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The biogenic methanobactin is an effective chelator for copper in a rat model for Wilson disease

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ABSTRACT

Copper is an essential redox-active metal ion which in excess becomes toxic due to the formation of reactive oxygen species. In Wilson disease the elevated copper level in liver leads to chronic oxidative stress and subsequent hepatitis. This study was designed to evaluate the copper chelating efficiency of the bacterial methanobactin (MB) in a rat model for Wilson disease. Methanobactin is a small peptide produced by the methanotrophic bacterium *Methylosinus trichosporium* OB3b and has an extremely high affinity for copper. Methanobactin treatment of the rats was started at high liver copper and serum aspartate aminotransferase (AST) levels. Two dosing schedules with either 6 or 13 intraperitoneal doses of 200 mg methanobactin per kg body weight were applied. Methanobactin treatment led to a return of serum AST values to basal levels and a normalization of liver histopathology. Concomitantly, copper levels declined to 45% and 24% of untreated animals after 6 and 13 doses, respectively. Intravenous application of methanobactin led to a prompt release of copper from liver into bile and the copper was shown to be associated with methanobactin. In vitro experiments with liver cytosol high in copper metallothionein demonstrated that methanobactin removes copper from metallothionein confirming the potent copper chelating activity of methanobactin.

Introduction

Copper is an essential trace element and an integral part of several enzymes. Due to its redox properties, which are exploited by, for example, oxidoreductases, copper can however also be toxic by generating deleterious oxygen radicals and, accordingly the homeostasis of the metal has to be carefully controlled [1]. The liver is the key organ for copper homeostasis which is maintained in particular by the excretion of the metal via bile. Biliary copper excretion is mediated by the copper transporter ATP7B [2]. Mutations in the ATP7B gene is one of the primary causes of Wilson disease [3]. Wilson disease is characterized by copper accumulation particularly in the liver, leading to hepatitis, jaundice and finally liver failure [3]. In some patients the first signs of the disease are neurological symptoms but these symptoms are secondary to high liver copper levels [3]. In the presymptomatic stages of (untreated) Wilson's disease copper in liver is mainly bound to cytosolic metallothionein, a small cysteine rich protein with high affinity for copper and zinc [4]. For the treatment of Wilson disease, copper chelators such as D-penicillamine, trientine and tetrathiomolybdate have been shown to be effective. D-Penicillamine is the first choice for treatment but patients suffer occasionally from side-effects like allergic reactions or the worsening of symptoms in the neurological form of Wilson disease [5]. These patients are then switched to trientine treatment [3]. Tetrathiomolybdate is still an experimental drug which is developed for the initial treatment of the neurological form of Wilson disease [6].

As an authentic animal model for Wilson disease the Long Evans Cinnamon (LEC) rat has been established previously [7]. The LEC rat has a deletion in the ATP7B gene leading to a non-functioning protein [7]. The animals steadily accumulate copper in liver and develop fulminant hepatitis at the age of about 3 months when liver copper is highest [8,9], accompanied by an acute increase in serum AST and bilirubin levels. If untreated, most of the animals die. One difference to the human disease is the absence of neurological symptoms in these rats. Recently, the LPP rat, a cross between LEC and Piebald Virol Glaxo (PVG) rats was established [10]. The phenotype of the LPP rats is identical to the LEC rats with respect to copper accumulation and the development of hepatitis.

Methanobactin, a small siderophore-like molecule with extremely high affinity to copper has been recently isolated from the growth media of methanotrophic bacteria [11–16].

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Methanobactin seems to be essential for bacterial copper uptake, regulation of methane monooxygenase expression, and protection against copper toxicity [16].

The present investigation addresses the capability and mode of action of methanobactin to chelate copper in rat liver. The suitability of methanobactin to ameliorate copper toxicity is followed in the LPP rat as a model of Wilson disease.

Materials and methods

Isolation and purification of methanobactin

Methylosinus trichosporium OB3b was cultured for methanobactin isolation in nitrate mineral media [17] amended with 0.2 μM $CuSO_4$ as previously described [12]. To obtain the quantities necessary for animal studies a new 4 step isolation process was applied.

Methanobactin was separated from cells in the culture medium using a Centramate™ PE tangential flow filtration system containing an OS030C10 Centramate 30,000 Da molecular mass filter cassette (Pall Corporation, Framingham, MA, USA). The filtrate was loaded directly on a 5.0 cm × 30 cm Dianion HP20 column (Sigma Chemical Co., St. Louis, MO, USA) and the column washed with H_2O. Methanobactin was eluted with 60% acetonitrile/40% H_2O and the eluate directly dropped into liquid nitrogen. The frozen sample was then freeze-dried as described by Choi et al. [14]. This isolation procedure proved to be superior to multi-step approaches [12,18]. It significantly reduces sample manipulation, processing times, sample loss, and produces a more consistent sample. Metal free methanobactin is stable in the freeze-dried state, at −20 °C, or when associated with a metal such as copper [12,18].

