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RESEARCH ARTICLE

Methanobactin rapidly facilitates biliary copper excretion in a Wilson disease rat model visualised by ^{64}Cu PET/MRI

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Background and Purpose: Methanobactins are peptides with high copper affinity and potential to treat Wilson disease. We examined how two methanobactins (ARBM101 and MB-OB3b) affected copper handling in the LPP *Atp7b*$^{-/-}$ Wilson disease rat model, compared to penicillamine or saline, by ^{64}Cu positron emission tomography/magnetic resonance imaging. Heterozygotes served as controls.

Experimental Approach: ^{64}Cu was administered i.v. to 19 LPP and four control rats. A baseline scan was performed 1 h later. LPP rats received one dose of saline, penicillamine, MB-OB3b or ARBM101 i.p. ($t = 100$ min), followed by a 90-min scan and a final scan at $t = 24$ h. Controls followed identical procedures without intervention. ^{64}Cu levels were evaluated as % injected dose (%ID) in the liver, kidney and 'abdominal-pelvic region' (intestines and other non-hepatic, non-renal organs).

Key Results: At baseline, hepatic %ID was ≈50% higher in LPP rats than in controls. Intraintestinal ^{64}Cu activity, indicating biliary excretion, was present in controls and absent in LPP rats. After methanobactin injection (but not saline or penicillamine), ^{64}Cu appeared in the small intestines of LPP rats within 10–15 min. Hepatic %ID increased over 24 h in saline-, penicillamine- and MB-OB3b-injected rats but decreased in control rats. ARBM101 almost normalised hepatic ^{64}Cu at 24 h.

Conclusions and Implications: A single i.p. methanobactin dose restored biliary copper excretion in LPP rats. The effect was more pronounced with ARBM101 than with MB-OB3b. Non-ATP7B transporters must be involved because ATP7B is absent in LPP rats. Methanobactin may have therapeutic potential in Wilson disease.

KEYWORDS
Chelation therapy, Positron-Emission Tomography, copper-binding protein, copper metabolism

Abbreviations: ID, Injected Dose; LPP, Modified Long-Evans Cinnamon; MB, Methanobactin; WD, Wilson disease.

1 | BACKGROUND

In Wilson disease (WD), mutations in the ***ATP7B*** gene cause malfunction in the copper-transporting ATPase, ATP7B, protein that is necessary for hepatic excretion of excess copper into the bile. As a consequence, copper accumulates in tissues, primarily the liver and brain, and Wilson disease patients typically present with hepatic and/or neurological symptoms (Ala et al., 2007; Bull et al., 1993). Untreated, the disease progresses to death (Ala et al., 2007). Current treatment options include chelating drugs such as **penicillamine** and trientine, which bind copper and increase urinary excretion, as well as zinc, which decreases intestinal copper uptake (Ferenci, 2004; Roberts & Schilsky, 2008). These treatment options generally work well. However, there are also important shortcomings: Early neurological worsening is seen in a substantial fraction of patients with the neurologic phenotype and is not always reversible (Kumar et al., 2022; Litwin et al., 2015; Mohr et al., 2023; Weiss et al., 2013), late progression of both neurological and hepatic disease is a clinical problem (Mohr et al., 2023) and current medications may not be sufficient to rescue patients with acute liver failure (Nazer et al., 1986; Roberts & Schilsky, 2008). While current treatments bring some degree of control over copper metabolism, none of them restore hepatic copper excretion, and hepatic copper content may still be elevated after decades of treatment. Histological normalisation is uncommon, and in a fraction of patients, the liver disease progresses (Cope-Yokoyama et al., 2010; Sini et al., 2013; Weiss et al., 2011; Wiggelinkhuizen et al., 2009). Thus, restoring the normal physiological route of copper excretion to control total body copper levels is a relevant treatment target in Wilson disease.

Methanobactins (MBs) are a group of small peptides with extremely high copper affinity and therapeutic potential in Wilson disease (DiSpirito et al., 2016; Kang-Yun et al., 2022; Krentz et al., 2010). Two groups of MBs have been described:- Group I MBs, the methanobactin from *Methylosinus trichosporium* OB3b (MB-OB3b), typically contain two oxazolone groups, each with an associated thioamide and an internal disulphide bridge (Choi et al., 2006) and, Group II MBs, from *Methylocystis* strain SB2 (MB-SB2, recently termed ARBM101) that contains one oxazolone group with an associated thioamide and one pyrazinedione or imidazolone group with an associated thioamide as well as a sulphate group (Bandow et al., 2012) (Figure 1).

Repeated dosing of MB in the LPP rat model of Wilson disease has been shown to reduce copper in hepatic homogenate and mitochondria, delay hepatic injury, and prolong survival (Lichtmannegger et al., 2016). Subsequent studies suggested that this was caused by biliary excretion of copper-MB (Einer et al., 2023). However, these studies were based on continuous dosing, and it remains to be studied how a single dose affects the elimination of copper through the liver and the extrahepatic distribution. Also, the quantitative difference between the effect of MB-OB3b and ARBM101 on the *in vivo* hepatic copper excretion needs further investigation.

The ^{64}Cu isotope is a positron-emitting tracer with a half-life of 12.7 h, which makes it ideal for studying short-term copper metabolism by positron emission tomography (PET, Munk et al., 2023). The method has been used to study the effects of zinc, trientine and penicillamine on intestinal copper uptake in humans (Kirk et al., 2023; Munk et al., 2022), of bis-choline tetrathiomolybdate on human copper excretion and distribution (Kirk et al., 2024), and of gene therapy in a Wilson disease mouse model (Murillo et al., 2022).

In this study, we employed ^{64}Cu PET/magnetic resonance imaging (^{64}Cu PET/MRI) to examine how a single dose of MB-OB3b or ARBM101 quantitatively affected copper metabolism in LPP rats and compared that to the effects of saline and penicillamine. We further investigated ^{64}Cu distribution differences between LPP rats and heterozygote (HZ) rats, which served as healthy controls because of the recessive nature of the disease.

What is already known?

- Methanobactin is a copper-binding peptide that improves survival in a Wilson disease rat model.

What does this study add?

- PET scans using 64-copper confirm the phenotypic differences between Wilson disease and control rats.
- Methanobactin facilitates biliary 64-copper excretion within minutes in Wilson disease rats.

