Pediatric RESEARCH

BASIC SCIENCE ARTICLE

Menkes disease: Oral administration of glyoxal-bis(*N*(4)-methylthiosemicarbazonato)-copper(II) rescues the macular mouse

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BACKGROUND: Menkes disease is a copper metabolism disorder caused by mutations in ATP7A, a copper-transporting P-type ATPase. In this study, oral copper supplementation via glyoxal-bis(*N*(4)-methylthiosemicarbazonato)-copper(II) (CuGTSM), a lipophilic copper complex, was investigated in male hemizygous macular ($Mo^{Ml/y}$) mice, a mouse model of Menkes disease.
METHODS: CuGTSM was administered by oral gavage on postnatal days 5, 8, 11, 17, 23, and 32. The copper levels in the organs and serum, copper-dependent enzyme activities in the brain, and ceruloplasmin (Cp) activity in the serum were measured at 15 days and 3 and 8 months of age. Histological analysis of the intestines and the rotarod test were also performed.
RESULTS: CuGTSM treatment extended the lifespan of $Mo^{Ml/y}$ mice and partly restored the copper concentrations and cytochrome oxidase and DBH activities in the brain; however, the rotarod test showed impaired motor performance. The treatment also increased copper concentrations and Cp activity in the serum. In suckling $Mo^{Ml/y}$ mice, CuGTSM treatment transiently induced diarrhea accompanied by copper accumulation and altered villus morphology in the ileum.
CONCLUSION: Oral administration of CuGTSM extended the lifespan of $Mo^{Ml/y}$ mice. Oral administration is attractive, but pharmaceutical studies are needed to reduce the adverse enteral effects.

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INTRODUCTION

Menkes disease (MIM 309400) is an X-linked disorder caused by mutations in ATP7A, a copper-transporting P-type ATPase.[1] ATP7A is expressed in most cells to transfer copper to copper-dependent enzymes in the trans-Golgi network and to efflux excess copper from cells.[2] ATP7A dysfunction causes severe problems in multiple aspects of copper transport, including intestinal absorption, renal excretion, and the transportation of copper into the brain across both the blood-brain and blood-cerebrospinal fluid barriers.[3] Therefore, Menkes disease results in reduced copper-dependent enzyme activity in multiple organs, including the brain.

Parenteral administration of copper-histidine ameliorates the natural manifestation of Menkes disease when started in early infancy.[4] However, the neurological results remain suboptimal despite several reported favorable outcomes.[1] To facilitate copper distribution to the brain, copper chelators such as diethyldithiocarbamate (DEDTC) and dimethyldithiocarbamate (DMDTC) have been proposed in combination with copper injections.[5–7] These compounds form lipophilic complexes with copper in the body and can penetrate the blood-brain barrier to increase copper levels and copper-dependent enzyme activity in the brain.

Copper bis(thiosemicarbazone) complexes (CuBTSCs) have been investigated as metallodrugs for medical applications such as radiopharmaceuticals, antitumor agents, antibiotics, and therapies for neurodegenerative diseases.[8–10] Pyruvaldehyde-bis(*N*(4)-methylthiosemicarbazonato)-copper(II) (CuPTSM), a neutral and lipophilic CuBTSC, was partially effective for a Menkes disease mouse model, although the utility of the compound was highly restricted by poor water solubility.[11] Recently, oral gavage of glyoxal-bis(*N*(4)-methylthiosemicarbazonato)-copper(II) (CuGTSM) was shown to increase copper levels in the brain and ameliorate brain dysfunction in Alzheimer's disease mouse models.[8] Therefore, oral gavage of CuGTSM may have therapeutic effects in Menkes disease without the burden of prolonged injections of copper.

In this study, we evaluated the effects of orally administered CuGTSM in macular mice. The macular mutation (S1382P) is located in the eighth transmembrane domain in ATP7A.[12] The mutation causes an aberrant distribution of the mutant ATP7A protein in the post-Golgi compartments and plasma membrane without phosphorylation, which reduces but does not eliminate copper-transporting activity.[13] Male hemizygous macular ($Mo^{Ml/y}$) mice have a perinatal lethal phenotype and provide a close model of Menkes disease.[14]

METHODS

Drug preparation

CuGTSM was synthesized as previously described.[15] All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO)

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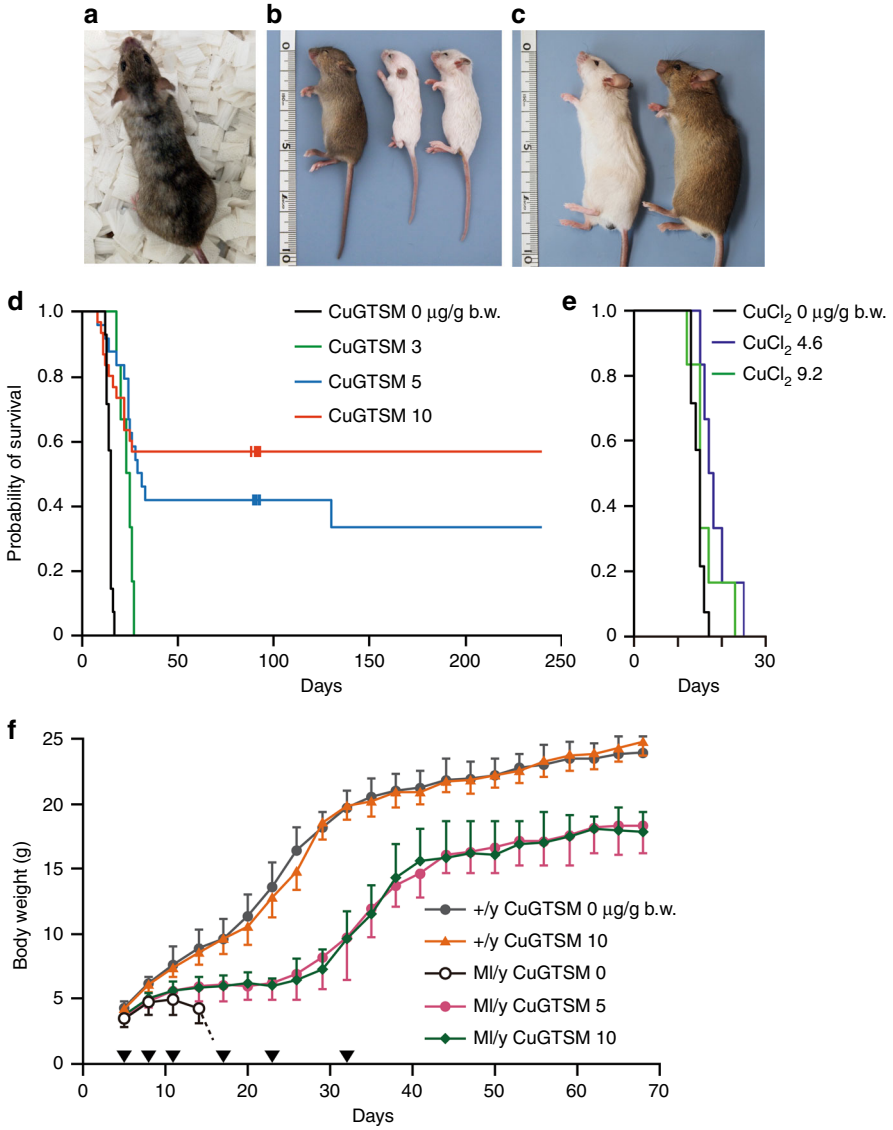

