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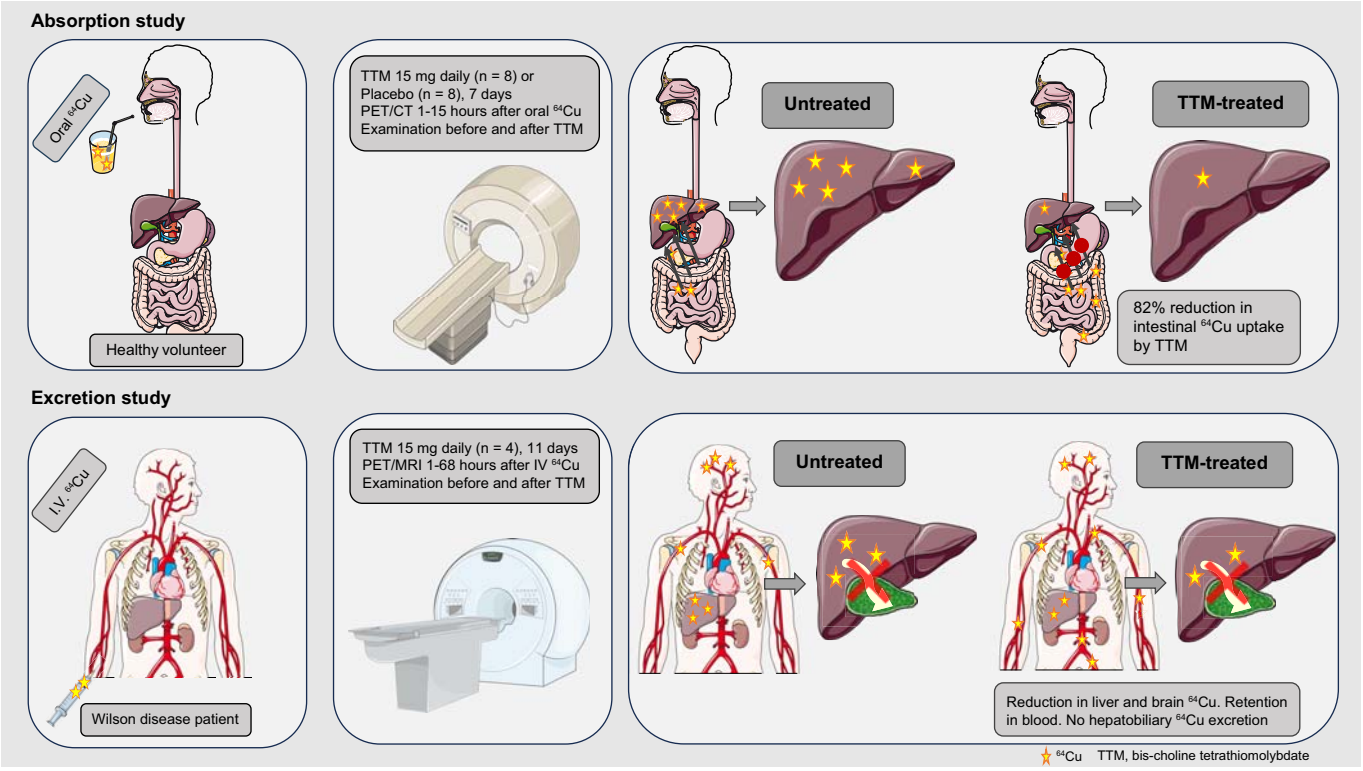
Graphical abstract



Highlights

- ^{64}Cu PET/CT/MRI can detect the uptake and distribution of ^{64}Cu in the body.
- TTM is a highly effective copper-binding agent.
- The mode of action depends on the compartment it is active in.
- TTM very efficiently reduces intestinal ^{64}Cu uptake.
- TTM rapidly binds ^{64}Cu in blood and reduces copper availability to the liver and brain.

Impact and implications

Bis-choline tetrathiomolybdate (TTM) is an investigational copper chelator being developed for the treatment of Wilson disease. In animal models of Wilson disease, TTM has been shown to facilitate biliary copper excretion. In the present human study, TTM surprisingly did not facilitate biliary copper excretion but instead reduced intestinal copper uptake to a clinically significant degree. Our study builds on our understanding of human copper metabolism and the mechanism of action of TTM.

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Effects of tetrathiomolybdate on copper metabolism in healthy volunteers and in patients with Wilson disease

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Background & Aims: In Wilson disease (WD), copper accumulates in the liver and brain causing disease. Bis-choline tetrathiomolybdate (TTM) is a potent copper chelator that may be associated with a lower risk of inducing paradoxical neurological worsening than conventional therapy for neurologic WD. To better understand the mode of action of TTM, we investigated its effects on copper absorption and biliary excretion.

Methods: In a double-blind randomized setting, hepatic ^{64}Cu activity was examined after orally administered ^{64}Cu by PET/CT in 16 healthy volunteers before and after seven days of TTM treatment (15 mg/d) or placebo. Oral ^{64}Cu was administered one hour after the final TTM dose. Changes in hepatic ^{64}Cu activity reflected changes in intestinal ^{64}Cu uptake. Additionally, in four patients with WD, the distribution of ^{64}Cu in venous blood, liver, gallbladder, kidney, and brain was followed after i.v. ^{64}Cu dosing for up to 68 hours before and after seven days of TTM (15 mg/day), using PET/MRI. Increased gallbladder ^{64}Cu activity was taken as evidence of increased biliary ^{64}Cu excretion.

Results: In healthy volunteers, TTM reduced intestinal ^{64}Cu uptake by 82% 15 hours after the oral ^{64}Cu dose. In patients with WD, gallbladder ^{64}Cu activity was negligible before and after TTM, while TTM effectively retained ^{64}Cu in the blood, significantly reduced hepatic ^{64}Cu activity at all time-points and significantly reduced cerebral ^{64}Cu activity two hours after the intravenous ^{64}Cu dose.

Conclusions: While we did not show an increase in biliary excretion of ^{64}Cu following TTM administration, we demonstrated that TTM effectively inhibited most intestinal ^{64}Cu uptake and retained ^{64}Cu in the blood stream, limiting the exposure of organs like the liver and brain to ^{64}Cu.