What is the clinical significance?

- Methanobactin removes copper from the liver via an ATP7B-independent mechanism.
- Methanobactin has therapeutic potential in Wilson disease.

2 | METHODS

2.1 | Animals

All animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). In addition these experiments were approved by the Danish Animal Experiments Inspectorate (permit 2021-15-0201-01069). The LPP rats are a well-established Wilson disease model originating from the Long-Evans Cinnamon (RRID: RGD_68066) rat strain (Ahmed et al., 2005; Reed et al., 2018). The rats are homozygote for a 13-kb deletion in the coding part of the *Atp7b* gene. This results in a disturbance in copper metabolism because of a defect in the copper transporting ATPase ATP7B, similar to the human disease. When the rats

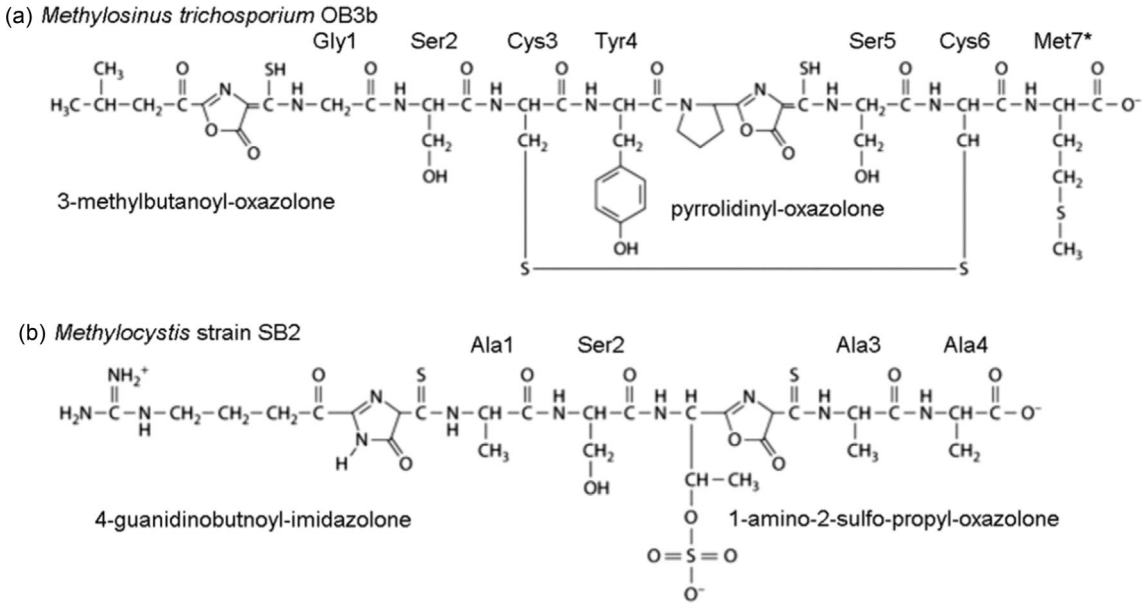


FIGURE 1 Structure of methanobactins.

are approximately 100–120 days old, they develop spontaneous hepatitis with increased liver enzymes, leading to liver failure and death.

Like humans with Wilson disease, the LPP rats have low serum ceruloplasmin, low serum copper and a high copper content in the liver. Unlike patients with Wilson disease, the LPP rats do not have detectable neurologic symptoms (Einer et al., 2023). Because of the recessive inheritance of the disorder, heterozygote *Atp7b*$^{+/-}$ rats have a normal phenotype and were used as controls in this study, ensuring a similar genetic background.

The animals used in this study were bred at the Helmholtz Centre in Munich. At least 1 week before the experiments in Aarhus, Denmark, the animals were transported from Munich to Aarhus for full acclimatisation.

In Aarhus, the animals were kept on a 12-h light/dark cycle. Temperature and relative humidity were 20 ± 1°C and 50 ± 5%, respectively, in accordance with European Recommendation 2007/526 EC. Animals were maintained *ad libitum* on Altromin® diet (1314, 13 mg kg^{-1} copper, Altromin, Germany) and treated under the guidelines for the care and use of laboratory animals at Helmholtz Munich. The rats were housed in an individually ventilated cage system.

2.1.1 | Experimental protocol

The rats were anaesthetised with isoflurane. During the scans, the rats body temperature was kept around 37°C, and heart rate and respiration were monitored to ensure stable anaesthesia levels. The study design is visualised in Figure 2. At time zero, 19 *Atp7b*$^{-/-}$ (LPP) rats and 4 *Atp7b*$^{+/-}$ (control) rats were injected with 2.5–12.4 megabecquerel (MBq) $^{64}CuCl_2$ in a tail vein. One hour later, the rats were placed in the prone position in the scanner, and a 20-min static scan was performed ($t = 60$ to $t = 80$ min).

2.1.2 | Intervention

At time point 180 min, 1 h and 40 min after the baseline scan, LPP rats were intraperitoneally (i.p.) injected with 1 ml 0.9% NaCl, MB-OB3b (150 mg kg^{-1} body weight), ARBM101 (110 mg kg^{-1} body weight), or penicillamine (100 mg kg^{-1} body weight). The *Atp7b*$^{+/-}$ control rats did not receive any interventions.

2.1.3 | First post-intervention scan

In the first six animals (one MB-OB3b, one saline, one control, two ARBM101 and one penicillamine), the dynamic 90-min post-intervention scan was started 1 h after the first intervention. This revealed a very fast excretion in the MB group, and consequently, we redesigned the study so that the remaining 17 animals were scanned dynamically immediately after the drug injection (time period 180–270 min, Figure 2). Thus, the copper content in the first 30 min of the dynamic scan protocol for the first six animals was comparable to the copper content in the last 30 min of the scan for the remaining animals. After this scan, the rats were allowed to recover from the anaesthesia and returned to their home cages.

2.1.4 | Second post-intervention scan

At time point 1440–1460 min (24 h), the animals underwent a final static PET scan lasting 20 min and were then killed by cervical dislocation under continued anaesthesia. No blood or tissue sampling was performed. During the studies, the animals had free access to food and water.