Fig. 1 Effects of CuGTSM treatment on the survival and growth of macular mutant mice, an animal model of Menkes disease. **a** Adult female heterozygote ($Mo^{Ml/x}$) with a 'macular coat'. **b** Wild-type male ($Mo^{+/y}$; left), male hemizygote ($Mo^{Ml/y}$) treated with vehicle (standard suspension vehicle; middle), and $Mo^{Ml/y}$ mice treated with CuGTSM (10 µg/g body weight [b.w.] per os; right) at 15 days old. The vehicle-treated $Mo^{Ml/y}$ mice had a white coat and were severely emaciated, whereas the coat color changed to pale gray, and emaciation was reduced in the CuGTSM-treated $Mo^{Ml/y}$ pups. **c** $Mo^{Ml/y}$ mouse treated with CuGTSM (10 µg/g b.w.; left) and wild-type male mouse (right) at 6 months old. The $Mo^{Ml/y}$ mice exhibited a white coat and were smaller than the wild-type mice. Mice were anesthetized using isoflurane in (**b**) and (**c**). Scale units in (**b**) and (**c**) are in cm. **d** Kaplan–Meier survival curves for $Mo^{Ml/y}$ mice treated with vehicle (standard suspension vehicle; $n = 14$) and treated with CuGTSM at a dose of 3, 5, or 10 µg/g b.w. ($n = 6$, 21, and 20, respectively). Small vertical bars on the survival curves indicate cessation of observation in a subset of mice that were used for experiments at 3 months old. **e** Kaplan–Meier survival curves for the $Mo^{Ml/y}$ mice treated with the vehicle (normal saline; $n = 14$) and treated with $CuCl_2$ at a dose of 4.6 or 9.2 µ/g b.w. ($n = 6$ in each group). **f** Body weight gain in the $Mo^{Ml/y}$ mice (designated as Ml/y) and wild-type littermates (+ /y) treated with vehicle or CuGTSM ($n = 6$ in each group). Triangular symbols indicate days when CuGTSM or vehicle was administered. Data are expressed as the mean ± SD

unless specified otherwise. The CuGTSM powder included cellulose fiber (<1% weight) derived from filter paper. For oral dosing, the CuGTSM powder was finely ground with a mortar and formulated in a standard suspension vehicle (SSV) containing 0.9% (w/v) NaCl, 0.5% (w/v) sodium carboxymethylcellulose, 0.5% (v/v) benzyl alcohol, and 0.4% (v/v) Tween 80 (Wako, Osaka, Japan). The suspension was then sonicated for 1 h and vortexed just before use. $CuCl_2$ $2H_2O$ (Wako) was dissolved in distilled water instead of SSV because SSV gelled with the free copper ions.

Animals and treatments

Male hemizygous macular ($Mo^{Ml/y}$) mice and wild-type littermates ($Mo^{+/y}$) were used for all experiments (Fig. 1b, c). CuGTSM was administered by oral gavage on postnatal days 5, 8, 11, 17, 23, and 32, which were determined from previous reports.[16] Mice that were sacrificed on day 15 for tissue sampling were administered the treatment on days 5, 8, and 11. The current doses for CuGTSM were determined from a previous report on a model mouse of Alzheimer's disease with an expanded dose range (3–10 µg/g body weight [b.w.]).[8] The pups were weaned at 4 weeks of age. A commercial stock food containing 0.95 mg copper/100 g food and 13.8 mg iron/100 g food (Charles River formula 1 [CRF-1], Oriental Yeast, Tokyo, Japan) and water were provided ad libitum. At the ages of 15 days, 3 months, or 8 months, mice were deeply anesthetized using isoflurane during collection of blood from the inferior vena cava and then

euthanized by cervical dislocation before tissue sampling. Blood hemoglobin concentrations (Hb) were measured using a veterinary hematology analyzer (Celltac α; Nihon Kohden, Tokyo, Japan). All procedures involving animals were approved by the animal care and use committee of the Teikyo University School of Medicine.

Catecholamine analysis

The posterior half of each cerebrum was homogenized in 10 volumes of homogenization buffer and centrifuged (4 °C, 800× *g*, 20 min). The homogenization buffer included 5.4 mM ethylenediamine-N,N,N′,N′-tetraacetic acid disodium salt, 0.4 M $HClO_4$, and 0.1 mM L-ascorbic acid in distilled water.[5] The levels of dopamine, noradrenaline, and adrenaline in the supernatant were measured using a catecholamine autoanalyzer (HLC-8030; TOSO Industry, Tokyo, Japan).