Introduction

Wilson disease (WD) is a rare inherited autosomal recessive disorder characterized by reduced excretion of copper in the bile and subsequent accumulation of toxic amounts of copper in the liver, brain and other organs. If left untreated, WD is fatal.[1–3] The disease is caused by a defect in the copper-transporting ATPase, ATP7B, primarily expressed in hepatocytes. ATP7B facilitates the intrahepatic incorporation of copper into ceruloplasmin (Cp) and promotes biliary copper excretion.[1]

Current therapies for WD act by either increasing urinary copper excretion, as occurs with D-penicillamine and trientine, or by reducing the intestinal absorption of dietary copper with zinc.[2] A feared side effect for patients treated with either D-penicillamine or trientine is paradoxical neurological worsening, a rapid clinical worsening of neurologic symptoms within months of treatment initiation, possibly caused by rapid mobilization of large amounts of copper.[3–5]

Copper deficiency is seen in sheep and cattle with diets rich in molybdenum and sulfur which creates tetrathiomolybdate in the animals' gastrointestinal tracts.[6] Inspired by this knowledge, ammonium tetrathiomolybdate was proposed as a copper chelation therapy in WD. Recently a new formulation, bis-choline tetrathiomolybdate (TTM), also known as ALXN1840 or WTX-101, was developed. In blood, TTM forms a stable tripartite complex (TPC) with albumin and copper, rendering the bound copper inert. TTM also rapidly reduces bioavailable copper in plasma not bound to Cp, presumably through the formation of the TPC.[7,8] *In vitro* studies also demonstrate that TTM can remove copper from metallothioneins (MT), an intracellular copper storage protein.[9,10] In human trials ammonium tetrathiomolybdate led to rapid copper control and neurological improvement and a reduced risk of paradoxical neurological worsening compared to conventional chelation therapy.[7,11,12]

The mode of action of TTM in WD is not completely understood. Early balance studies with ammonium tetrathiomolybdate

Keywords: Copper absorption; copper excretion; 64 copper; randomized; placebo; rare disease; tetrathiomolybdate; PET; WTX-101; ALXN1840; copper chelation.
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in patients showed increased fecal copper output that could be caused by either inhibition of intestinal uptake or increased biliary excretion.[13] While human studies are missing, rat studies reported conflicting results regarding changes in biliary copper excretion following TTM administration.[14–17]

To gain further knowledge on TTM's mode of action, we conducted two studies in humans. We first used PET/CT to examine the effect of TTM on the intestinal absorption of orally administered ^{64}Cu (the absorption study). Because the ATP7B protein is not involved in intestinal uptake, these experiments could be carried out in healthy volunteers (HVs) in a double-blind, randomized, placebo-controlled study. We next examined the effect of TTM treatment on the fate of intravenously injected ^{64}Cu in patients with WD, with a specific focus on following the biliary excretion of ^{64}Cu and the distribution of radiocopper to other organs (the excretion study). Because ATP7B is critical for hepatic copper transport, these experiments were carried out in patients with WD where background biliary copper excretion is known to be low so that any changes in biliary copper excretion would be demonstrable.

Patients and methods

In this paper, we present data from the absorption study (Clinical trial no. 2020-005832-31, EudraCT) and from the excretion study (clinical trial no. 2021-000102-25, EudraCT).

Study design

Absorption study: Investigation using PET/CT to study the effects of TTM *vs*. placebo (PLA) on the absorption of an oral ^{64}Cu dose in HVs.

The study design of this randomized, placebo-controlled, double-blinded study is illustrated in Fig. 1A. Sixteen HV were initially examined by PET/CT one hour (1H) and 15H after an oral 30 megabecquerel dose of ^{64}Cu. ^{64}Cu activity in venous blood was assessed 30 minutes post ^{64}Cu and shortly before the 15H PET/CT scan. Participants were then randomized 1:1 to receive either a delayed-release, oral tablet containing 15 mg of TTM once daily or PLA once daily for seven days before repeating the examinations. The PLA and TTM tablets were identical in design and were both supplied by Alexion, AstraZeneca Rare Disease, Boston, MA, USA. The last TTM dose was administered one hour before administration of the oral ^{64}Cu tracer. HVs fasted for one hour before and after medication intake and for six hours prior to ^{64}Cu ingestion, they were put on individualized controlled diets. TTM dosage was determined as the lowest starting dose used in a previous phase II trial.[7]

Excretion study: Investigation using PET/MRI to study the effect of TTM on the fate of an i.v. ^{64}Cu bolus in patients with WD.

The study design of this controlled, open-label study is illustrated in Fig. 1B. Five patients with WD initially paused their usual WD medication for 72 hours. They were then examined in an "untreated" state by PET/MRI 1, 2, 6, 20, 48, 54 and 68 hours after an i.v. 75 megabecquerel dose of ^{64}Cu. The patients were then given seven days of TTM treatment, 15 mg once daily, and the examinations were repeated. TTM treatment continued until the final day of PET/MRI examinations for a total of 11 days with the final dose taken on the morning of the 68 hour scan.

Patients were instructed to fast for one hour before and after intake of study medicine. Study medication adherence was self-recorded using a printed form. Participants fasted (water allowed) for four hours before each PET/MRI scan to ensure the gallbladder was visible. ^{64}Cu activity in venous blood was assessed 5, 10, 15, 60, 120, 300 and 600 seconds after injection and shortly before each PET/MRI scan.

Participants

Absorption study: HVs were recruited through online and newspaper advertisements and were >18 years old with BMI <30. Exclusion criteria were breastfeeding, claustrophobia, participation in a medical trial including a PET scan within the last year, known hypersensitivity to ingredients in the ^{64}Cu tracer or study medications and ineligibility assessed by a medical doctor based on blood samples collected at baseline.

Excretion study: patients with WD were recruited from the outpatient clinic at the Department of Hepatology and Gastroenterology, Aarhus University Hospital after clinical assessment of disease stability. The diagnosis was confirmed in accordance with the Leipzig criteria.[2]

Inclusion criteria included treatment with either D-penicillamine or trientine for at least 1 year and age >18. Exclusion criteria were known biliary tract pathology or prior cholecystectomy, breastfeeding, claustrophobia, participation in a medical trial including a PET scan within the last year. None had decompensated cirrhosis, a model of end-stage liver disease score >11, or a modified Nazer score (Revised King's score) >6,[2] and none were treated with zinc within the last year.

For both studies, fertile women were required to use safe contraception during the study and for up to 1 month following participation and supply a negative pregnancy test on each day of the ^{64}Cu administration. There were no stipulations on contraception for male participants.

All participants gave informed written consent prior to inclusion in the studies.

Five patients with WD were included, of whom one withdrew from the study before the second examination without explanation, hence four patients with WD completed the study. Only data from the four complete examinations were included in the analysis.

Descriptive biochemical parameters for both HVs and patients with WD were collected at the time of inclusion and on the day of the final PET scan.

Randomization and masking

In the absorption study, HVs were randomized to either TTM or PLA. TTM and PLA were visually indistinguishable to both patients and investigators. PET image analysis and data analysis were performed prior to unblinding. At the time of inclusion, each participant received an inclusion number and after the first scan, a randomization number with subsequent treatment modality was provided by the hospital pharmacy at Aarhus University Hospital. The medication was handed out following the baseline PET/CT scan.

^{64}Cu PET imaging

PET/MRI and PET/CT examinations were performed at the Department of Nuclear Medicine and PET-center, Aarhus University Hospital, Aarhus, Denmark.