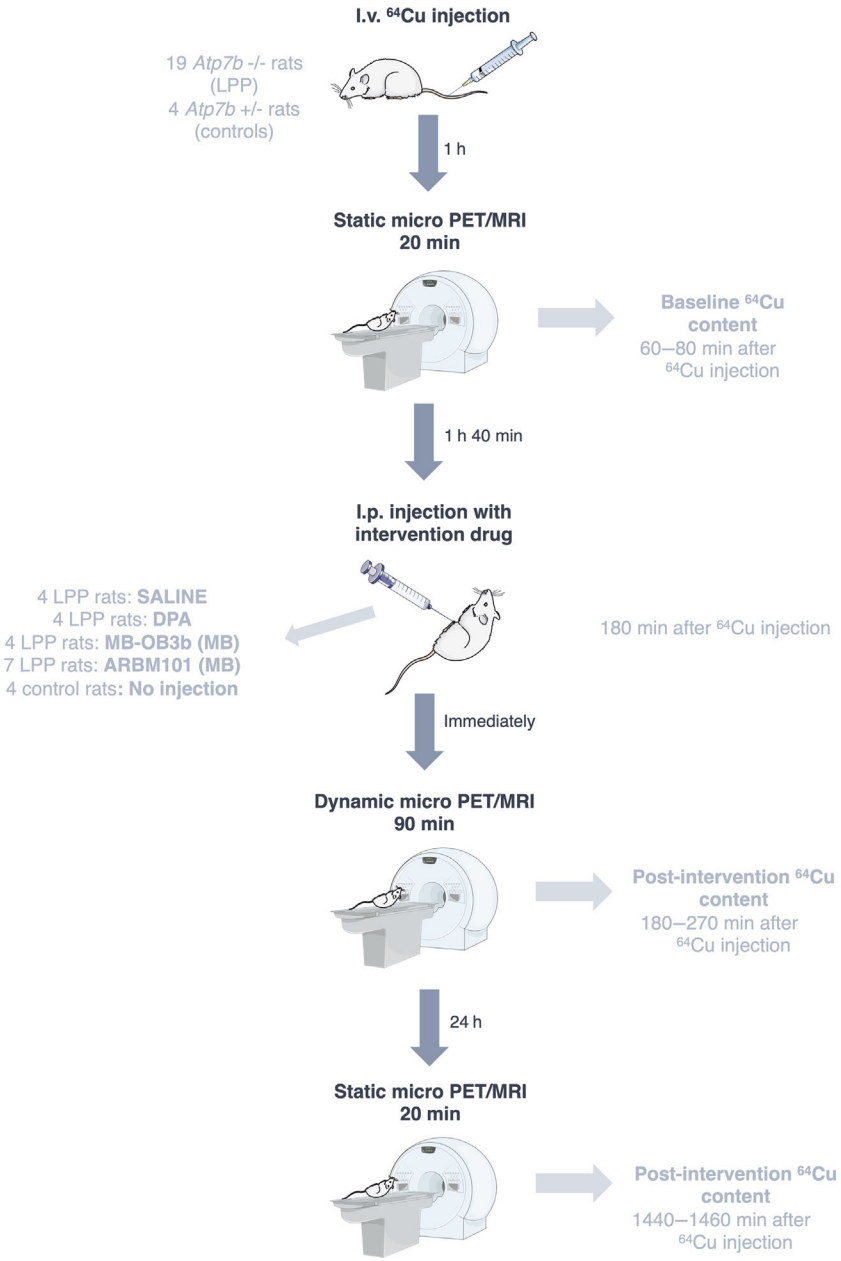


FIGURE 2 Study design.

2.2 | Image acquisition and reconstruction

Animals were scanned using PET (Mediso Medical Imaging Systems, Budapest, Hungary) after injection with the tracer $^{64}CuCl_2$. The PET scan was combined with micromagnetic resonance (micro-MR, 1-Tesla). The PET images were reconstructed with a three-dimensional ordered subset expectation algorithm (Tera-Tomo 3D; Mediso Medical Imaging Systems) with four iterations, six subsets and a voxel size of $0.6 \times 0.6 \times 0.6$ mm^3. Data were corrected for dead time, decay and randoms using a delayed coincidence window without corrections for attenuation and scatter.

2.3 | PET data handling

The PET scans were analysed using PMOD (version 4.0, PMOD Technologies LLC, RRID: SCR_016547). The PET data were supported by an MRI scan to visualise the anatomy. It was possible to separately analyse ^{64}Cu content in the liver, kidneys and the 'abdominal-pelvic region' that included the colon, small intestine, pancreas, spleen, stomach, large blood vessels and female reproductive organs. The latter was used because the small intestines and colon were difficult to separate because of the MRI resolution. Data are presented as the percentage of the injected dose (%ID) or standard uptake value (SUV; g ml^{-1}) in each organ or region. We defined the injected dose (ID) as

the total activity of the rat on the whole-body scan at baseline (60 min after the ^{64}Cu injection). Standard uptake value is a measure of mean activity per millilitre and is normalised to the ^{64}Cu dose per gram body weight and corrected for radioactivity decay and was used for visualisation of ^{64}Cu distribution.

We calculated the %ID in each organ by measuring the mean radioactivity (kBq ml^{-1}) in a volume of interest (VOI) covering the entire organ and correcting the radioactivity for decay and injected dose as defined earlier. Only the left kidney was analysed to avoid spill-over from the liver, and the %ID was calculated for both kidneys by multiplying by two.

Blinding was not implemented during image analysis, as radioactivity quantification was based on standardised whole-organ volume of interests. This approach minimised the risk of selection bias, as no subjective delineation of sub-regions was required.

2.4 | Statistics

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2025). Data were transferred from PMOD to GraphPad Prism 10 for macOS (GraphPad Software LLC, version 10.0.02, RRID: SCR_002798). Because of the high cost of tracer and PET/MRI technology and the limited availability of LPP rats, it was necessary to restrict the number of experimental animals to a minimum. Accordingly, the primary outcome was determined to be binary, whether MB stimulated biliary copper excretion in LPP rats. Power calculation was performed yielding a need for at least three rats per group, with an estimated effect on the abdominal-pelvic ^{64}Cu and alpha = 0.05, power = 0.90. To account for unforeseen variations, we aimed for 4–5 animals per group. The normality of data was tested by inspecting QQ plots. Differences between three or more groups were tested with one-way analysis of variance (ANOVA) and between two groups with Student's *t*-test because the data were normally distributed. Post-hoc pair-wise comparisons were performed only when the ANOVA was statistically significant. Only the primary outcome was subject to comparative statistical analysis because of the small sample size. Secondary outcomes were assessed, but no comparative statistical analyses were performed, and these data must be considered exploratory in accordance with the British Journal of Pharmacology's guidance on design and analysis. Data are presented as means with standard deviations or for individual animals. A threshold of $P < 0.05$ was used to indicate statistical significance.

