respectively]. Comparison with addition of kojic acid, a reference inhibitor of tyrosinase [blue trace, (h)],<sup>[18]</sup> indicated that partial inhibition of tyrosinase mediated oxidation occurred only for concentrations of TDMQ22 as high as 1 or 2 mM [Figure 7, red dashed and full traces (f) and (g), respectively]. This result evidence that inhibition of tyrosinase by TDMQ20 or TDMQ22 is unlikely to occur *in vivo*.



**Figure 7.** Oxidation of L-tyrosine to L-dopachrome (475 nm) (phosphate buffer, pH 7.4) by tyrosinase, in the presence of TDMQ20 [green traces, (b) 1 mM, (c) 2 mM], or in the presence of TDMQ22 [red traces, (d) 0.3 mM, (e) 0.5 mM, (f) 1 mM, (g) 2 mM], added at 10 min. Oxidation of L-tyrosine by tyrosinase without any additive [black trace (a) = full activity of tyrosinase], or in the presence of kojic acid 0.3 mM [blue trace (h), full inhibition of tyrosinase] is given for comparison. See Experimental Section, Tyrosinase activity, conditions (a).

The tyrosinase activity was also monitored after incubation of TDMQ20 or TDMQ22 during 1 h at 37 °C. Under these conditions, the full activity of tyrosinase was retained in the presence of 25  $\mu\text{M}$  TDMQ chelators, while concentrations of TDMQs of 62  $\mu\text{M}$  resulted in an enzyme inhibition below 10–20%. At 125  $\mu\text{M}$  of TDMQ20 or TDMQ22, the inhibition of tyrosinase activity was we observed 35–39% (Figure S7a and S7b, for TDMQ20 and TDMQ22, respectively). These concentrations are 10 to 100 times higher than the concentration measured *in vivo* for the copper chelators clioquinol and PBT2.<sup>[15]</sup>

## Conclusions

TDMQ20 and TDMQ22, two strong tetradentate copper chelators designed as drug-candidates for Alzheimer's disease, are unable to modify metalloenzymes or metal-containing cofactors. Despite the fact that there are able to chelate cobalt(II) [but not Co(III)], they have been found unable to demetallate vitamin B12, even in the presence of millimolar concentrations of glutathione. On the other hand, the  $\text{IC}_{50}$  values of TDMQ chelators for mushroom tyrosinase were higher or equal to 2 mM for TDMQ20 and TDMQ22, respectively. The  $\text{IC}_{50}$  values of TDMQ20 and TDMQ22 for bovine Cu,Zn-SOD were 1.5 mM and higher than 1 mM, respectively. So, these chelators did not prevent the biological activity of three essential metalloenzymes or cofactor involved in brain biochemistry or regulation of oxygen metabolism. These “negative results” are a very positive issue supporting the development of TDMQ chelators as anti-AD drug-candidates.

## Experimental Section

**Chemicals and methods.** All solvents and commercially available reagents were purchased from usual chemical suppliers, and were used without further purification. Tyrosinase from mushroom (EC number 1.14.18.1, Sigma T3824-25KU, 2680 units/mg solid) and recombinant bovine Cu,Zn-SOD expressed in *E. coli* (EC number 1.15.1.1, Sigma S9697-15KU, 6100 units/mg protein) were used. The Cu,Zn-superoxide dismutase (SOD) colorimetric activity kit was from ThermoFisher scientific / Invitrogen (Ref. EIASODC) or from Sigma (Ref. 19160-1KT-F). TDMQ20 and TDMQ22 were prepared according to Reference 2a. UV-visible spectra were recorded from 200 to 600 nm, at room temperature, on Agilent 8453 or Evolution 300 (Thermo Fisher Scientific) spectrophotometers equipped with magnetic stirring. The stock solutions were as prepared as follows: TDMQ20 or TDMQ22 (45 mM, 20 mM, 10 mM, 5 mM, 3 mM or 0.5 mM in  $\text{H}_2\text{O}$ ), Hepes buffer 100 mM, pH 7.4, phosphate buffer 100 mM, pH 7.4, vitamin B12 (0.5 mM),  $\text{Co}^{\text{II}}\text{Cl}_2$  (3 mM or 30 mM in  $\text{H}_2\text{O}$ ),  $\text{Co}^{\text{III}}(\text{NH}_3)_6\text{Cl}_3$  (300 mM in  $\text{H}_2\text{O}$ ), L-tyrosine (2.5 mM in  $\text{H}_2\text{O}$ ), mushroom tyrosinase (2000 units/mL in  $\text{H}_2\text{O}$ ), kojic acid (45 mM in  $\text{H}_2\text{O}$ ), bovine Cu,Zn-SOD (100 units/mL in the assay buffer of the Cu,Zn-SOD kit, KCN (300 mM in  $\text{H}_2\text{O}$ ).