In the absorption study, PET images from the clavicula to the upper femur were acquired through continuous bed motion with

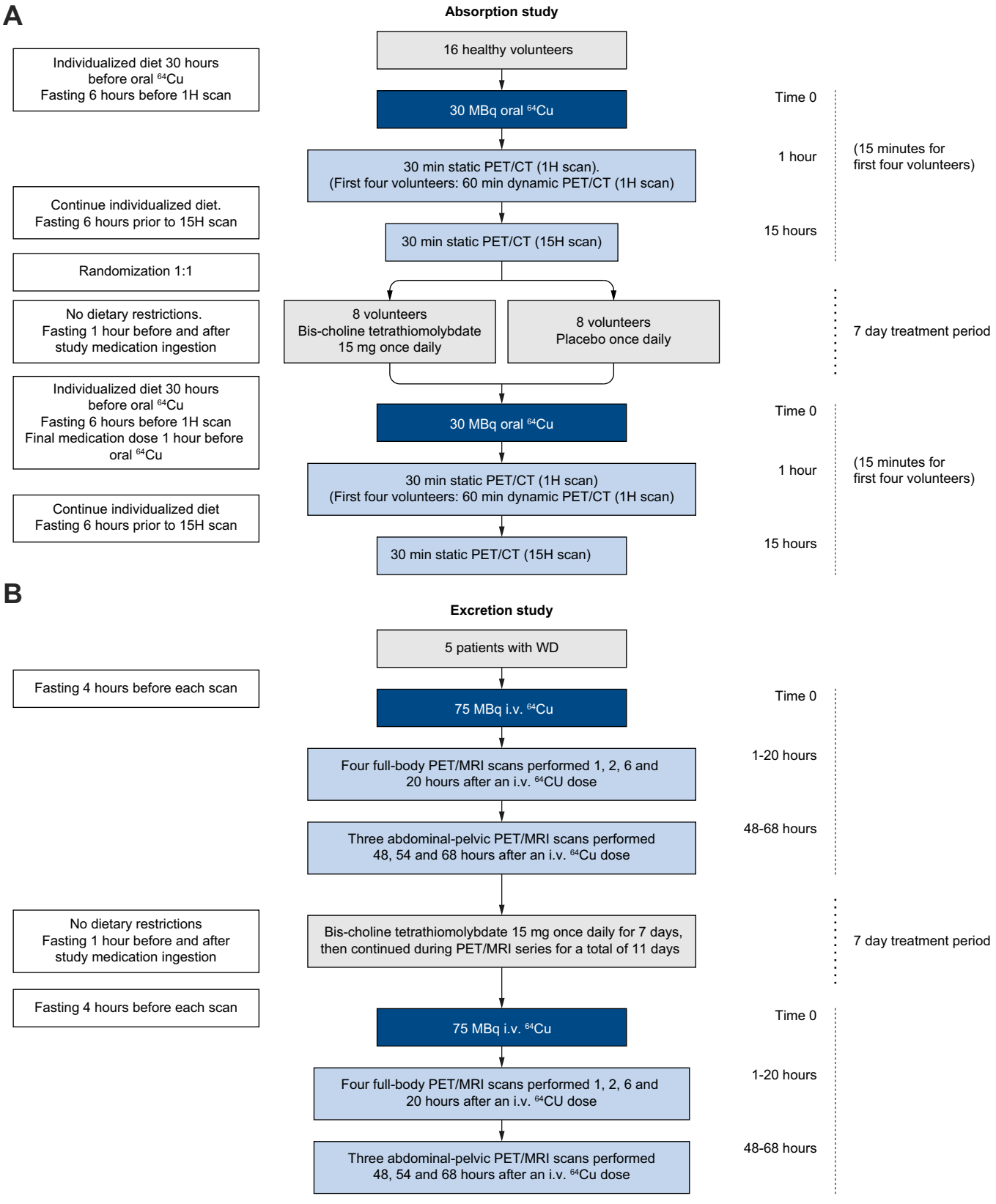

Fig. 1. Study designs. (A) Absorption study. (B) Excretion study. H, hour; MBq. megabecquerel.

a scan speed of 0.4 mm/second allowing for good count statistics. The PET/CT procedure, image analysis, blood analysis and radiotracer preparation have been detailed elsewhere.[18,19]

In the excretion study, patients with WD were positioned in a supine position in the PET/MRI scanner (GE Signa, GE Healthcare, Milwaukee, WI, USA). An MRI scan was performed prior to each PET scan to define anatomical structures and for PET attenuation correction.

Four full-body PET/MRI scans were performed (top of skull to upper thigh; six bed positions) at 1, 2, 6 and 20 hours post ^{64}Cu injection (duration of 1.5, 1.5, 3 and 4.5 minutes per bed). At 48, 54 and 68 hours post ^{64}Cu injection, three abdominal-pelvic PET/MRI scans were performed (top of liver to symphysis pubica; three bed positions) (duration of 10 minutes per bed). The images were reconstructed using TOF (time of flight), with two iterations, 16 subsets and a 128 x 128 matrix.

Blood analysis and radiotracer preparation have been previously described.[18]

The PET/CT and PET/MRI images were analyzed using PMOD (version 4.0, PMOD Technologies LLC, Zürich, Switzerland) as previously described.[20]

Five spherical volumes of interest (VOI), each of 4 ml, were placed in the right liver lobe, accounting for a total of approximately 1.5% of liver volume. This approach has been used previously and eliminates signal spill-out from other organs such as the colon, large blood vessels within the liver and the effect of respiratory motion.[21] A single 0.5 ml VOI was placed centrally within the gallbladder to estimate biliary ^{64}Cu content.

In the excretion study, a hand-drawn VOI was created to include only the kidney parenchyma without renal pelvis or calyces – only the left kidney was analyzed to avoid spill-out from the liver. For the first four PET/MRI images, a single 100 ml VOI was placed centrally in the occipital lobe of the brain. Brain ^{64}Cu activity was corrected for blood ^{64}Cu activity – assuming 4% blood volume in brain tissues, as has been previously described.[22]

VOIs were placed by two trained ^{64}Cu-PET examiners in collaboration.

^{64}Cu activity from the PET scans are given as mean standard uptake value (SUV), where

$$SUV\ \left(g \cdot mL^{-1}\right) = A\frac{BW}{D}$$

A: Mean activity concentration in Bq · ml^{-1} (Calibrated pixel value).
BW: Patient weight in g.
D: Ingested dose in Bq decay corrected.

Venous ^{64}Cu radio counts in kBq/ml were similarly corrected for bodyweight and administered dose.

To better quantify ^{64}Cu redistribution we calculated the percentage of injected dose (%ID) in the excretion study, as previously described.[19,22]

Metallothionein 1 and *SLC30A1* expression

Venous blood samples were collected on both days of ^{64}Cu administration, prior to ingestion or injection. Peripheral mononuclear blood cells (PBMCs) were isolated using BD Vacutainer® cell preparation tubes. RNA was extracted using Qiagen RNAeasy plus micro kits. The mRNA expression, relative to a stably expressed reference gene was determined for metallothionein 1 (*MT1*), (gene isoforms *MT1A, MT1G, MT1H* and *MT1X*) and zinc transporter 1 (ZNT1) encoded by the *SLC30A1* gene, as previously described.[23]

Outcomes

To quantify the inhibition of intestinal ^{64}Cu uptake in the absorption study by the two treatments, hepatic ^{64}Cu activity was measured before and after treatment. The inhibition of intestinal uptake was estimated by the reduction of hepatic SUV after TTM at 1 hour and 15 hours after the oral ^{64}Cu dose as described by Munk *et al*.[21]

Secondary endpoints included changes in ^{64}Cu distribution as measured by the SUV in different organs including the gallbladder as well as changes in MT and *ZNT1* gene expression.