2.5 | Materials

2.5.1 | Copper chelators

Both methanobactins (MBs) were provided by Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, USA, and sent via the Institute of Toxicology and Environmental Hygiene, Munich, Germany to Aarhus in lyophilised form. Methanobactin OB3b (MB-OB3b) was isolated from *M. trichosporium* OB3b and ARBM101 from a proprietary strain as previously described (Bandow et al., 2012; Krentz et al., 2010). The purity of the MBs was 98%, and MB was dissolved in 0.9% sodium chloride to a 25 mg ml^{-1} concentration for MB-OB3b and 16 mg ml^{-1} for ARBM101. The solutions were stored at −20°C in 1-ml cryotubes.

Penicillamine was likewise provided by the Institute of Toxicology and Environmental Hygiene, Munich, Germany, and sent to Aarhus in powder form. The drug was dissolved in sterile water to 16 mg ml^{-1} and stored at 4°C.

2.5.2 | Radiochemistry

The ^{64}Cu radioisotope was obtained from Hevesy Laboratory, Danish Technical University Nutech, Risø, Roskilde, Denmark. The ^{64}Cu tracer solution (a sterile acetate-buffered solution of $^{64}CuCl_2$) was subsequently prepared and quality-controlled at our centre as previously described (Sandahl et al., 2022).

Details of other materials and suppliers are provided in specific sections.

2.6 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/ (Alexander et al., 2023).

3 | RESULTS

Twenty-three rats [19 LPP (*Atp7b*$^{-/-}$) and four controls (*Atp7b*$^{+/-}$)] were scanned from March 2022 through March 2023. There were no differences regarding age, weight or injected ^{64}Cu dose in the five groups (Table S1). One LPP rat treated with ARBM101 died during the second scan, most likely because of an anaesthesia overdose.

3.1 | The LPP rat versus controls

On the baseline scans, performed 1 h after the i.v. injection of ^{64}Cu, the hepatic %ID was ≈50% higher in the liver of LPP rats (51.9 ± 6.2% ID) compared to controls (31.2 ± 4.9%ID, Figure 3a). At later time points, this difference increased (Figure 3b), and at 24 h, the hepatic %ID was ≈85% in LPP rats and ≈20% in controls. The observation suggested a Wilson disease-like phenotype with dysfunction of the ATP7B protein and no biliary excretion. In accordance with differences in biliary excretion, the %ID in the abdomino-pelvic region at the baseline scan was significantly lower in the LPP rats (7.9 ± 1.7%)

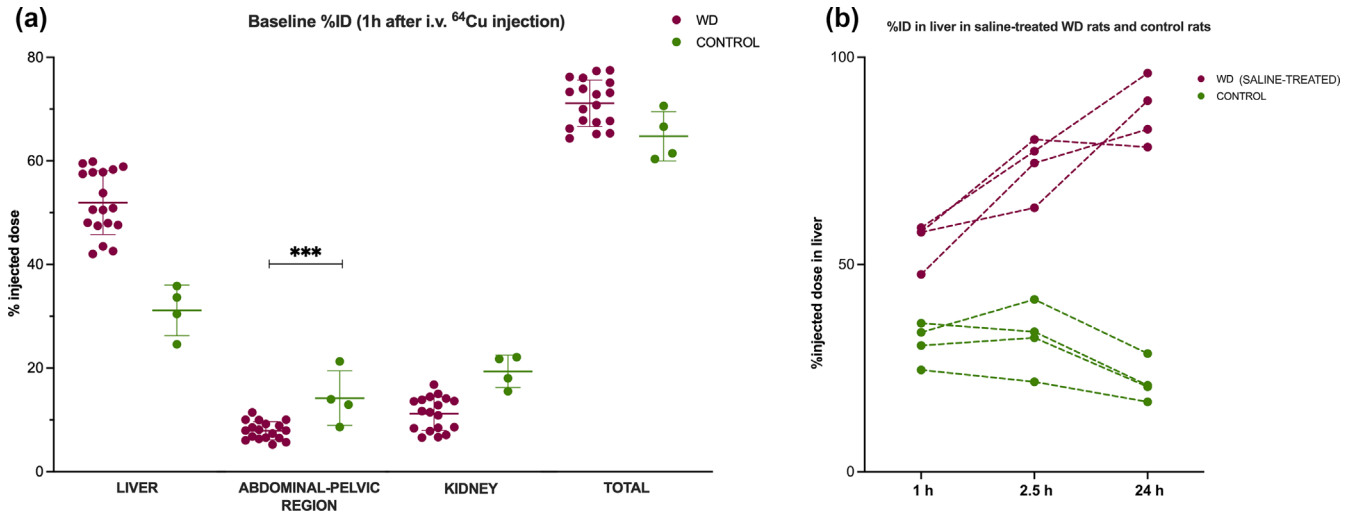


FIGURE 3 % injected dose (ID) in organs. (a) %ID in liver, abdominal-pelvic region, kidney and total accounted for in these organs in Wilson disease (WD) and control animals at baseline (1 h after i.v. tracer injection, prior to intervention). (b) %ID in liver in saline-treated WD animals and control animals at 1, 2.5 and 24 h after i.v. tracer injection. Lines indicate mean and standard deviation. (a) Because of a technical error in the baseline scan, one ARBM101-treated animal is not included (n=18 in the WD group, n=4 in the control group). (b) Only saline-treated LPP rats are included (n=4 in the WD group, n=4 in the control group).***$P < 0.001$.

than in the control rats (14.2 ± 5.3%, $P = 0.0003$, Figure 3a). As seen in the baseline PET scans (Figure S1), the distribution of ^{64}Cu in the abdomino-pelvic region was more diffuse in the LPP rats than in controls. In the controls, there was localised activity in the small intestines which was not seen in the LPP rats, again highly suggesting the absence of biliary excretion in the LPP rats. The remaining abdomino-pelvic activity in LPP rats most likely represents the distribution in blood and in the intestinal wall. The renal %ID was ≈50% lower in LPP rats (11.2 ± 3.2%ID) than in controls (19.4 ± 3.11%ID, Figure 3a).

