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1,1′-Xylyl bis-1,4,8,11-tetraaza cyclotetradecane: A new potential copper chelator agent for neuroprotection in Alzheimer's disease. Its comparative effects with clioquinol on rat brain copper distribution

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Abstract—Dysfunction of copper metabolism leading to its excess or deficiency results in severe ailments. Recently, neurodegenerative disorders such as Alzheimer's disease have been associated with copper metabolism. Compounds having the ability to reduce copper levels in brain or to affect its distribution could have neuroprotective effects, mainly through a downregulation of the transcription of amyloid peptide precursor (APP). We report here the biological effect of compound 1,1′-xylyl bis-1,4,8,11-tetraaza cyclotetradecane, which specifically affects copper concentration in the brain cortex region. Its copper homeostatic activity is compared with that of clioquinol, a well-known drug, which has been recently reported as an active Aβ-peptide clearance drug in vivo for Alzheimer's patients.

A central, unresolved question in the pathophysiology of Alzheimer's disease (AD) relates to the role of metal ions in plaque formation and neurodegeneration. AD plaques containing fibrils composed of the 39–42 residue amyloid-β (Aβ) peptides are thought to be linked to neurodegeneration in AD.[1,2] Metal ions have been proposed to play a significant role in the assembly and neurotoxicity of AD fibrils.[3] Administration of a metal ion chelator decreases deposition of Aβ in the brains of transgenic mice[4] and releases soluble Aβ from preformed amyloid deposits,[5] supporting the hypothesis that metal ions are incorporated in plaque architecture in vivo. Selective chelating therapy to combat AD is a promising strategy. It has been observed through Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog) that patients with low Cu levels had significant by higher ADAS-cog values than patients with medium Cu levels, who exhibited lower ADAS-cog scores. This finding supports the hypothesis of a mild Cu deficiency in most AD patients.[6]

An hydrophobic moderate metal chelator (5-chloro-7-iodo-8-hydroxy-quinoline, known as clioquinol), termed as a metal–protein-attenuating compound (MPCA), has exhibited a promising effect in a phase II clinical trial of moderately severe AD patients.[7] More recently, it has been shown that in mammals clioquinol–copper complexes can form in the instestinal tract and cross the blood–brain barrier (BBB) to enter the brain, and this explains why soluble copper and zinc levels were increased by 15% in mice brain upon clioquinol treatment. Therefore, clioquinol–copper complexes could selectively and markedly elevate copper levels in the brain of individuals with AD and counterbalance the changes in copper levels observed in AD; most probably mediated through the amyloid peptide precursor (APP) export function.[8] It should be underlined that clioquinol was removed from the US market in 1971 because of a link with subacute myelo-optic neuropathy (SMON), an

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uncommon neurological syndrome largely confined to Japan.[9] These issues may be important in understanding the integration of copper homeostasis in AD and with other physiological processes such as aging.

Because of their interesting biological and physicochemical properties, saturated macrocyclic polyamines such as 14-membered tetramine derivatives have attracted the attention of biologists, mainly through the discovery and development of bicyclam, a chemokine receptor antagonist, which has highlighted the therapeutic potential of this compound in HIV infection,[10] inflammatory diseases, cancer and stem-cell mobilization.[11] In the present work, our aim was to study the influence of such bicyclam analogues **1** and **2** (named as JLK 169 and JLK 1291) on the distribution of copper in rat brains in comparison with other metal chelating agents such as **3** (Clioquinol) or **4** (D-Penicillamine), and to discuss the role of bicyclam analogues in the copper homeostasis.

Bicyclam **1** was synthesized according to known procedure.[12] Its Cu^{2+} complex **2** was synthesized as described under Ref. 13. Compounds **3** and **4** were commercially available. The structures of analogues **1**–**4** are presented in Figure 1.

All animal experiments are described under Ref. 14. Copper ion determination was carried out using atomic absorption as described under Ref. 17.

Compounds **1**–**4** were injected in rats according to the above animal experiment protocol. The obtained results are summarized in Table 1.

As is observed, **4** did not affect copper concentrations in blood or CSF or indeed in the studied brain regions. It is known that **4**–Cu(I) complexes have very poor aqueous solubility in the pH range of 1.9–7.6 due to the formation of neutral complexes.[18] Moreover, **4** which preferentially binds Cu(I) is less likely to extract Cu(II) involved in non redox structural capacity in proteins.[19] In contrast, **1** mainly affects copper concentration in the brain cortex, compared to its corresponding copper complex **2** or to **3**, while copper concentrations in blood, CSF or corpus callossum are similar to those found in untreated rats.

Figure 2 shows the comparative effects of **1** and **3** on copper ion distribution in various brain regions and blood and CSF with respect to the control.