In the excretion study, the primary endpoint was if TTM treatment changed the gallbladder SUV in patients with WD after an i.v. ^{64}Cu dose. Detection of ^{64}Cu in the gallbladder would imply biliary ^{64}Cu excretion. Secondary endpoints included TTM's effects on ^{64}Cu distribution as measured by the SUVs of the venous blood, liver, kidney and brain during the time frame of the study. Additional secondary endpoints were changes in *MT* and *ZNT1* gene expression in PBMCs.

Statistical analysis

The normality of data was tested using histograms, QQ plots, and the Shapiro-Wilk test. Percentage changes are calculated per individual and given as median values.

As data were generally not normally distributed, data were analyzed using the Mann-Whitney *U* test or Wilcoxon signed-rank test where appropriate. A p value <0.05 was considered statistically significant. Data is presented as median (IQR) unless otherwise specified.

STATA version 17.0 (StataCorp LP, College Station, TX) was used to perform statistical analysis and Graphpad Prism version 9.5.1 (Graphpad Software, San Diego, CA) was used to generate figures. See CTAT table for further details.

Sample size

The sample size for the absorption study was based on experience from a previous similar study using zinc therapy with 10 participants in each group.[21] We improved the methodology using dynamic PET/CT to select the best starting time point and introduced individualized controlled diets. Given the high copper affinity of TTM, we estimated that clinically relevant changes would be detectable using eight participants per group.

For the excretion study, the goal was to investigate a dichotomous outcome, as biliary copper excretion is largely non-existent in WD and was believed to normalize and thus increase many-fold on TTM, allowing for use of a small sample size.

Ethics, approvals and registration

The protocols and all amendments were approved by national regulatory authorities (Absorption study: 2021061093, Excretion study: 2021014329) and the local ethics committee (Absorption study: 1-10-72-343-20, Excretion study: 1-10-72-25-

21). The studies are registered with EudraCT (Absorption study: 2020-005832-31, Excretion study: 2021-000102-25). The Good Clinical Practice unit at Aarhus and Aalborg University Hospitals monitored the studies.

Results

Absorption study

All 16 HVs completed the study. Baseline parameters and select blood parameters are presented in Table 1. Compliance in relation to the study drug was 100% in both the TTM and PLA group. In both groups, compliance for fasting in relation to study medication, before each scan and before ^{64}Cu ingestion, was 100%.

Table 2 and Fig. 2 show hepatic SUVs 1 hour and 15 hours after oral ^{64}Cu. Per participant changes in hepatic SUV are shown in Fig. S1. Seven days of TTM treatment reduced the hepatic SUV by 92% at 1 hour and 82% at 15 hours post ^{64}Cu (Table 2). In comparison, hepatic SUV did not change in the PLA group. Gallbladder SUV was similarly reduced by 88% at 1 hour and 94% at 15 hours post ^{64}Cu by TTM (p <0.02), but not by PLA. Accordingly, SUVs in the liver and gallbladder were significantly lower after TTM than after PLA (p <0.05).

Venous ^{64}Cu activity is shown in Fig. 2. Per participant changes of venous ^{64}Cu activity are shown in Fig. S2. At the early time point 30 minutes after ingestion, TTM reduced venous ^{64}Cu activity by approximately 90% (p = 0.18), likely reflecting inhibition of intestinal uptake. At the late timepoint 15 hours after ingestion, an approximately 45% reduction (p = 0.27) was observed, suggesting possible copper redistribution. In comparison, PLA did not affect venous ^{64}Cu activity.

Seven days of TTM treatment in HVs did not significantly increase MT expression in PBMCs, Table S1. *ZNT1* expression was slightly, and statistically significantly inhibited, p = 0.04 (Table S1).

Excretion study

Baseline parameters and select blood parameters of the four patients with WD who completed the study are presented in Table 1. Compliance in relation to the study drug as well as fasting was 100% for all participants.

TTM significantly reduced hepatic SUV at all time-points up to and including 68 hours post ^{64}Cu, Table 3 and Fig. 3. A fused PET/MRI scan showing the liver, gallbladder and kidneys 6 hours post ^{64}Cu is shown in Fig. 4.

Gallbladder SUV trended lower after TTM treatment in accordance with lower hepatic ^{64}Cu availability. This effect was significant only 6 hours post ^{64}Cu, Table 3 and Fig. 3.

Renal SUV slowly increased on TTM treatment and plateaued significantly higher on treatment from 20 to 68 hours post ^{64}Cu, Table 3 and Fig. 3.

Brain SUV corrected for blood ^{64}Cu activity trended towards lower values on TTM treatment (Table 3 and Fig. 3). This effect was statistically significant at 2 hours post ^{64}Cu and less clear at the final brain PET scan at 20 hours post ^{64}Cu.

TTM increased venous ^{64}Cu activity (Fig. 5). As early as 10 minutes after ^{64}Cu injection, venous ^{64}Cu activity trended higher on TTM and this finding was consistent and statistically significant from 1 hour to 48 hours (p = 0.02). Thus, TTM retained ^{64}Cu in the blood, compared to measurements before treatment. Individual venous data points, including those in the first 10 minutes, are presented in Fig. S3.

Thus, TTM treatment retained a substantial amount of ^{64}Cu in the blood stream for several hours and a comparatively smaller amount over the observation days. Retention of ^{64}Cu in the bloodstream seemed to limit organ availability except for the kidneys, as illustrated by reduced hepatic and cerebral contents after TTM. Gallbladder SUV showed a trend towards a reduction with TTM, with no signs of increased biliary excretion of ^{64}Cu.

Table 1. Baseline characteristics and select blood parameters collected at inclusion.

	Absorption study		Excretion study	
	HV: TTM (n = 8)	HV: Placebo (n = 8)	Patients with WD (n = 4)	Normal range
Age (years)	25.5 (19.5)	24.0 (39.0)	45 (24.50)	
Sex (male)	4	6	3	
BMI	23.80 (2.31)	22.97 (3.86)	22.31 (3.44)	
Alanine aminotransferase (U/L)	17.50 (13.00)	20.00 (11.50)	39.50 (33.50)	10–70
Bilirubin (μmol/L)	8.50 (5.00)	11.00 (4.50)	9.50 (9.50)	5-25
Albumin (g/L)	41.50 (3.50)	43.00 (4.50)	40.50 (4.50)	36–45
Creatinine (μmol/L)	72.00 (28.00)	70.5 (8.00)	63.00 (14.00)	60–105
Total copper (μmol/L)	16.2 (5.75)	14.90 (4.50)	2.45 (4.00)	7.9–23.6

Table includes data from HV in either treatment group as well as patients with WD. Data presented as median (IQR).
HV, healthy volunteers; TTM, bis-choline tetrathiomolybdate; WD, Wilson disease.

Table 2. Absorption study: ^{64}Cu activity in organs of interest before and after treatment, following an oral ^{64}Cu dose in HV.