Tissue copper measurements

Tissue samples were digested by high-purity HNO_3 in polypropylene vessels (DigiTUBEs, SCP SCIENCE, Quebec, Canada) at 105 °C for 4 h with a DigiPREP apparatus (SCP SCIENCE), while serum samples were digested by high-purity HNO_3 in small vessels (tetrafluoroethylene [TFM] insert; Milestone, Sorisole, Italy) at 170 °C for 5 min with a microwave digestion system (ETHOS 1, Milestone). Then, copper concentrations in the digested samples were measured using inductively coupled plasma-mass spectrometry (ICP-MS; iCAP Qc, Thermo Fisher Scientific, Waltham, MA) in the kinetic energy discrimination (KED) mode with helium gas to minimize polyatomic interferences.

Cytochrome oxidase histochemistry

In cytochrome oxidase (CO) histochemistry, tissue CO catalyzes the oxidation of cytochrome *c*, which couples to the polymerization of 3,3′-diaminobenzidine, resulting in brown deposits. The deposit density strongly correlates with regional CO activity.[17,18] CO histochemistry was performed using previously described methods with modifications. Briefly, fresh frozen brains were coronally sectioned (20 µm thickness) using a cryostat microtome at −20 °C and mounted on silanized slides. The slides were then stored at −80 °C. Triplicate series of the sections from each brain were prepared to determine an average. All the sections were simultaneously incubated at 37 °C for 2 h in the dark in a reaction medium consisting of 120 mL of 0.1 M phosphate buffer (pH 7.4), 60 mg of 3,3′-diaminobenzidine, 18 mg of cytochrome *c*, and 4.8 g of sucrose (Wako). Sections were then dehydrated in ethanol, cleared in xylene, and covered.

The processed sections were synoptically reviewed with an entity microscope (SZ 61, Olympus, Tokyo, Japan; Fig. 3a). For quantitative analysis, color digital images of the processed sections and a grayscale step tablet (Photographic Step Tablet No. 2; Eastman Kodak, Rochester, NY) were obtained using a microscope (BX-61, Olympus, Tokyo, Japan) equipped with a digital camera (DP-70, Olympus) in the same optical setting. The blue color plane was extracted from the digital images for subsequent analyses.[19] Regions of interest (ROIs) were set at whole layers of the frontal lobe primary somatosensory (S1) cortex, with the help of a mouse brain atlas. After background subtraction, pixel values within the ROIs were averaged and transformed to optical density (OD) using a standard curve method with the help of the grayscale step tablet representing known OD values. These images and numerical data were processed with LabVIEW software incorporating the Vision Development Module (National Instruments, Austin, TX).

Ceruloplasmin (Cp) activity

Serum Cp activity was measured using a Cp colorimetric activity kit (Arbor Assays, Ann Arbor, MI) based upon the published method of Curzon and Vallet.[20]

Pathology of the intestines

The small intestine and colon were fixed in 10% neutral buffed formalin and embedded in paraffin. The tissues were then sectioned at 3 µm and stained with hematoxylin and eosin (H&E). Images of the stained sections were captured with a slide scanner (Nanozoomer-XR, Hamamatsu Photonics, Hamamatsu, Japan), and the villus length and width in ~50 villi of the ileum sections were measured and averaged in each animal.

Rotarod test

The accelerating rotarod test was performed with a rotarod treadmill (ENV-576M, Med Associates, St. Albans, VT) as previously described. For training, each mouse was placed on the rotating beam at a constant speed of 4 rpm for 120 s for three consecutive days. For testing, each mouse was placed on the beam, and the rotation speed was increased linearly from 4 to 40 rpm over 5 min. The rotation speeds at which the mice fell from the beam were recorded. The measurements were performed three times for each mouse at 30-min intervals. The rotating beam was covered with a rubber sheet to prevent mice from clinging.[21]

Statistical analysis

Numerical data are shown as the mean ± SD. Statistical analyses comparing three groups were performed using the Kruskal–Wallis test. For multiple comparisons among the groups, the Mann–Whitney test was used, in which raw *p* values were adjusted using the Holm method. Statistical analyses comparing two groups were performed using the Mann–Whitney test. Survival rates were estimated by the Kaplan–Meier method and were compared using the log rank test. When survival curves obviously crossed, the log rank test was not performed. For all tests, values of $P < 0.05$ were considered statistically significant. All statistical analyses were performed using R software (The R Foundation for Statistical Computing) with Easy R, a graphical interface for R.[22]

RESULTS

Effects of CuGTSM treatment on survival and weight gain in $Mo^{Ml/y}$ mice

All of the $Mo^{Ml/y}$ mice treated with the vehicle died before weaning with a median survival of 15 days (Fig. 1d). CuGTSM at a dose of 3 µg/g b.w. extended the lifespan to a median survival of 24 days, but none survived to weaning. Doses of 5 and 10 µg/g b.w. CuGTSM increased the survival of the mice with survival rates at 8 months of age of 33% (95% confidence interval [CI] 14–54%) and 57% (95% CI 37–72%), respectively. The survival curves for CuGTSM treatment with different doses intersected at approximately day 25. Oral gavage of $CuCl_2$ did not rescue the $Mo^{Ml/y}$ mice (Fig. 1e). Oral gavage of 4.6 µg/g b.w. $CuCl_2$, which contained the same amount of copper as the 10 µg/g b.w. dose of CuGTSM, significantly extended the lifespan of the $Mo^{Ml/y}$ mice with a median survival of 18 days. However, $CuCl_2$ at an increased dose of 9.2 µg/g b.w. did not further extend the lifespan of the $Mo^{Ml/y}$ mice.

Without CuGTSM treatment, all the $Mo^{Ml/y}$ mice exhibited a reduction in body weight from ~11 days old until death (Fig. 1f). The body weight gain in the CuGTSM-treated $Mo^{Ml/y}$ mice was stagnant until they were 1 month old. Then, the body weight rapidly increased until day 40 and subsequently plateaued at a lower weight than that of the wild-type littermates. There were no significant differences in the mean growth curves between the mice receiving 5 µg/g b.w. and those receiving 10 µg/g b.w. of CuGTSM. In the wild-type mice, CuGTSM (10 µg/g b.w.) did not affect the body weight gain in comparison with the body weight gain in the vehicle-treated wild-type mice.