It can be observed that 1 h after injection the effects of **1** and **3** are quite similar since both significantly decreased by about 70% copper concentration in the CSF with respect to the control and only very slightly in blood. They do not affect copper concentration in the corpus callosum. (It should be remembered that copper concentration in CSF does not necessarily correlate with copper concentration in brain department). In contrast, the effect of **1** compared to that of **3** on the observed increase of copper concentration in cortex is significantly different since **3** has a very low effect on copper cortex concentration, while in contrast **1** increased copper concentration with respect to the control by about 90%. It should be underlined that our results concerning **3** are different from those of other reports[20] which indicated a 15% overall increase of copper in whole mice brain upon **3** injection.

When copper analysis was performed on tissue samples or blood and CSF of animals decapitated 2 h, instead of 1 h, after drug treatment (**1** and **3**), the effects on copper distribution in the cortex or in CSF observed after 1 h were almost abolished, with respect to the control.

Moreover, copper–bicyclam complex **2** injected for 1 h has almost no significant effect on copper concentration on the whole brain samples as well as in CSF with respect to the control.

Several parameters need to be taken into account to explain the observed differences in the effects of these two drugs on copper distribution in the brain.

- First, the binding affinities for copper between **1** and **3** are quite different. Their log K_s values are, respectively, 27 and 10[21]:

  (K_s constant being defined as follows:

  $K_s = [\text{complex}]/[\text{ligand}] \cdot [Cu^{2+}]$)

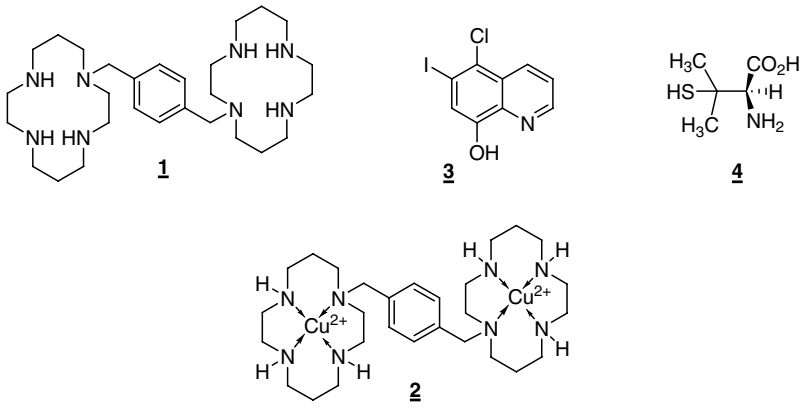
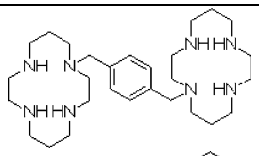
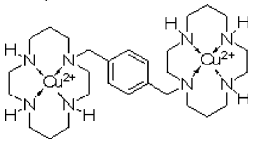
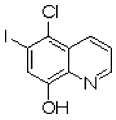
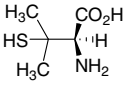


Figure 1. Molecular structures of the different metal chelators.

Table 1. Effects of various metal chelators on the concentrations of copper in blood, CSF and various brain regions, after 1 h of drug injection in rats

Compound	Structure	Blood (μg/100 ml)	CSF (μg/l)	Cortex[a] (μg/g)	Corpus callosum[a] (μg/g)
1		142 ± 10	6.1 ± 2	30.6 ± 8	10.2 ± 2
2		139 ± 15	13.9 ± 4	15.5 ± 4	9.1 ± 2
3		126 ± 12	7.7 ± 3	11.6 ± 3	10.5 ± 2
4		135 ± 15	n.d.	15.4 ± 5	10.1 ± 1
P.P.I[b]	Vehicle	127 ± 7	8.3 ± 2	10.0 ± 2	11.0 ± 1

n.d., not determined.
[a] Cu concentrations were evaluated as μg per g of dry brain tissue.
[b] Injection vehicle (0.5% DMSO in water). The obtained Cu concentrations represent the average concentrations of copper found in untreated rats.

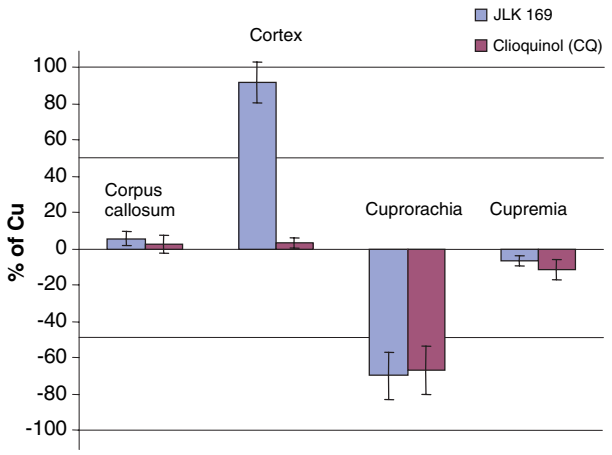


Figure 2. Comparative effects of **1** and **3** on the concentration of copper in various regions of rat brain as well as in blood and cerebro spinal fluid 1 h after injection.