**Interaction between vitamin B12 and TDMQ chelators.** In a UV-vis cuvette containing Hepes buffer 100 mM, pH 7.4 (750  $\mu\text{L}$ ) under magnetic stirring, were added  $\text{H}_2\text{O}$  (750  $\mu\text{L}$ ) and vitamin B12 (0.5 mM, 60  $\mu\text{L}$ ). The TDMQ20 or -22 solution was then added (5 mM, 6  $\mu\text{L}$ , 1 mol equiv with respect to B12), and the mixture was allowed to stand at room temperature for 30 min. The spectrum was then recorded. Final concentrations were:  $[\text{B12}] = [\text{TDMQ chelator}] = 20 \mu\text{M}$ . Control experiments were carried out using B12 alone, or the TDMQ chelator alone.

**Titration of TDMQ chelators by Co(III).** In a UV-vis cuvette containing Hepes buffer 100 mM, pH 7.4 (750  $\mu\text{L}$ ) under magnetic stirring, were added  $\text{H}_2\text{O}$  (630  $\mu\text{L}$ ) and TDMQ20 or -22 (0.5 mM, 120  $\mu\text{L}$ ). This mixture was titrated by addition of  $\text{Co}^{\text{III}}(\text{NH}_3)_6\text{Cl}_3$  aliquots, and the spectrum was recorded from 200 to 600 nm, after addition of each aliquot. Final concentrations were:  $[\text{TDMQ}] = 40 \mu\text{M}$ ,  $[\text{Co}^{\text{III}}] = 0.8 \text{ mM} = 1\text{--}200 \text{ mol equiv}$ ;  $\Delta v < 2.7 \text{ vol\%}$  in the cuvette.

**Titration of TDMQ chelators by Co(II).** In a UV-vis cuvette containing Hepes buffer 100 mM, pH 7.4 (750  $\mu\text{L}$ ) under magnetic stirring, were added  $\text{H}_2\text{O}$  (630  $\mu\text{L}$ ) and TDMQ20 or -22 (0.5 mM, 120  $\mu\text{L}$ ). This mixture was titrated by addition of  $\text{CoCl}_2$  aliquots, and the spectrum was recorded from 200 to 600 nm, after addition of each aliquot. Final concentrations were:  $[\text{TDMQ}] = 40 \mu\text{M}$ ,  $[\text{Co}^{\text{II}}] = 0\text{--}200 \mu\text{M} = 1\text{--}5 \text{ mol equiv}$ ;  $\Delta v < 2.5 \text{ vol\%}$  in the cuvette.

**Assay of demetallation of vitamin B12 by TDMQ chelators in the presence of glutathione.** In a UV-vis cuvette containing Hepes buffer 100 mM, pH 7.4 (750  $\mu\text{L}$ ) under magnetic stirring, were added  $\text{H}_2\text{O}$  (690  $\mu\text{L}$ ), vitamin B12 (0.5 mM, 30  $\mu\text{L}$ ), and TDMQ20 or -22 (0.5 mM, 30  $\mu\text{L}$ ). This mixture was titrated by addition of 2  $\mu\text{L}$  aliquots of the 150 mM GSH solution (each aliquot = 20 mol equiv of GSH with respect to B12). The spectrum was recorded from 200 to 600 nm, after addition of each aliquot. Final concentrations were:  $[\text{B12}] = [\text{TDMQ}] = 10 \mu\text{M}$ ,  $[\text{GSH}] = 0\text{--}2 \text{ mM} = 0\text{--}200 \text{ mol equiv}$ ;  $\Delta v < 1.3 \text{ vol\%}$  in the cuvette.

**Cu,Zn-SOD activity.** Conditions (a). This activity was measured using the ThermoFisher scientific / Invitrogen kit, with a modified protocol as follows. Xanthine oxidase 1X and substrate 1X (= chromogenic tetrazolium) were prepared according to the instructions of the supplier. A solution of bovine Cu,Zn-SOD (Sigma S9697-15KU) at 100 units/mL was prepared in the Assay buffer of the kit. The reaction mixture was prepared in a UV-visible cuvette under magnetic stirring, and contained substrate 1X (750  $\mu\text{L}$ ), assay buffer (260  $\mu\text{L}$ ), Cu,Zn-SOD 100 units/mL (15  $\mu\text{L}$ ),  $\text{H}_2\text{O}$  (90–100  $\mu\text{L}$ ). Xanthine oxidase 1X (375  $\mu\text{L}$ ) was then added, to start the reaction. After 10 min, TDMQ ligand or KCN solution (5 or 10  $\mu\text{L}$ ) was added. The reaction was monitored at 450 nm for 45 minutes. Final volume was 1.5 mL. Variation of volume upon addition of TDMQ or KCN was  $\leq 5 \text{ vol\%}$ .