During the course of the CuGTSM treatment, diarrhea transiently occurred at 2 weeks of age after the third

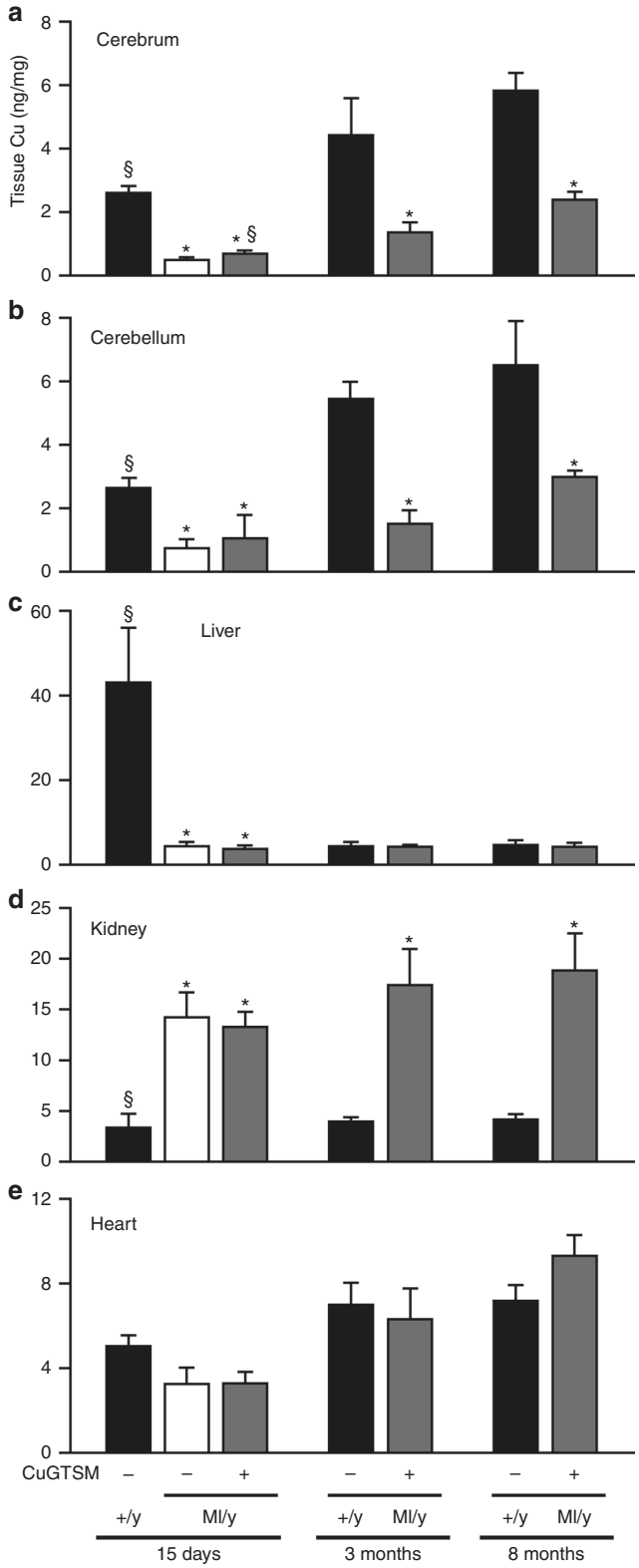

Fig. 2 Effects of CuGTSM treatment on tissue copper concentrations. Copper concentrations (ng/mg tissue [wet weight]) in the cerebrum (**a**), cerebellum (**b**), liver (**c**), kidney (**d**), and heart (**e**) were measured in wild-type mice (+/y) and in $Mo^{Ml/y}$ mice (Ml/y) treated with vehicle or CuGTSM (10 µg/g b.w.) at 15 days and at 3 and 8 months of age ($n = 9$, 7, and 5 in each respective age group). Bars depict the mean ± SD. *$P < 0.05$ vs. the wild-type mice; §$P < 0.05$ vs. the vehicle-treated $Mo^{Ml/y}$ mice

administration of CuGTSM in the $Mo^{Ml/y}$ mice. The diarrhea incidence rates in mice treated with 3, 5, and 10 µg/g of CuGTSM were 0, 36, and 55%, respectively. The duration of diarrhea in the 5 and 10 µg/g treatment groups was 4 ± 3 days and 6 ± 4 days, respectively. One out of six mice exhibited diarrhea both in the 4.6 and 9.2 µg/g b.w. $CuCl_2$-treated $Mo^{Ml/y}$ mouse groups. Diarrhea was not observed in the wild-type mice when they received CuGTSM (10 µg/g b.w., $n = 6$) in the same protocol. In separate experiments, 1-month-old $Mo^{Ml/y}$ mice rescued by CuGTSM treatment (10 µg/g b.w. at postnatal days 5, 8, 11, 17, 23, and 32) did not exhibit diarrhea when an additional administration of CuGTSM (10 µg/g b.w.) was serially given at postnatal days 35 and 38 ($n = 6$).

Effects of CuGTSM treatment on the distribution of copper in the major organs

Figure 2 illustrates the copper concentrations in the major organs of wild type and $Mo^{Ml/y}$ mice with vehicle or CuGTSM treatment (10 µg/g b.w.) at 15 days, 3 months, and 8 months of age. Only data from the wild type and CuGTSM-treated $Mo^{Ml/y}$ mice at 3 and 8 months of age were compared because all the vehicle-treated $Mo^{Ml/y}$ mice died by 17 days of age.

At 15 days old, copper concentrations in the cerebrum of the CuGTSM-treated $Mo^{Ml/y}$ mice were significantly higher than those in the vehicle-treated $Mo^{Ml/y}$ mice but were still lower than the levels in wild-type mice. At both 3 and 8 months of age, the copper concentrations in the CuGTSM-treated $Mo^{Ml/y}$ mice were still lower than those of the wild-type mice, although copper concentrations in the CuGTSM-treated $Mo^{Ml/y}$ mice tended to increase with age despite the cessation of CuGTSM administration. Copper concentrations in the cerebellum showed a similar tendency to those in the cerebrum, although significant differences were not detected between the CuGTSM-treated and vehicle-treated $Mo^{Ml/y}$ mice at 15 days of age.