The clioquinol–copper complex required a two to one stoechiometry to form a tetradentate ligand complex, while the tetradendate–copper complex **2** is a one-to-one stoechiometry complex.

Moreover, binding energy (BE) for Cu^{2+}–clioquinol complex was rather high (685.26 kcal/mol) and comparable with that of Cu^{2+}–Aβ-complex (752.26 kcal/mol).[22] The calculated BEs for polyazamacrocycles are found to be higher than that of clioquinol[23] and quite comparable to that of Cu^{2+}–Aβ-complex.

- Second, the drugs do not have the same permeation properties that are the capacity to cross membranes and, this is important in particular for the blood–brain barrier. The calculated log *P* values (octanol/water calculated from ACD software) for **1** (HCl free) and **3** were, respectively, 0.2 ± 0.7 and 4.75 ± 0.83. Moreover, a good correlation has been reported[24] between lipophilicity values and the permeability of metal chelators. This is particularly true for chelators belonging to the clioquinol family. This observation that lipophilicity is determinative for BBB permeation is supported here, by the fact that the bicyclam–copper complex **2** which had no effect on copper distribution in brain is a very polar complex, whose log *P* value cannot be calculated with ACD log *P* software.
- Third, the observed effects on copper distribution in brain are closely related to the duration of the time between drug injection and animal sacrifice. Optimum responses were observed for copper tissues analysis 1 h after drug injection.

Why could bicylam compound **1** be of interest in selective chelator therapy for diseases related to copper homeostasis?

This compound has already been entered in clinical phases for other applications: HIV infection, inflammatory disease, cancer, and stem-cell mobilization.[11] It could be considered as a safe drug with only weak toxicity. In contrast, clioquinol, whose activity on Aβ-peptide clearance has already been investigated in vivo on AD patients, displays some severe side effects.

From the above results, it appears that injection of **1** favours accumulation of copper mainly in the cortex region. It should be recalled that Cu concentrations are lower in the hippocampus and the globus pallidus than in other brain regions; in particular in the cortex where Aβ-peptide is found to accumulate.[25] In contrast

comparatively clioquinol has in comparison only a weak effect on copper accumulation.

A crucial point remains unclear: what could be the effect of copper accumulation in the cortex on the clearance of Aβ-peptide? Indeed, it has been recently reported by Treiber et al.,[8] that using a yeast model, clioquinol drastically increased the intracellular copper concentration. Through these experiments the researchers suggest that clioquinol can act therapeutically by changing the distribution of copper or facilitating copper uptake rather by decreasing copper levels.

Our experiments do not allow us to answer the question of the role of compound **1** on copper uptake, but these preliminary results encourage us to study the possible activity of compound **1** on the Aβ-peptide clearance using transgenic animal models. To our knowledge, this is the first time that bicyclam **1** has been identified as a possible candidate for Aβ-peptide clearance AD therapy.

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13. Dissolving **1** in water and passing through an Amberlite anion-exchange column in order to obtain the free biscyclam **1**. Then, $CuCl_2.6H_2O$ was added in hot EtOH to free compound **1**, the copper complex was precipitated by adding ether to the solution. After filtration, the residue was dissolved in H_2O and CH_3COONa was added to the aqueous solutions to afford the corresponding acetate salt **2**.
14. Animal experiments were carried out in compliance with the Guidelines for the Care and Use of Laboratory animals ECC directive 86/609 and APA ethical principles for animal experimentations. Five normal Wistar female rats (average weight 250 g) were used for each compound. Each rat received a 500 μl intravenous injection in the tail vein of a compound solution (dissolved in 1% DMSO/PPI) corresponding to a dose of 5 mg/kg. One hour after injection, animals were anesthetized with a mixture of ketamine and xylazine at 80 and 10 mg/kg, respectively, and submitted to fluid collection. Cerebro spinal fluid (CSF) was collected from the cisterna magna using a 26-gauge needle attached to a syringe according to the technique previously described by Lebedev et al.[15] CSF was carefully aspirated avoiding blood contamination and stored in Eppendorf tubes at −80 °C. Blood samples were also collected from the left ventricles and centrifuged at 4 °C, 13.000*g* during 15 min to separate plasma from whole blood. Plasma and CSF were submitted to cupremia analysis. Then, animals were sacrificed by decapitation and dissected for excision of the brain. The cortex and corpus callosum regions of the brain were identified and separated according to the stereotaxic atlas of the brain reported by Paxinos and Franklin [16] Both tissues were analyzed for copper ion determination.
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