	TTM		PLA	
Time post ^{64}Cu (hours)	1 hour	15 hours	1 hour	15 hours
Liver SUV, before treatment	7.42 (2.63)	13.22 (4.89)	6.31 (3.79)	13.62 (3.61)
Liver SUV, after treatment	0.48 (0.79)†	3.05 (4.52)†	6.12 (4.74)	15.91 (6.24)
Liver SUV before *vs.* after treatment	p <0.02	p <0.02	n.s.	n.s.
Gallbladder SUV, before treatment	1.29 (1.51)	26.12 (23.54)	2.45 (1.31)	16.71 (11.06)
Gallbladder SUV, after treatment	0.17 (0.43)†	1.91 (2.37)†	1.51 (0.42)	12.39 (9.99)
Gallbladder SUV before *vs.* after treatment	p <0.02	p <0.02	n.s.	n.s.

Data presented as median (IQR). n.s., not significant. Wilcoxon signed-rank test used to compare activity before vs after treatment. The Mann-Whitney U test was used for between group comparison. HV, healthy volunteers; PLA, placebo; SUV: standard uptake value (a measure of ^{64}Cu activity); TTM, bis-choline tetrathiomolybdate.
†p <0.05 compared to placebo. Mann-Whitney U test was used for statistical analysis.

Eleven days of TTM treatment in patients with WD did not significantly increase *MT* or *ZNT1* expression in PBMCs, Table S1. However, a trend towards an inhibition of *ZNT1* expression was observed, $p = 0.16$.

Safety measurements and adverse events

In the absorption study, blood tests at the end of the trial in HVs were similar to baseline values (Table S2). Small but statistically significant differences in the active treatment groups were within normal ranges and were not of clinical significance. Fifteen treatment-emergent adverse events were reported by eight participants (four TTM, four PLA) and none of these were considered severe. Six were considered possibly related to the study treatment (five TTM/one PLA), (Table S3). All treatment-emergent adverse events resolved after conclusion of the trials.

In the excretion study, there were no significant changes in biochemical parameters before and after treatment, nor were any adverse events reported. All measured parameters are available in the supplementary materials (Table S4). For the patients with WD, 11 days of TTM treatment increased total serum copper from 2.45 (4.00) μmol/L to 3.25 (2.55) and reduced exchangeable serum copper from 0.61 (0.30) μmol/L to 0.47 (0.08) and relative exchangeable copper from 14.50 (27.90) % to 11.50 (3.60) %. However, several measurements were below the cut-off value for our analysis and results should be interpreted with caution.

Discussion

While the therapeutic options for WD are lifesaving for patients, the choices are currently limited, leaving intolerant patients with very few options. The development of new treatments is therefore warranted. TTM has potential for the treatment of patients with WD, but its mode of action is not clear. TTM increases fecal copper, but it is unknown whether that is because of intestinal copper uptake inhibition or a result of increased biliary excretion.[13] Also, it is unclear what effect the formation of the TPCs has on bioavailable non-ceruloplasmin-bound copper in plasma and its hepatic uptake. We studied these questions using ^{64}Cu PET/CT and PET/MRI examinations before and after 7 days of TTM 15 mg/day.

Since the intestinal uptake of copper does not depend on ATP7B, the effect of TTM treatment on intestinal ^{64}Cu absorption could be studied in HVs in a small randomized-controlled double-blind study (absorption study). In that study, TTM reduced hepatic uptake of ^{64}Cu in HVs by ≈ 90% 1 hour after an oral ^{64}Cu intake and by ≈80% at 15 hours. Gallbladder excretion was similarly reduced. Blood ^{64}Cu activity was also reduced but not to the same extent. These data

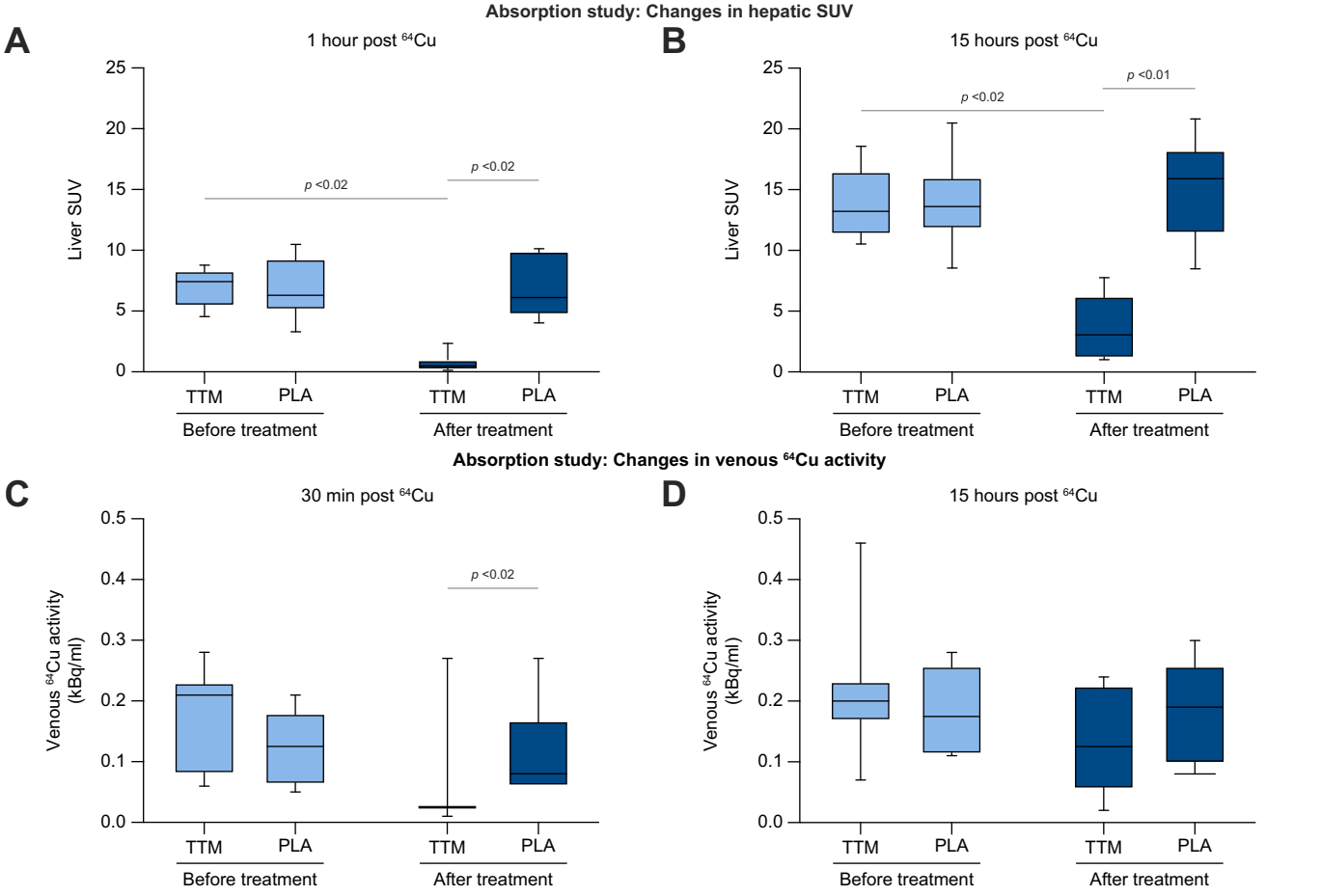

Fig. 2. Absorption study. Top panel: Liver SUV at (A) 1 hour and (B) 15 hours post ^{64}Cu, before and after treatment with either TTM or PLA. Lower panel: Venous ^{64}Cu activity 30 minutes (C) and 15 hours (D) post ^{64}Cu dose, before and after treatment with either TTM or PLA. Wilcoxon signed-rank test used to compare activity before vs after treatment. The Mann-Whitney U test was used for between group comparison. PLA, placebo; SUV, standard uptake value (a measure of ^{64}Cu activity); TTM, bis-choline tetrathiomolybdate.