3.2 | Effects of the intervention

After the baseline scan, LPP rats were treated in four groups: Saline, penicillamine, MB-OB3b and ARBM101 given intraperitoneally, followed by a 90-min dynamic scan.

Observations in the saline- and the penicillamine-treated LPP rats were similar during the 90-min post-intervention scan and at 24 h (Figures 4–6). ^{64}Cu was not at any timepoint visible in the lumen of the small or large intestines in penicillamine- or saline-treated LPP rats (Figure 5). The renal %ID was also not affected by penicillamine when compared to the saline-treated LPP rats (Figure S2).

In contrast to penicillamine, ARBM101 reduced the hepatic %ID at 24 h when compared to the saline- and penicillamine-treated LPP rats, but only partially normalised hepatic %ID when compared to controls (Figure 7). MB-OB3b did not reduce the hepatic %ID at 24 h; although during the 90-min post-intervention scan, the abdomino-pelvic %ID rose to normal levels with both MB-OB3b and ARBM101 (Figures 4 and 5). This effect was visible already 5–20 min after the i.p. intervention (Figure 4a), demonstrating a very fast action of the MBs. The hepatic copper excretion during the 90-min dynamic scan was not statistically significantly different between ARBM101 and MB-OB3b ($P = 0.1$). The apparent difference between the curves in Figure 4a was caused by a single MB-OB3b treated animal with reduced ^{64}Cu excretion to the gut, likely because of variable MB uptake rates following the i.p. injection.

LPP animals in all treatment groups had comparable levels of ^{64}Cu in the kidneys, which remained lower than controls (Figure S2A).

Figure 6 shows the total %ID accounted for in the liver, kidneys and the remaining abdominal-pelvic region at baseline and 24-h post-intervention in the five groups. In the saline- and penicillamine-treated LPP rats, total %ID was high, 98.1 ± 12.9% and 91.3 ± 12.9% of the injected dose, respectively, while it was lower in control rats (42.8 ± 8.9%). Based on earlier findings, this illustrates the excretion rate of accumulated tracer in the body (Munk et al., 2023), which was severely reduced in the LPP animals. The MBs partly normalised total %ID in LPP rats to 77.9 ± 9.1% after MB-OB3b and even to 67.4 ± 3.6% with ARBM110. Thus, MB-treated animals appeared to have lower ^{64}Cu content in these organs after 24 h compared to saline- and penicillamine-treated animals, most likely because the ^{64}Cu had been excreted in dropped faeces.

4 | DISCUSSION

In this ^{64}Cu PET/MRI study in LPP (Wilson disease) and control animals, we showed that one i.p. injection with MB (MB-OB3b or ARBM101) induced a rapid biliary excretion of i.v.-injected ^{64}Cu to the gut in LPP animals. This was accompanied by decreased hepatic ^{64}Cu content in the ARBM101-treated animals 24 h after the injection. These effects almost normalised ^{64}Cu kinetics to that of control animals. The results support that ARBM101 restores biliary copper

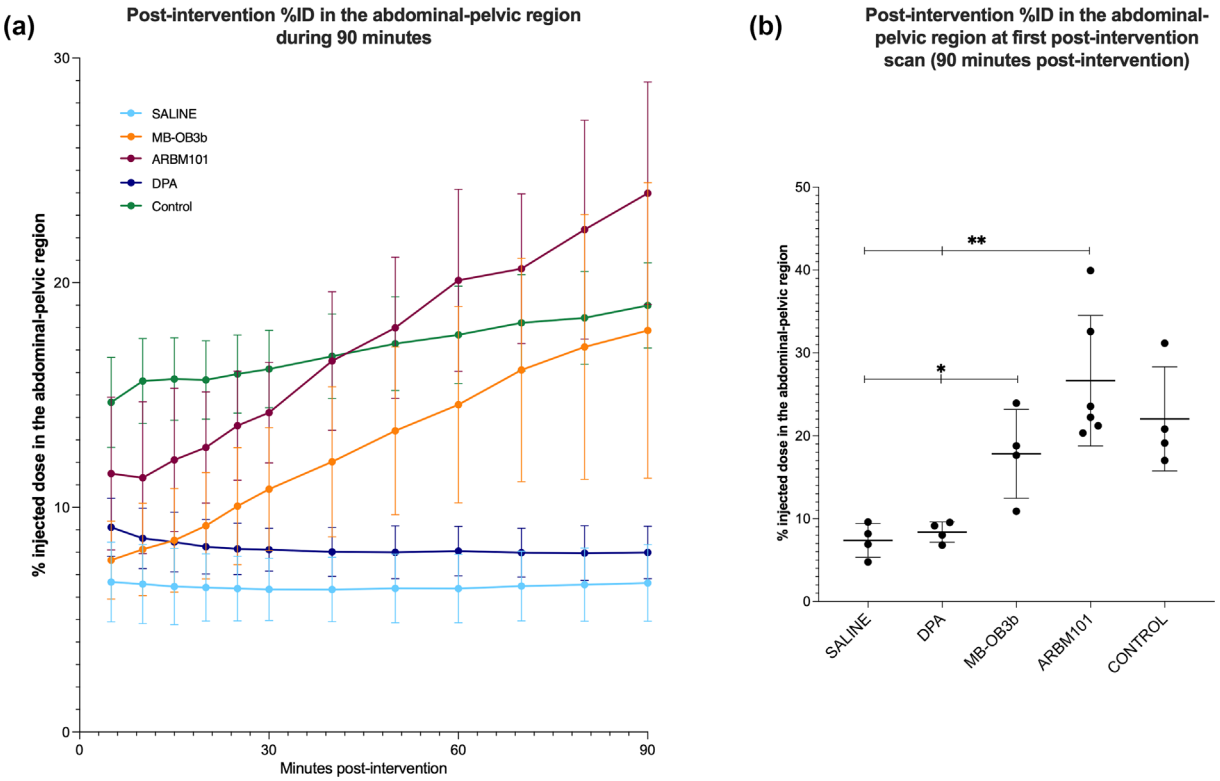


FIGURE 4 Abdominal-pelvic % injected dose (ID). (a) %ID 0–90 min post-intervention. (b) Dot plot of %ID 90 min post-intervention. (a) Dots indicate the mean, and error bars the standard deviation. The figure includes animals scanned immediately after the intervention, and one ARBM101-treated animal died during the dynamic scan, thus not included in this figure (n=5 in the ARBM101 group, n=3 in the other groups). (b) Middle lines indicate the mean, and error bars the standard deviation. One ARBM101-treated animal died during the dynamic scan, thus not included in this figure (n=6 in the ARBM101 group, n=4 in the other groups). $^{*}P < 0.05$. $^{**}P < 0.01$.

excretion in this Wilson disease rat model and, by one single injection, removes copper from the liver to a degree that is quantifiable with ^{64}Cu PET and comparable to findings in non-Wilson disease control rats.