SOD activity. Condition (b) using Sigma-Aldrich Cu,Zn-SOD assay kit. Reagent working solution (WST-1 tetrazolium salt) and enzyme working solution (xanthine oxidase, XO) were prepared according to the supplier instructions. The Cu,Zn-SOD stock solution was prepared at 100 units/mL by dilution of Cu,Zn-SOD (Sigma S9697) in the dilution buffer of the kit. In an aliquot of this Cu,Zn-SOD solution (15  $\mu$ L), were added in the following order: (i) TDMQ ligand (suitable volume of 5 mM, 15 mM or 50 mM stock solutions), (ii) dilution buffer of the kit (65  $\mu$ L), and (iii) water to adjust the volume to 180  $\mu$ L. The mixture was incubated under stirring for 1 h at 37 °C. The concentration of TDMQ during incubation was 0.83 mM to 8.3 mM. After incubation, reagent working solution (1200  $\mu$ L) and enzyme working solution (XO, 120  $\mu$ L) were added. The absorbance of formazan was immediately monitored at 450 nm for 45 minutes, under magnetic stirring (800 rpm). Final volume in the cuvette was 1.5 mL. Final concentrations in the cuvette were [Cu,Zn-SOD] = 1 unit/mL and [TDMQ ligand] = 100  $\mu$ M, 500  $\mu$ M or 1mM. Control experiments without TDMQ ligand ("fully active SOD" curve) and without Cu,Zn-SOD ("no SOD" curve) were also performed in the same conditions.

Tyrosinase activity. Conditions (a). Under continuous stirring, were added in the UV-visible cuvette containing phosphate buffer 100 mM, pH = 7.4 (750  $\mu$ L), H<sub>2</sub>O (427.5  $\mu$ L), and tyrosinase (22.5  $\mu$ L, 2000 units/mL). The reaction started upon addition of L-tyrosine (300  $\mu$ L), and the reaction was monitored at 475 nm during 10 minutes. Then, a suitable volume of the 45 mM TDMQ20 or TDMQ22 solution was added to achieve a final concentration of 0-2 mM ( $\Delta v < 5$  vol% in the cuvette). The absorbance at 475 nm was monitored for 20 min further. Final volume was 1.5 mL. The final concentrations were [phosphate buffer] = 50 mM, [L-tyrosine] = 0.5 mM, [tyrosinase] = 30 units/mL. For control, inhibition of tyrosinase was carried out by addition of kojic acid (10  $\mu$ L, final concentration = 0.3 mM) instead of the TDMQ chelator.

Tyrosinase activity. Conditions (b). In a UV-visible cuvette, were added (i) tyrosinase (2000 units/mL, 22.5  $\mu$ L), (ii) variable concentrations of TDMQ ligand (suitable volume of 5 mM or 15 mM of stock solutions), (iii) phosphate buffer pH 7.4 (100 mM, 750  $\mu$ L), and (iv) water to adjust the volume at 1200  $\mu$ L. The mixture was incubated under stirring for 1 h at 37 °C. During incubation, the concentration of TDMQ was 0-125  $\mu$ M. After incubation, L-tyrosine (2.5mM, 300  $\mu$ L) was added and the absorbance was immediately monitored at 475 nm for 30 minutes. Final volume in the cuvette was 1.5 mL. Final concentrations in the cuvette were [L-tyrosine] = 0.5 mM, [tyrosinase] = 30 units/mL, [TDMQ ligand] = 10  $\mu$ M, 20  $\mu$ M, 50  $\mu$ M or 100  $\mu$ M.

## Acknowledgements ((optional))

This work was supported by the NSFC (grant 21502023), the Guangdong Province (Program for Innovative Research Teams and Leading Talents Introduction, grant 2050205), GDUT (grant 220418037), and the CNRS.

**Keywords:** Alzheimer's disease • Copper chelator • Cu,Zn-superoxide dismutase • Cyanocobalamin • Tyrosinase

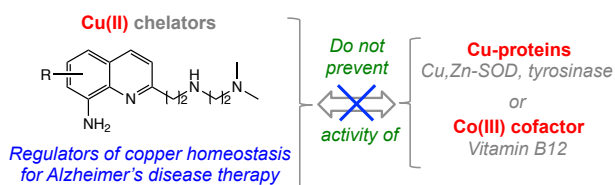
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## Entry for the Table of Contents (Please choose one layout)

Layout 2:

## COMMUNICATION

**Copper homeostasis in Alzheimer's disease\***

Ju Huang, Michel Nguyen, Yan Liu,\*  
Anne Robert,\* Bernard Meunier

Page No. – Page No.

**The TDMQ Regulators of Copper Homeostasis Do Not Disturb Cu,Zn-SOD and Tyrosinase Activity, nor the Co(III) Cofactor Vitamin B12.**

The Cu(II) chelators TDMQ20 and TDMQ22 have been designed as regulators of copper homeostasis to inhibit oxidative stress in Alzheimer's disease. These chelators did not interfere with the activity of copper enzymes such as Cu,Zn-superoxide dismutase and tyrosinase, and cobalt cofactor vitamin B12. These data are essential to consider a safe development of these drug-candidates.

\*one or two words that highlight the emphasis of the paper or the field of the study