Table 3. Excretion study: ^{64}Cu activity before and after treatment, in organs of interest following an i.v. dose of ^{64}Cu in patients with WD.

	TTM						
Time post ^{64}Cu (hours)	**1 hour**	**2 hours**	**6 hours**	**20 hours**	**48 hours**	**54 hours**	**68 hours**
Liver SUV, before treatment	17.77 (4.53)	21.77 (4.69)	27.12 (7.78)	33.57 (8.62)	37.71 (6.05)	36.41 (9.37)	38.66 (8.87)
Liver SUV, after treatment	7.44 (3.01)	8.76 (2.82)	14.30 (3.52)	22.59 (6.70)	28.93 (2.90)	27.22 (4.41)	28.48 (6.07)
Liver SUV before *vs.* after treatment	*p* <0.021	*p* <0.021	*p* <0.021	*p* <0.021	*p* <0.021	*p* <0.021	*p* <0.021
Gallbladder SUV, before treatment	2.17 (1.89)	3.44 (1.78)	3.10 (0.20)	3.27 (1.74)	2.80 (0.80)	3.03 (1.50)	2.15 (2.58)
Gallbladder SUV, after treatment	1.31 (1.03)	2.06 (0.98)	1.98 (1.27)	2.44 (2.48)	2.55 (0.61)	3.05 (1.26)	2.78 (1.60)
Gallbladder SUV before *vs.* after treatment	n.s.	n.s.	*p* <0.021	n.s.	n.s.	n.s.	n.s.
Kidney SUV, before treatment	7.23 (2.66)	5.59 (3.72)	4.81 (3.13)	2.79 (1.49)	1.63 (0.73)	1.73 (0.43)	1.45 (0.83)
Kidney SUV, after treatment	6.87 (2.99)	6.78 (2.73)	9.05 (3.38)	10.59 (2.11)	12.72 (2.77)	12.77 (4.49)	11.77 (1.36)
Kidney SUV before *vs.* after treatment	n.s.	n.s.	n.s.	*p* <0.021	*p* <0.021	*p* <0.021	*p* <0.021
Brain SUV$^{\phi}$, before treatment	0.091 (0.04)	0.067 (0.03)	0.085 (0.04)	0.098 (0.03)			
Brain SUV$^{\phi}$, after treatment	0.023 (0.05)	0.024 (0.03)	0.040 (0.05)	0.090 (0.02)			
Brain SUV$^{\phi}$ before *vs.* after treatment	*p* = 0.08	*p* = 0.02	*p* = 0.08	*p* = 0.56			

The brain was not assessed after 20 h. Data presented as median (IQR). n.s., not significant. Wilcoxon signed-rank test was used for statistical analysis.
SUV, standard uptake value; TTM, bis-choline tetrathiomolybdate.
$^{\phi}$Brain SUV was corrected for blood ^{64}Cu activity.

demonstrate that TTM strongly inhibits intestinal uptake of ^{64}Cu but also that the hepatic handling of ^{64}Cu is affected by TTM treatment. In addition, the data contradicts the belief that TTM would increase biliary excretion, at least in human HVs.

In the second study (excretion study) we examined the effect of TTM on the fate of ^{64}Cu after i.v. injection. Since we were specifically interested in the hepatic handling of bioavailable copper, in which ATP7B is involved, this study had to be carried out in patients with WD. In this study, venous TTM concentrations should have reached ≈75-85% of steady state levels and illustrate effects during long-time administration based on data on half-life from previous studies conducted by Alexion (Data not publicly available).

Blood ^{64}Cu activity was significantly increased on TTM by ≈600% 1 hour after i.v. injection and ≈240% after 20 hours. Thus, TTM treatment somehow retained ^{64}Cu in plasma. TTM is known to form TPCs with copper and albumin. Our data strongly suggest that ^{64}Cu in these complexes inhibits the removal of