In the wild-type mice, copper was largely stored in the liver at 15 days of age, and the liver copper levels decreased at 3 and 8 months of age. This copper storage was not observed in the $Mo^{Ml/y}$ mice, and CuGTSM treatment did not increase copper levels in the liver.

In the kidney, copper concentrations in the $Mo^{Ml/y}$ mice were much higher than those in the wild-type mice. There were no significant differences between the CuGTSM-treated and vehicle-treated $Mo^{Ml/y}$ mice at 15 days of age. There were no significant differences in heart copper concentrations between the groups.

Effect of CuGTSM on brain metabolism and function

Figure 3a shows photographs of brain slices processed for CO histochemistry. CO activity, detected as the density of brown reaction product, was recognized mainly in the gray matter, which was low in the brain tissues from the $Mo^{Ml/y}$ mice at 15 days of age. As summarized in Fig. 3b, the CO activity in the cortex of the CuGTSM-treated $Mo^{Ml/y}$ mice was significantly higher than that in the vehicle-treated $Mo^{Ml/y}$ mice at 15 days of age. The CO activity in the CuGTSM-treated $Mo^{Ml/y}$ mice gradually increased with age from 3 to 8 months.

The ratio of noradrenaline to dopamine (NA/DA ratio) in brain extracts, representing dopamine β hydroxylase (DBH) activity, was markedly reduced in the $Mo^{Ml/y}$ mice at 15 days of age, but the ratio was improved in the CuGTSM-treated mice, albeit slightly (Fig. 3c). The NA/DA ratios in the CuGTSM-treated mice were increased at 3 and 8 months of age, although the ratios remained lower than those in the wild-type mice at both ages.

At 15 days old, the brain weights of the vehicle-treated $Mo^{Ml/y}$ mice were significantly lower than those of the wild-type mice, while no significant differences in the brain weight were detected between the wild-type and CuGTSM-treated $Mo^{Ml/y}$ mice (Fig. 3d). At 3 and 8 months of age, there were no significant differences between the wild-type and CuGTSM-treated $Mo^{Ml/y}$ mice.

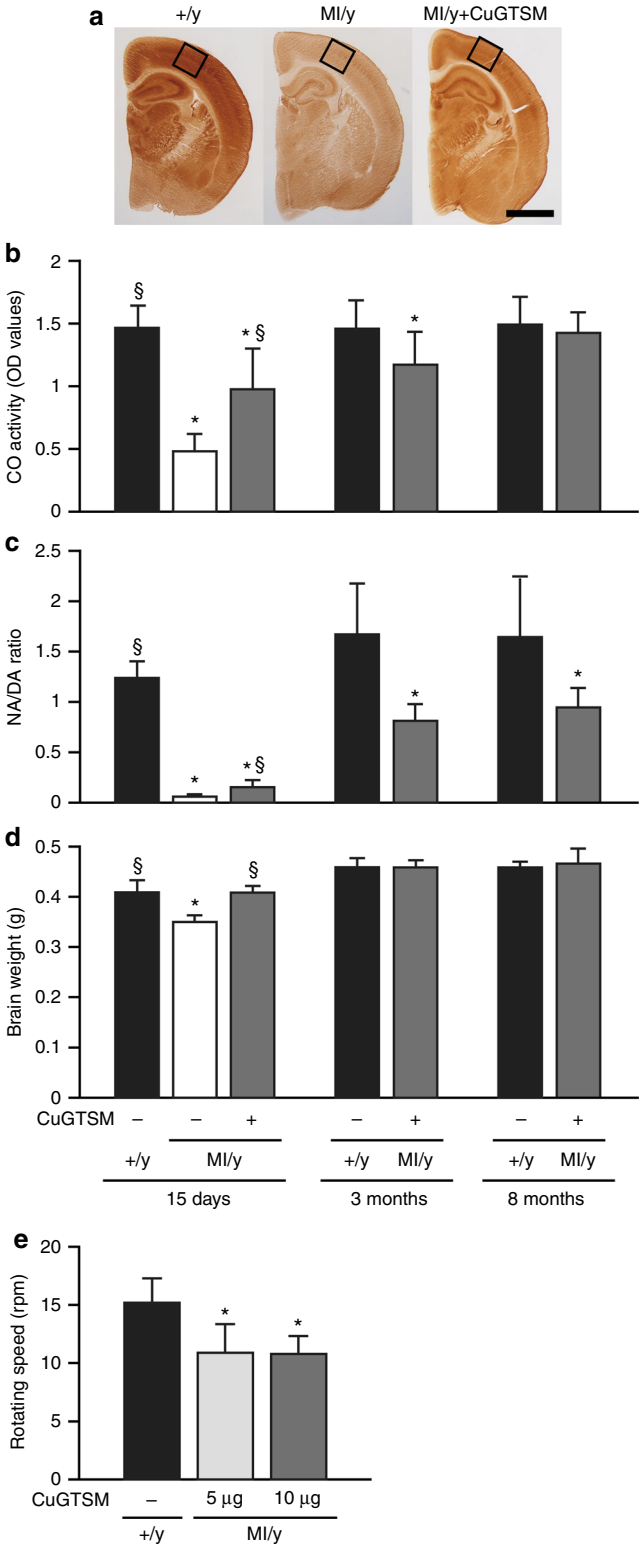

Fig. 3 Effects of CuGTSM treatment on brain metabolism and motor function. **a** Photographs of brain slices processed for cytochrome oxidase (CO) histochemistry. Processed brain slices from the wild-type (+/y) mice, the vehicle-treated $Mo^{Ml/y}$ mice (Ml/y) and the CuGTSM-treated $Mo^{Ml/y}$ mice (Ml/y + CuGTSM) at 15 days old are shown. Rectangular boxes indicate the regions of interest (ROIs) for optical density (OD) measurements. Scale bar: 3 mm. **b**–**d** The relative CO activity in the cortex expressed as OD values (**b**), the ratios of noradrenaline to dopamine (NA/DA ratios) in brain extracts (**c**) and the brain weights (**d**) were measured in the wild-type mice and $Mo^{Ml/y}$ mice treated with vehicle or CuGTSM (10 µg/g b.w.) at 15 days and at 3 and 8 months of age ($n = 9$, 7, and 5 in each respective age group). **e** The rotarod test results show the rotation speed at which the mice fell from the accelerating rotarod, as measured at 3 months of age in the wild-type mice (+/y) and $Mo^{Ml/y}$ mice (Ml/y) treated with CuGTSM at a dose of 5 or 10 µg/g b.w. ($n = 9$, 6 and 7, respectively). rpm, rotations per min. Bars depict the mean ± SD. $^{*}P < 0.05$ vs. the wild-type mice, $^{§}P < 0.05$ vs. the vehicle-treated $Mo^{Ml/y}$ mice