We initially confirmed the phenotypic difference between LPP and control animals after i.v. injection of ^{64}Cu and showed that LPP animals, as expected, did not excrete ^{64}Cu to the gut but instead accumulated the tracer in the liver. Our findings can be compared to a ^{64}Cu PET study in *Atp7b* knock-out (KO) mice, where 30% of the injected dose was present in the liver 1 h after i.v. injection, compared to only 15%ID in wild-type mice (Peng et al., 2012). In another study, the hepatic tracer content was 60–80%ID in *Atp7b* KO mice after 72 h and 15–20%ID in wild-type and heterozygote mice (Murillo et al., 2022). These results are very similar to ours, with ≈50%ID in the liver of Wilson disease rats and ≈30%ID in heterozygote control animals 1 h after injection and ≈85%ID and ≈20%ID, respectively, after 24 h. The amount of ^{64}Cu in the liver after i.v. injection is also similar to humans, where Wilson disease patients accumulate ≈45%ID in the liver after 90 min and ≈65%ID after 20 h (Munk et al., 2023; Sandahl et al., 2022).

One hour after the ^{64}Cu injection in the LPP rats, the tracer content in the liver, kidneys and abdominal-pelvic region together accounted for ≈70%ID. The remaining ^{64}Cu is likely distributed among blood, skeletal muscle, skin and subcutaneous fat, as described in wild-type rats and humans (Munk et al., 2023; Weiss & Linder, 1985). In saline-treated LPP animals, the hepatic %ID increased from baseline (1 h after the injection) to the end of the first post-intervention scan (≈4–5 h after the injection), a result of extrahepatic copper slowly being delivered from other organs to the eliminating organ, the liver, similar to observations in humans (Munk et al., 2023).

We found that control animals had higher renal ^{64}Cu levels than LPP animals. This was not seen in humans (Munk et al., 2023) but has been reported in ^{64}Cu PET mice studies (Gray et al., 2012; Murillo et al., 2022; Peng et al., 2012). Taken together, these observations support that the ^{64}Cu PET method effectively reproduced the characteristic disturbances of copper metabolism in the LPP Wilson disease rat model.

When the ^{64}Cu content of the liver, kidneys and abdominal-pelvic region was combined, it increased to nearly 100%ID in saline- and penicillamine-treated animals during the 24-h scan protocol. In contrast, it declined over time in control rats to ≈40%, to ≈65% in ARBM101-treated rats and to ≈80% with MB-OB3b. This reflects the rate of elimination of ^{64}Cu from the body (Munk et al., 2023; Weiss & Linder, 1985) which was partly normalised by MB. Although penicillamine is an effective chelator that increases the amount of copper excreted in the urine, we saw no decline in the %ID in the liver, kidneys and abdominal-pelvic region 24 h after the ^{64}Cu injection, most likely because the quantitative removal is too small to detect a