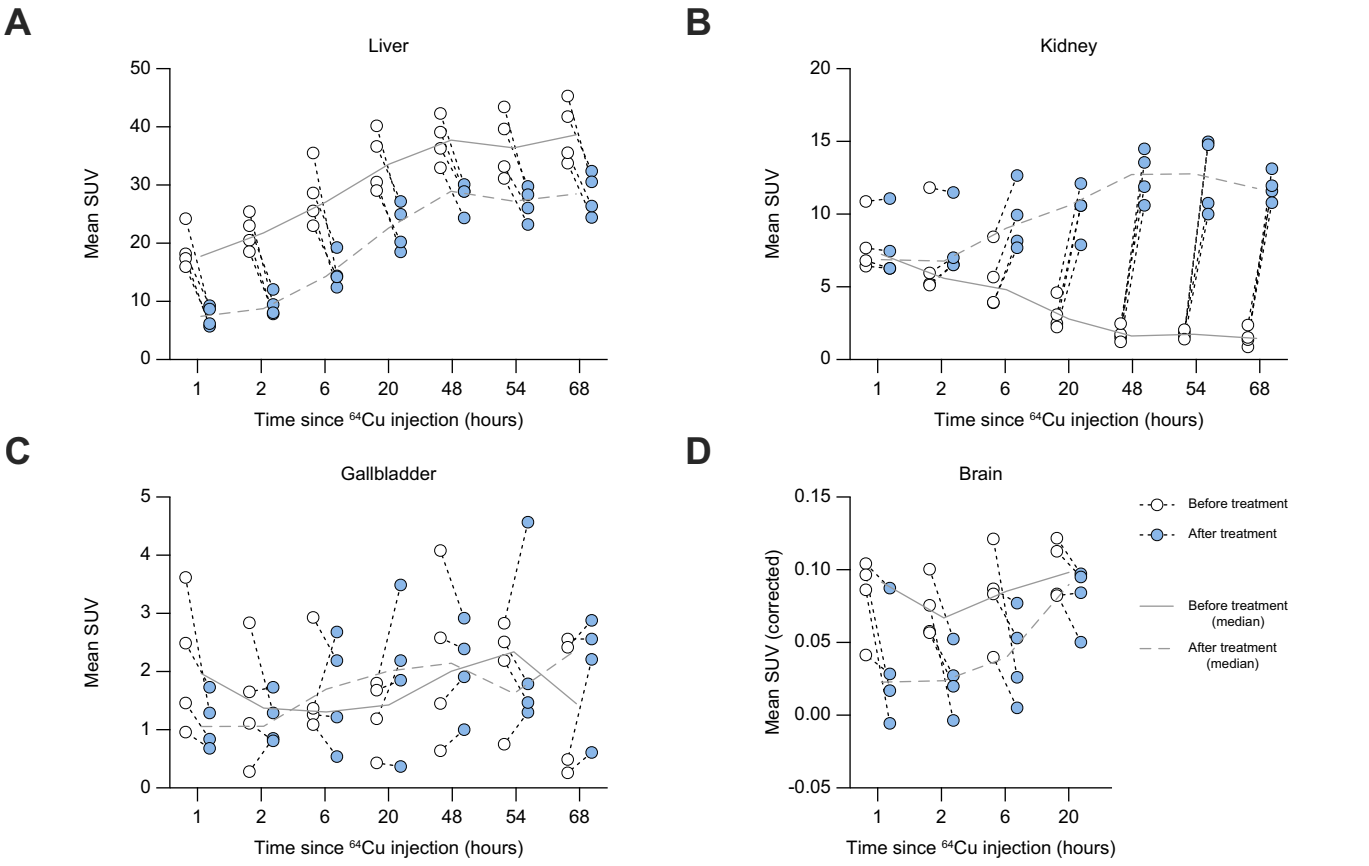

Fig. 3. Excretion study: ^{64}Cu activity in the liver, kidney, gallbladder and brain 1 – 68 hours after ^{64}Cu injection. Liver SUV 1 to 68 hours post ^{64}Cu injection, before and after TTM treatment (A). Kidney SUV (B). Gallbladder SUV (C). Brain SUV corrected for blood ^{64}Cu levels (D). Note different Y-axes and non-linear X-axes. SUV, standard uptake value (a measure of ^{64}Cu activity); TTM, bis-choline tetrathiomolybdate.

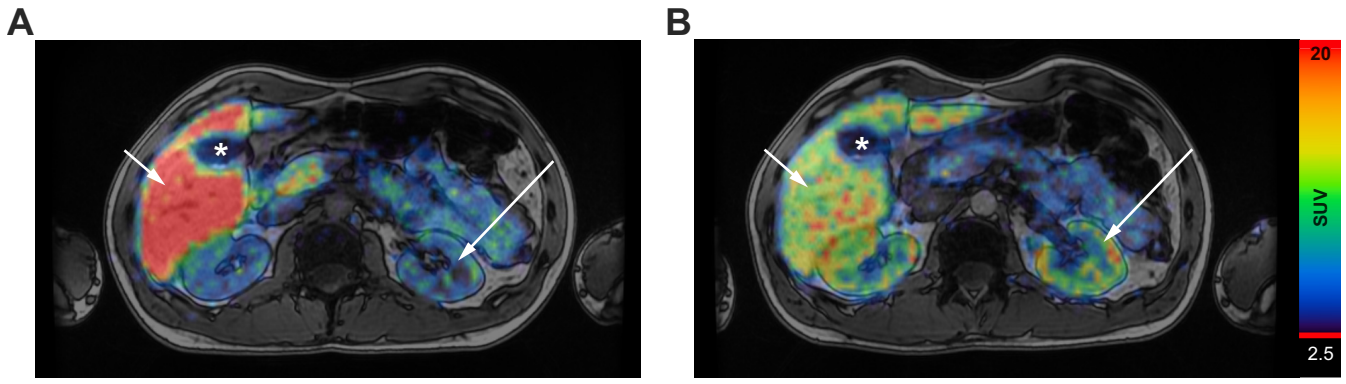

Fig. 4. Fused axial abdominal PET/MRI image showing the liver (short arrow), gallbladder (*) and kidney (long arrow), 6 hours post ^{64}Cu injection. Images shown before treatment (A) and after TTM treatment (B). SUV scale 2.5 (Black) – 20 (Red). SUV, standard uptake value (a measure of ^{64}Cu activity); TTM, bis-choline tetrathiomolybdate.

^{64}Cu from the blood stream. In accordance, even though blood ^{64}Cu was higher after TTM, hepatic SUV was reduced by ≈50-60% within the first 6 hours and by ≈30-40% from 20–68 hours after i.v. ^{64}Cu administration. Similarly, brain SUV was lower after TTM, reaching statistical significance at the 2-hour time point. Gallbladder SUVs were very low, as would be expected in patients with WD, and were not affected by TTM, suggesting no enhancement of biliary copper excretion. Thus, the excretion study showed that TTM retains ^{64}Cu in the blood – likely due to formation of TPCs – and inhibits copper uptake by the liver and other organs, including the brain.

Since ^{64}Cu was not quantitatively detected in bile or urine, our data raised the question of where it was actually distributed to. A secondary analysis showed that the total ^{64}Cu within the PET field-of-view (from the top of liver to the upper femur) at the 48-hour and 68-hour time-points accounted for 80-90% of the injected dose. The remaining 10-20% was likely distributed outside the field-of-view. This was very constant across patients (<5% variation) and time-points (<10% variation) and only slightly modified by treatment (Table S5). The organs with a significant amount of ^{64}Cu at the 68-hour timepoint, accounted for ≈80% of injected dose (%ID) before treatment (liver 78%ID, blood 2%ID and kidneys 0.5%ID) but only ≈ 60%ID during TTM treatment (liver 53%ID, blood 2%ID, and kidneys 4%ID) (Fig. S4). This supports the interpretation that TTM did not facilitate excretion of copper, but rather redistributed it dispersedly in the body. In a similar study, we showed distribution into muscle and fat but in the present study we could not identify meaningful pre- to post-treatment differences in these tissues, possibly due to weak signals.[22]

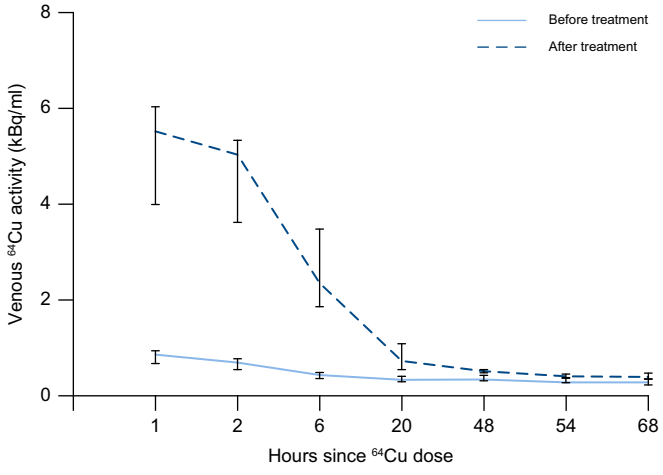

Fig. 5. Excretion study: Venous blood ^{64}Cu counts (kBq/ml) corrected for dose and bodyweight 1 - 68 hours after ^{64}Cu injection. Median line (IQR) before and after TTM treatment. Note non-linear X-axis. TTM, bis-choline tetrathiomolybdate.