Motor coordination was assessed using a rotarod test at 3 months of age (Fig. 3e). CuGTSM-treated mice performed worse than the wild-type mice did. There were no differences between mouse groups treated with 5 and 10 µg/g b.w. CuGTSM.

Effects of CuGTSM treatment on serum copper levels, Cp activity, and blood hemoglobin concentrations

Serum copper concentrations were reduced in the $Mo^{Ml/y}$ mice but increased in the CuGTSM-treated $Mo^{Ml/y}$ mice at 15 days of age (Fig. 4a). Serum copper concentrations remained low in the $Mo^{Ml/y}$ mice at 3 and 8 months. As shown in Fig. 4b, serum Cp activity was reduced in the $Mo^{Ml/y}$ mice at 15 days old, but it was significantly improved in the CuGTSM-treated mice. At 3 and 8 months of age, the serum Cp activity was lower in the $Mo^{Ml/y}$ mice than that in the wild-type mice.

Hb levels in the vehicle-treated $Mo^{Ml/y}$ mice were significantly higher than those in the wild-type mice (Fig. 4c). No significant differences in Hb were detected between the wild-type and CuGTSM-treated $Mo^{Ml/y}$ mice at 3 and 8 months of age.

Effect of CuGTSM on copper concentrations and histology of the intestines

As shown in Fig. 5a, oral gavage of CuGTSM did not increase the tissue copper concentrations in the duodenum, jejunum, or ileum of the wild-type mice at 15 days of age. This indicated that CuGTSM grains were fairly well removed from the intestinal lumen.

In the $Mo^{Ml/y}$ mice at 15 days of age, the copper concentrations in the ileum were prominently higher than those in the wild-type mice. Here, CuGTSM-treated $Mo^{Ml/y}$ mice exhibited higher copper concentrations than those in the vehicle-treated $Mo^{Ml/y}$ mice, although a significant difference was not detected between these mouse groups. In the $Mo^{Ml/y}$ mice, the copper concentrations in the duodenum in the Cu-treated mice were significantly higher than those in the vehicle-treated $Mo^{Ml/y}$ mice. At 3 months of age, the CuGTSM-treated mice showed significantly higher copper concentrations than those in the wild-type mice in all the three parts of the small intestine.

Because diarrhea was observed in the CuGTSM-treated $Mo^{Ml/y}$ mice before weaning, histology of the small intestine (ileum) and colon was examined at 15 days of age (Fig. 5b). The villi in the ileum were less developed in the $Mo^{Ml/y}$ mice treated with both vehicle and CuGTSM than those in the wild-type mice, whereas no obvious histological differences were noted in the colon. Signs of inflammation were not observed in the ileum or colon in all the mouse groups. The villi of the ileum in the vehicle-treated $Mo^{Ml/y}$ mice were shorter and thinner than those in the wild-type mice, and the villus length was further shortened by the CuGTSM treatment, whereas the villus width was rather increased (Fig. 5c, d).

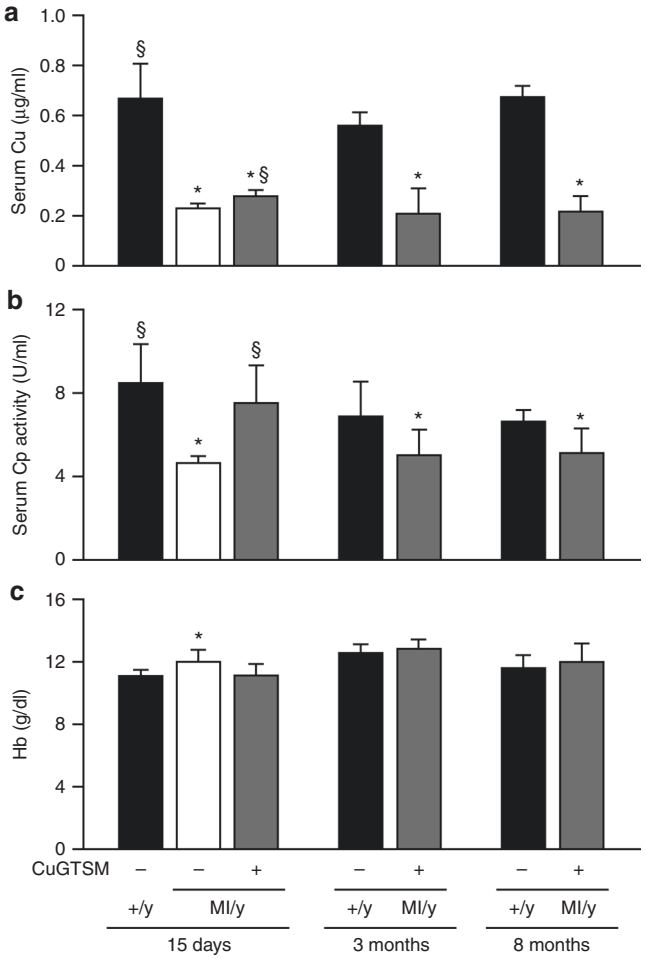

Fig. 4 Effects of CuGTSM treatment on serum copper concentration and Cp activity. Serum copper concentrations (**a**), serum Cp activity (**b**), and Hb (**c**) were measured in the wild type (+ /y) and $Mo^{Ml/y}$ mice (Ml/y) treated with vehicle or CuGTSM (10 μg/g b.w.) at 15 days and at 3 and 8 months of age ($n = 9$, 7, and 5 in each respective age group). Bars depict the mean ± SD. *$P < 0.05$ vs. the wild-type mice; §$P < 0.05$ vs. the vehicle-treated $Mo^{Ml/y}$ mice