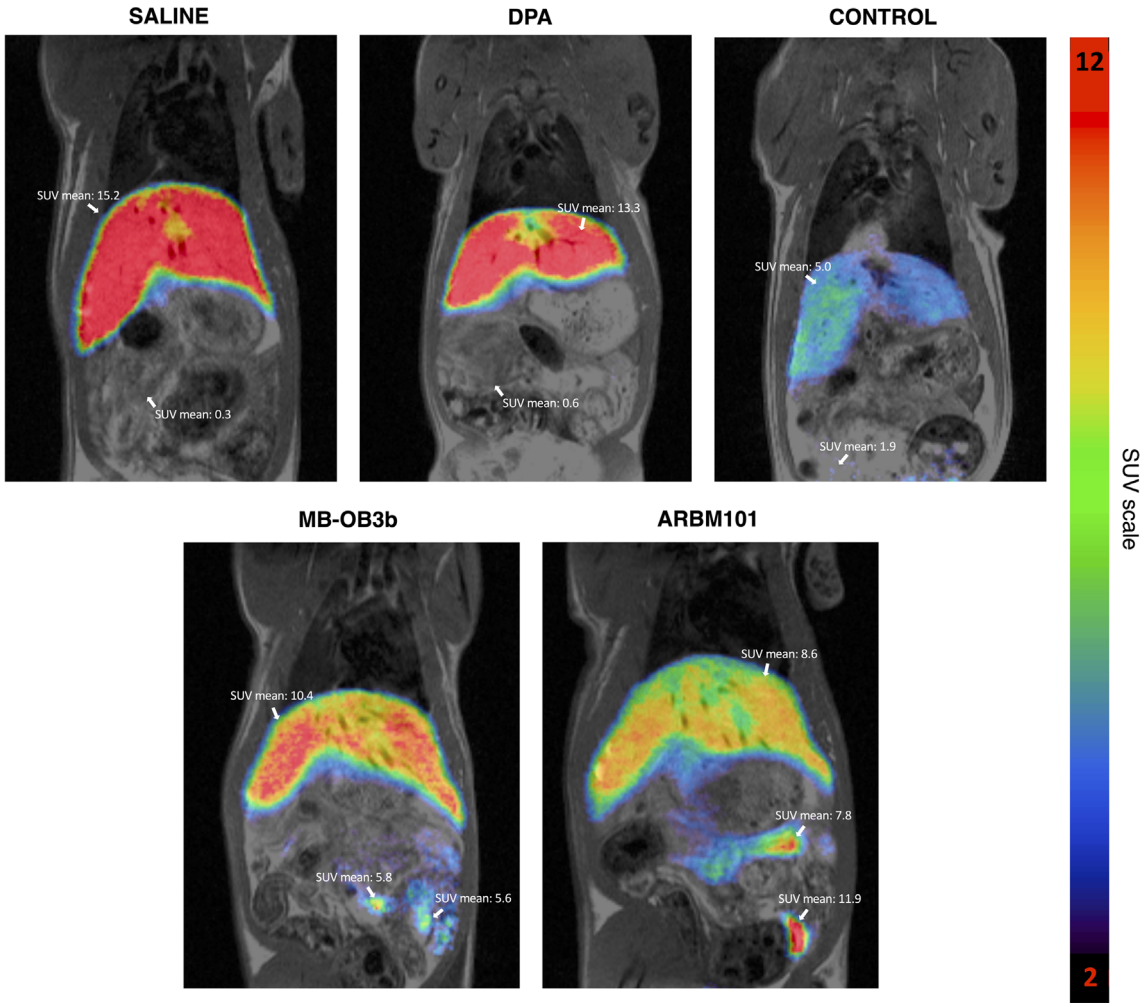


FIGURE 5 First post-intervention PET/MRI scans (accumulated activity 90 min post-intervention displayed as standard uptake value).

difference within this short timeframe. Further, penicillamine was administered 180 min after the ^{64}Cu injection, where most of the ^{64}Cu will be distributed in the tissues, and only ≈2–4% is available for chelation by penicillamine in plasma. Einer et al. found that while urinary copper was higher with penicillamine compared to MB treatment in LPP rats (0.5 μmol/24 h), this was a small quantity compared to the faecal excretion of copper with MB treatment (8–10 μmol/24 h) (Einer et al., 2023). In this study, we found a less convincing decrease in hepatic %ID in the MB-OB3b-treated rats compared to the ARBM101-treated rats, although MB-OB3b did cause significant biliary copper excretion, as seen in Figure 4, and the %ID in the liver, kidneys and abdominal-pelvic region after 24 h appeared lower than in saline- and penicillamine-treated rats (Figure 6). In accordance, Einer et al. observed that ARBM101 treatment caused a more sustained delivery of copper to the bile over 96 h with twice-daily treatments, compared to MB-OB3b (Einer et al., 2023), which may explain the differences in hepatic ^{64}Cu content between the two MBs in our study.

Our study demonstrated a very fast effect of MB on ^{64}Cu excretion to the gut within 10–15 min of i.p. injection. Such fast biliary excretion is also found in wild-type animals (Bissig et al., 2005; Owen, 1965), and the MBs seemingly imitate normal physiology. However, the transport of copper after MB treatment must be different from wild-type. In wild-type animals, hepatic uptake of copper is via the copper transporter CTR1, and biliary copper excretion and incorporation into the main copper-carrying protein ceruloplasmin are mediated by ATP7B in interaction with the trans-Golgi network. The transport of MB and MB-copper complexes into the liver, binding of MB to intrahepatic copper, and final excretion to bile are mediated by other transporters. That can be of importance, that is, concerning interactions with other drugs. We also note that, just like all the currently available treatments, MB does not restore the incorporation of copper into ceruloplasmin (Einer et al., 2023). In this study, we injected the drug intraperitoneally, which allows fast absorption into the systemic circulation. Like intraperitoneal administration, intravenous administration is effective at de-coppering LPP rats even at the acute liver failure stage (Lichtmannegger et al., 2016). Because MB is a peptide, oral administration is not intended because of gastrointestinal degradation. Therefore, intravenous or subcutaneous administration could be the preferred route for Wilson disease patients. How that will affect copper metabolism remains to be studied.

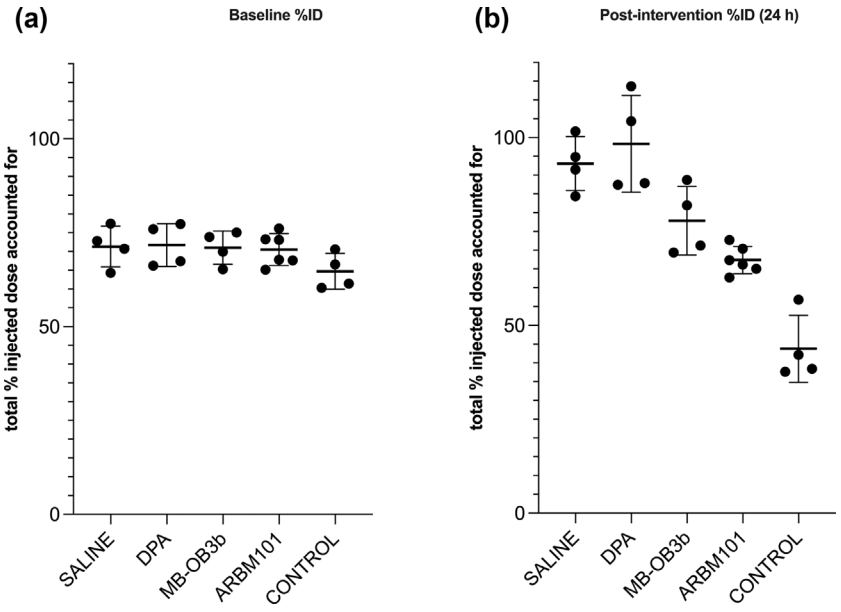
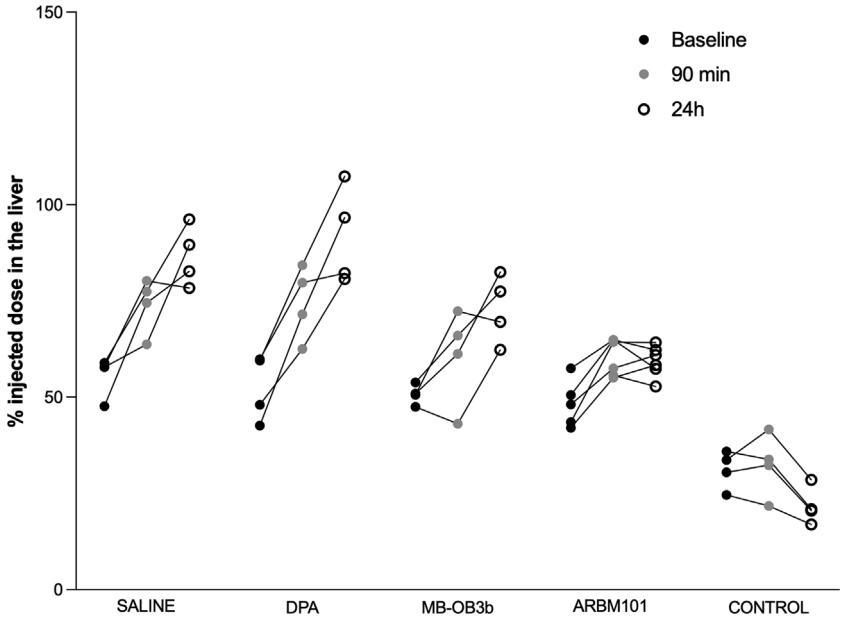