The finding that TTM did not increase biliary excretion of ^{64}Cu was surprising, as that has been considered as a primary mode of action. Previous studies reported increased fecal copper during treatment with ammonium tetrathiomolybdate in patients with WD but it was unclear whether this was due to reduced absorption or increased biliary copper excretion.[13] Some studies in a rat WD model reported significantly increased biliary copper excretion after TTM was administered i.v. in large doses (approximately 10 mg/kg)[15,16] while others found that inhibition of intestinal copper absorption was more important.[17,24] With our data we can conclude that formation of TPCs in plasma does not enhance hepatic excretion of copper. We can also conclude that TTM does not stimulate biliary excretion of copper that is taken up by the liver from the pool of bioavailable copper in plasma within the time frame of our study. The PET/CT assessment cannot follow non-radioactive copper so from a theoretical point of view, TTM could have stimulated excretion of “cold” copper in the liver. There are different compartments of copper in the liver, yet we find this possibility highly unlikely.

We showed that TTM effectively inhibited intestinal copper uptake in humans (absorption study). The inhibition of intestinal ^{64}Cu absorption by 80% on TTM is greater than the 50% reported with zinc.[21] Since the normal copper absorption is up to 50%, a further 80% inhibition will reduce absorption to 10% of intestinal copper.[25] Because the intestinal juices contain around 2 mg of copper, approximately equivalent to the dietary uptake of 2 mg/day, such an effect will theoretically create a negative copper balance of 1.8 mg (28 μmol)/day which is meaningful for therapeutic purposes. Our data did not explain how TTM inhibited intestinal uptake of copper. Treatment with zinc inhibits intestinal uptake of copper by induction of MT. We did not measure intestinal MT but since TTM did not induce MTs in PBMC it is unlikely that TTM inhibited intestinal uptake by the same mechanism as zinc. A likely alternative explanation is that chelation of copper by TTM in the intestinal lumen

prevents absorption. In such a case, the time from TTM to ^{64}Cu administration may be important (1 hour in the absorption study) and maximal effect on dietary copper intake may require dosing before each meal. In the randomized comparison of ammonium tetrathiomolybdate *vs*. trientine,[12] 20 mg of the tetrathiomolybdate formulation was administered 6x per day (3x with meals and 3x between meals). It is likely that inhibition of intestinal copper absorption contributed to the treatment effect, although doses cannot be directly compared to those of TTM. In the present study peroral ^{64}Cu was given 1 hour after the last TTM dose to achieve maximal effect. Our data do not provide information on how this effect will change with time after the last dose.

Data suggests TTM can mobilize intracellular copper.[26–28] Since MT is important for intracellular copper binding, we examined if TTM changes mRNA expression of *MT* and other metal binding proteins in PBMCs, which correlates well with changes in the liver.[29] There was a trend that TTM increased *MT* mRNA expression and reduced *ZNT1* mRNA expression. These changes were not sufficient to support further speculation, but more studies into these effects seem warranted. Taken together, our data suggest a dual action of TTM involving inhibition of intestinal uptake and retention in the blood of bioavailable copper, leading to a reduction in the copper exposure of the liver, and likely also the brain.

An unanswered question is the further fate of the TPC. Formation of this complex likely explains that TTM increased plasma ^{64}Cu by ~600% 1 hour and 2 hours after i.v. dosing. At the 68-hour time point, ^{64}Cu in blood was still higher after TTM but reduced to 200%, so the complexed copper somehow left the blood stream. We were not able to localize where this ^{64}Cu was distributed to. As seen in Fig. 3, the hepatic SUV was reduced by the same amount at different time-points, so the TPC did not seem to be distributed into the liver.

Interestingly, renal SUV rose and plateaued at statistically significantly higher levels on TTM treatment after an i.v. ^{64}Cu dose, whereas bladder ^{64}Cu activity was unchanged. Consequently, TTM did not seem to increase urinary excretion of ^{64}Cu as also supported by other studies.[12,13] Kidney SUV plateaued and did not decrease in parallel to blood ^{64}Cu activity, most likely because the TPC has specific affinity for this organ. The increase in renal ^{64}Cu accounted for approximately 4%ID and does not by itself explain the ≈25%ID decrease in liver ^{64}Cu content, see Fig. S4. The effect of renal accumulation of TPC is unclear. Our findings are in line with a recent study which showed renal accumulation of copper and molybdenum in TTM-treated rats, but no kidney damage was seen over 3 months.[30] To our knowledge, renal damage after TTM has not been reported in humans, nor was there any biochemical evidence of this in the present study.

Brain scans were only included in the excretion study where cerebral SUV was significantly reduced by TTM 2 hours after the i.v. ^{64}Cu dose and trended to be lower at 1 hour and 6 hours. The low signal level makes any interpretation uncertain. However, protection against cerebral uptake of bioavailable copper may explain the rapid beneficial effects of TTM on neurological status and why TTM appears less likely to cause paradoxical neurological worsening.[7,11,12]

In summary, TTM did not facilitate biliary copper excretion in humans but seemingly had a dual mode of action. TTM inhibited intestinal copper absorption by 80-90%. Once TTM itself was absorbed it was able to rapidly bind and retain copper in the blood, reducing the exposure of organs like the liver and brain to copper.

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Abbreviations

%ID, percentage of injected dose; HV, healthy volunteer; MT, metallothionein; MT1, metallothionein 1; PBMCs, peripheral blood mononuclear cells; PLA, placebo; SUV, standard uptake value; TPC, tripartite complex; TTM, bis-choline tetrathiomolybdate; VOI, volume of interest; WD, Wilson disease; ZNT1, zinc transporter 1.

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Conflict of interest

FTK received grants from Alexion, AstraZeneca. ESS and AMQ are employed by and own stock with Alexion, AstraZeneca. AL is the chairperson for the Danish Society of Pharmacology. MLS advises Arbormed and DepYmed, he received grants from Orphalan, Vivet, DepYmed, Alexion, AstraZeneca and the Wilson Disease Association. PO consults for Orphalan, he advises Ultragenyx, Vivex and Yaqrit. TDS consults for Arbormed, Vivet and Ultragenyx, he advices and received grants from Alexion, AstraZeneca, he is on the speaker's bureau for Orphalan. The remaining authors have no conflicts to report.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

FTK, DEM, ESS, AMQ, PO, and TDS. conceptualized the study and designed the protocol. FTK, DEM, and MV performed the PET experiments. AL performed metallothionein analysis. FTK performed the formal analysis. FTK, PO, and TDS did the original draft preparation. DEM, ESS, AMQ, MV, and MLS reviewed and edited the manuscript. FTK, PO and TS acquired the funding. All authors have read and agreed to the final version of the manuscript. FTK and TDS verified the data.

Data availability statement

The data that support the findings of this study are available from the corresponding author, [author initials], upon reasonable request.

Role of funding source

The studies were part of a study series on the effects of drugs in WD and financed by Alexion, AstraZeneca Rare Disease, Boston, MA, USA. Two authors, ESS and AMQ, are employees of the funding source. The study design was agreed upon by all investigators, and ESS and AMQ participated in data interpretation. However, the investigators (FTK, DEM, MV, AL, MLS, PO and TDS) maintained full control of the manuscript.

Clinical trial number

Data is presented from two clinical trials: Absorption study: EudraCT 2020-005832-31; Excretion study: EudraCT 2021-000102-25.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2023.11.023.

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Author names in bold designate shared co-first authorship

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