DISCUSSION

CuGTSM, a CuBTSC, is a neutral lipophilic compound that is capable of crossing cell membranes.[23] Once inside a cell, Cu(II) is reduced by intracellular reductants to Cu(I), which is readily liberated from the ligand. The water solubility of CuGTSM is poor but higher than that of other CuBTSCs.[23] CuGTSM is stable in gastric juices and partly dissolves in gastrointestinal fluids to be absorbed from the gut.[8]

Previous studies using Menkes disease mouse models demonstrated that copper deficiency diminished the activity of copper-dependent enzymes such as CO and DBH in the brain, which was improved by a parenteral copper supply.[16,24] In line with these studies, copper concentrations in the brain were low in the present work, causing a malfunction of CO and DBH in the 15-day-old $Mo^{Ml/y}$ mice. CuGTSM treatment improved CO and DBH activities, indicating that CuGTSM delivered bioavailable copper to neurons, as both CO and DBH activities are mainly seen in neurons.[18,25] Copper concentrations in the brain increased and was accompanied by further increases in CO and DBH activities despite the cessation of copper supply at 3 and 8 months old. The results were similar to previous biochemical studies in the brain of Menkes disease model mice.[26,27] Copper might gradually accumulate by residual activity of mutated ATP7A and be recycled within the brain.

The rotarod test is widely used to estimate neuromotor performance.[21] Although the 3-month-old $Mo^{Ml/y}$ mice were able to walk on the rod, their performance was impaired, and this was independent of the CuGTSM dose. In mosaic (Atp7a mo-ms) mice, another mouse model of Menkes disease, neuromotor deterioration manifests at 2 weeks old but is ameliorated by prenatal copper supplementation.[6] In this study, CuGTSM treatment started at 5 days old, which may be late for intact brain development. Starting the CuGTSM medication earlier may further improve neuromotor outcomes.

During the third trimester of gestation, copper is transferred from the maternal to fetal circulation via the placenta and is stored in the fetal liver.[28] Full-term wild-type pups use this store for systemic copper metabolism until weaning.[29] In the placenta, ATP7A is expressed on the basal side of syncytiotrophoblast and fetal vascular endothelial cells to mediate copper transfer to the fetus.[30] Since these cells are derived from the fetus, copper transfer to $Mo^{Ml/y}$ fetuses is impaired. In fact, the hepatic copper store was not observed in the 15-day-old $Mo^{Ml/y}$ mice in our study, indicating that immediate copper supplementation after birth is critically important. Although CuGTSM treatment did not apparently increase hepatic copper levels at 15 days old, the copper in CuGTSM may preferentially transfer to Cp protein (apo-Cp) rather than accumulate in the liver.

Cp serves as a copper vehicle by shuttling copper from the liver to other organs, as well as functioning as a multicopper ferroxidase enzyme.[31] Cp is mainly formed in hepatocytes, where apo-Cp is incorporated with copper with the help of ATP7B.[31] Dietary or genetic copper deficiency (brindled [$Mo^{Br/y}$] mice) reduces serum Cp activity in mice.[32] As manifested in aceruloplasminemia, a genetic disease, loss of Cp activity can cause mild anemia although mouse models of aceruloplasminemia do not always manifest anemia.[33] However, suckling $Mo^{Br/y}$ mice paradoxically exhibit high Hb levels despite reduced Cp activity.[32] In the present study, at 15 days old, Hb levels in the $Mo^{Ml/y}$ mice without CuGTSM treatment were higher than those in the wild-type mice. This might be a result of dehydration because the surviving vehicle-treated $Mo^{Ml/y}$ mice became inactive at this age. CuGTSM treatment improved Cp activity, which is beneficial for physiological copper delivery and iron metabolism. However, this implies that CuGTSM is partly metabolized within the liver. This hepatic first-pass metabolism would reduce the bioavailability of CuGTSM as a copper vehicle to the brain.

Copper accumulation in the kidney can cause mild renal tubular dysfunction in patients with Menkes disease, and urinary β2-microglobulin increases with age, whereas other biochemical indicators of renal function remain normal.[34] In our study, renal copper accumulation in $Mo^{Ml/y}$ mice did not deteriorate after CuGTSM treatment at 15 days old. In previous studies, copper supplementation with CuDMDTC or CuPTSM caused lower copper accumulation in the kidneys of a mouse model of Menkes disease compared to supplementation with $CuCl_2$.[6,11] The reduced renal copper accumulation is a possible benefit of these lipophilic copper complexes.

Menkes disease mouse models exhibit delayed body weight gain during the suckling period despite the improved biochemical data obtained after parenteral copper injection, suggesting impaired nutritional absorption.[16,35] In this study, oral gavage of CuGTSM elicited diarrhea along with reduced body weight gain, which was specific to the $Mo^{Ml/y}$ mice before weaning. Given that oral gavage of $CuCl_2$ also induced diarrhea in the $Mo^{Ml/y}$ mice, the diarrhea was conceivably caused by copper overload to the intestines.