FIGURE 6 Total % injected dose (ID) accounted for in liver, abdominal-pelvic region and kidneys. (a) Baseline scan. (b) 24 h post-intervention. The middle line indicates the mean, and the error bars indicate the standard deviation. (a) Because of a technical error in the baseline scan, one ARBM101-treated animal is not included (n=6 in the ARBM101 group, n=4 in the other groups). (b) One ARBM101-treated animal died during the dynamic scan, thus not included (n=6 in the ARBM101 group, n=4 in the other groups).

FIGURE 7 Change in % injected dose (ID) in the liver from the baseline scan to 90 min post-intervention and 24 h post-intervention.
NB. Control baseline value low because of pre-intervention ^{64}Cu excretion.



The study has limitations. We used heterozygote *Atp7b*$^{+/-}$ rats as controls because these animals do not show any signs of hepatic impairment because of the recessive nature of the disease (Einer et al., 2023; Lichtmannegger et al., 2016). We recognise that heterozygotes may not be entirely representative of normal copper physiology as it has been shown in both humans and mice that they may have affected biliary copper excretion (Murillo et al., 2022; Sandahl et al., 2022). Nonetheless, as the heterozygote controls show no signs of liver disease, we can reasonably conclude that the excretion rate in these animals effectively maintains copper levels sufficiently low to prevent disease, similar to observations in humans. This is supported by a report that administration of an adeno-associated vector-based gene therapy in Wilson disease mice effectively prolonged survival in the mice, although the treatment stimulated lower rates of ^{64}Cu excretion to bile and faeces than in WT mice (Murillo et al., 2019; Murillo et al., 2022), again implicating that fully restored biliary excretion is not necessary to maintain normal copper levels.

Another limitation is that because MB also binds non-radioactive copper (cold copper) already present in the animals, some cold copper was likely also excreted as seen in earlier studies where MB reduced

copper contents of liver homogenate and hepatic mitochondria (Einer et al., 2023; Lichtmannegger et al., 2016). In a previous rat study, we have shown a faecal excretion rate of ≈380 μg cold copper/24 h in LPP rats of comparable age using similar ARBM101 dosage while we only inject a tracer dose of ^{64}Cu equalling a maximum of 0.0003 μg per animal. Thus, the ^{64}Cu is not saturating the ARBM101 dose. The radioactive copper likely does not distribute like existing cold copper in the hepatocyte within the short timeframe from injection to scans. This is supported by the 30% reduction in hepatic ^{64}Cu within 24 h in the present study and the 50% reduction in cold hepatic copper demonstrated by Einer et al., in animals treated for 4 days with ARBM101 (Einer et al., 2023). Because of our study's nature, it is impossible to establish how much cold copper was removed and from which compartments. The ultimate goal of treatment in Wilson disease is decoppering the affected organs, primarily the brain and liver. While we demonstrate fast removal of hepatic ^{64}Cu, we realise that accumulated copper in the brain may be very difficult to mobilise, as the exchange of copper between blood and brain is a very slow process (Munk et al., 2023). Standard treatment, including chelators and zinc, slowly reduces cerebral copper, as seen by the disappearance of Kayser–Fleisher rings over several years (Telinius et al., 2019). Still, with current available treatments, the hepatic copper content stays elevated for decades despite adequate treatment (Schilsky et al., 1991). Our short-term study cannot predict these long-term effects of MB as a potential drug in Wilson disease, but we note that in contrast to other drugs, MB can remove hepatic copper.

This ^{64}Cu PET/MRI study in LPP (Wilson disease) and control animals confirmed a substantial biliary copper excretion to the gut by means of non-ATP7B transporters after a single dose of methanobactin. This effect was more pronounced with ARBM101 than with MB-OB3b. In addition, the findings demonstrated the rapidness of this effect, with quantifiable excretion happening within minutes. The data provide further support for the therapeutic potential of MBs in Wilson disease.

AUTHOR CONTRIBUTIONS

Emilie Munk Lynderup: Conceptualization (equal); data curation (lead); formal analysis (lead); funding acquisition (supporting); investigation (lead); project administration (lead); visualization (equal); writing—original draft (equal). **Mikkel Holm Vendelbo:** Investigation (equal); methodology (equal); resources (equal); supervision (supporting); writing—review and editing (equal). **Frederik Teicher Kirk:** Formal analysis (equal); visualization (equal); writing—original draft (equal). **Karina Højrup Vase:** Investigation (supporting); methodology (equal); writing—review and editing (equal). **Aage Kristian Olsen Alstrup:** Investigation (supporting); methodology (equal); resources (equal); writing—review and editing (equal). **Tamara Rieder:** Methodology (equal); resources (equal); writing—review and editing (equal). **Alan DiSpirito:** Resources (equal); writing—review and editing (equal). **Jeremy D. Semrau:** Resources (equal); writing—review and editing (equal). **Tea Lund Laursen:** Supervision (equal); writing—review and editing (equal). **Peter Ott:** Supervision (equal); writing—original draft (equal). **Hans Zischka:** Conceptualization (equal); resources (equal); writing—review and editing (equal). **Thomas Damgaard Sandahl:** Conceptualization (equal); funding acquisition (lead); methodology (equal); supervision (lead); writing—original draft (equal).

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CONFLICT OF INTEREST STATEMENT

HZ, AAD, JDS and TDS are scientific consultants for ArborMed Co. Ltd. The remaining authors disclose no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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