Dietary copper is taken up mainly from the duodenum in mature mice.[2] However, copper is avidly absorbed in the ileum rather than the duodenum in pups, and the absorption is dose-dependent and does not saturate.[29] Hence, copper is largely

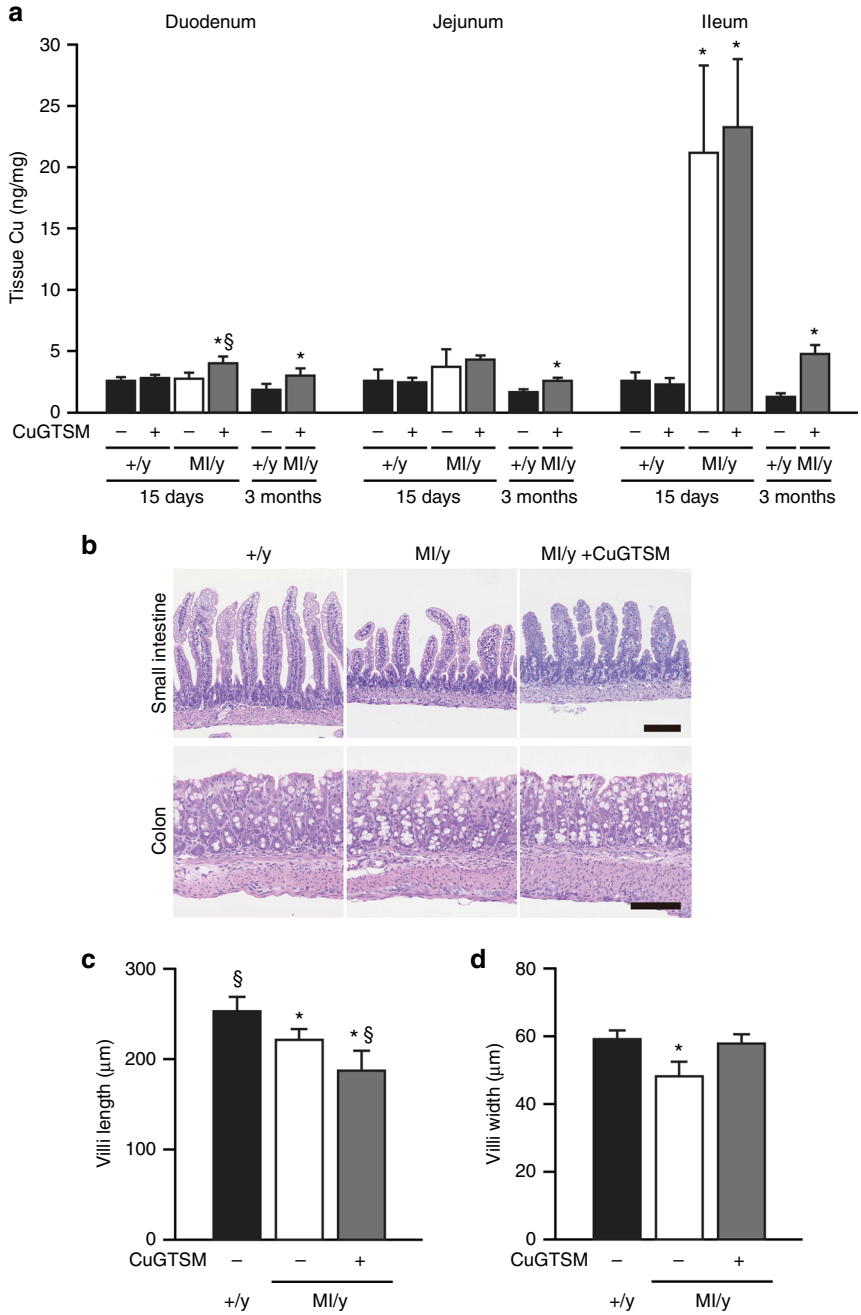

Fig. 5 Effects of CuGTSM treatment on the copper concentrations and morphology in the intestines. **a** Tissue copper concentrations (ng/mg tissue [wet weight]) in the small intestine in the wild-type mice (+ /y) and $Mo^{Ml/y}$ mice (Ml/y) treated with vehicle or CuGTSM (10 μg/g b.w.) at 15 days and 3 months of age ($n = 6$ in each group). **b** Representative images of H&E-stained small intestine (ileum; upper panel) and colon (lower panel) in the wild-type mice (+ /y) and the $Mo^{Ml/y}$ mice (Ml/y) treated with vehicle or CuGTSM (10 μg/g b.w.; Ml/y + CuGTSM) at 15 days of age. Scale bar: 100 μm. **c**, **d** A comparison of the length (**c**) and width (**d**) of ileal villi in the wild-type mice and $Mo^{Ml/y}$ mice treated with vehicle or 10 μg/g b.w. CuGTSM at 15 days of age ($n = 6$ in each group). Bars depict the mean ± SD. *$P < 0.05$ vs. the wild-type mice; $^{§}P < 0.05$ vs. the vehicle-treated $Mo^{Ml/y}$ mice

accumulated in the ileum when ATP7A activity to expel copper to the portal circulation is diminished.[36] In the 15-day-old $Mo^{Ml/y}$ mice, copper was prominently accumulated in ileal tissues and tended to be higher in the CuGTSM-treated group than the ileal copper accumulation in the vehicle-treated group, although statistical significance was not detected. The rapid turnover rate of enterocytes (~2 days) in mice might mask additional copper accumulation caused by CuGTSM.[37] Dietary copper load can shorten the villus length of the small intestine, owing to oxidative stress by copper.[38] In the present study, the villus length in the ileum was reduced in the 15-day-old $Mo^{Ml/y}$ mice, and the value was further decreased by CuGTSM treatment, implying additional stress by the treatment. The ileal copper accumulation may be related to the slowed body weight gain and the diarrhea during the suckling period. The Kaplan–Meyer curves for CuGTSM treatment with different doses intersected, indicating an increased risk for suckling $Mo^{Ml/y}$ mice that received high doses of CuGTSM, which might be a result of the adverse enteral effect. Therefore, it is beneficial to lessen enteral copper overload during CuGTSM therapy.

Administration of penicillamine reduces intestinal copper accumulation during internal radiotherapy with ^{64}CuBTSC.[39] Thus, combined administration of penicillamine with CuGTSM may reduce copper overload in the ileum. In addition, dietary copper supplementation combined with zinc reduced the incidence and severity of diarrhea, which may applicable to CuGTSM treatment.[40] Another option is to initially start CuGTSM treatment parenterally after birth, followed by oral CuGTSM treatment when the ileal copper level is adequately decreased.

In conclusion, oral administration of CuGTSM can rescue Menkes model mice. CuGTSM provided bioavailable copper to the brain. Oral administration is attractive for prolonged copper supplementation. However, further pharmaceutical studies are needed to reduce adverse enteral effects.

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ADDITIONAL INFORMATION

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