Purity of the isolated methanobactin was determined by UV–vis absorption spectroscopy [12], high performance liquid chromatography (HPLC), electrospray ionization time-of-flight (ESI-TOF) mass spectroscopy [14], and ^{1}H-total correlation spectroscopy (^{1}H-TOCSY) [13] and found to be >95%. ICP-AES analysis confirmed that methanobactin is essentially copper free (molar ratios: Cu:MB = 0.00023, Fe:MB = 0.01307, Zn:MB = 0.00563). Fig. A in the online supplement shows the respective spectra from ESI-TOF-MS, ^{1}H-TOCSY, and UV–vis spectroscopy of the methanobactin preparation.

Animals and treatment

The LPP rat strain was kindly provided by Jimo Borjigin, University of Michigan [10]. These animals are a crossbred between LEC rats, which have a deletion in the ATP7B gene [7] and PVG rats. LPP rats accumulate copper in liver with age and show symptoms typical of individuals with Wilson disease. The animals were maintained on ad libitum Altromin 1314 diet (Altromin, Lage, Germany) and on tap water. The copper content of the rat diet was 13 mg/kg. When the animals (ATP7B−/−) developed slightly elevated AST and bilirubin levels, treatment with methanobactin was started. All animals were treated under the guidelines for the care and use of laboratory animals of the Helmholtz Zentrum München.

Methanobactin (copper free, purity >97%) was dissolved in 0.9% NaCl at a concentration of 12.5 mg/mL. Due to its instability at pH below 4.5 [18], the compound was injected intraperitoneally. The applied dose was 200 mg/kg body weight. In a short term experiment animals were dosed on 5 consecutive days and another dose after two days and killed 2 days later. In a long term experiment animals received 13 doses, 3 times a week, every other day and without treatment during weekends. Three days after the last dose the animals were killed.

Cannulation of the bile duct was performed in an animal with slightly elevated AST and bilirubin levels (655 U/L and 0.652 mg/dL, respectively) under isofluran anesthesia. Methanobactin was infused during 15 min via the femoral vein as a solution of 12.5 mg/mL and bile fractions were taken for 4 h. Control bile was obtained from a LPP (ATP7B+/+) rat.

Plasma was prepared from heparinized blood taken from the sublingual vein under light ether anesthesia. AST activity and bilirubin concentration in plasma were measured with a Reflotron system (Roche, Mannheim, Germany). Liver homogenate and cytosol were obtained by subfractionation of liver as described earlier [19].

For histopathologic evaluation, liver tissue was fixed in 4% buffered formalin, embedded in paraffin and 2 μm sections were stained with hematoxylin/eosin.

In vitro analyses

Liver cytosol from LPP ATP7B (−/−) rats containing Cu almost exclusively bound to metallothionein [8,9] (Cu concentration 50.8 μg/mL) was incubated with methanobactin in a 10-fold molar excess over copper for 30 min at 37 °C. The samples then were kept at −80 °C until analysis.

Copper, iron, and zinc in liver homogenate, cytosol and bile were analyzed by ICP-AES (Ciros Vision, Spectro, Kleve, Germany) after wet ashing the samples with 65% nitric acid (Suprapur Merck, Darmstadt, Germany).

Liver cytosol and bile from in vitro experiments and bile were analyzed using a two-dimensional chromatographic approach being based on a size exclusion chromatographic (SEC) separation of MB from MT followed from a reversed phase chromatographic (RPC) purification/identification. As a prerequisite, first the elution times of metallothionein and methanobactin on both separation systems were elucidated by consecutive chromatography of pure single standard solutions of both compounds and monitoring respective UV and Cu chromatograms for each standard at both separation methods. Subsequently, liver cytosol samples were first separated by SEC on Fractogel TSK HW 55 F and Fractogel TSK HW 40 S columns (Tosoh, Stuttgart, Germany) with Tris–HCl 5 mM pH 7.4/5% methanol as an eluent. Fractions were collected and analyzed for copper by ICP-AES. Respective SEC fractions collected at the elution time of metallothionein (55–60 min) and methanobactin (70–75 min) were rechromatographed by RPC on a Capcell RP18 column (Shiseido, Japan) with Tris–HCl 5 mM/NaCl 25 mM, pH 7.4 against methanol in a gradient elution. The eluate was directly introduced into the ICP-AES instrument (Optima 7300, PerkinElmer Rodgau, Germany) via a Meinhard nebulizer with a cyclone spray chamber and copper was monitored online at 324.754 nm. Certified copper, iron, and zinc stock standards (1000 mg/L) were purchased from CPI (Santa Rosa, CA, USA). Standards and samples were diluted with deionized water (18.2 MΩ cm) prepared by a Milli-Q system (Millipore, Bedford, MA, USA).

As reference standards for the separation on the SEC as well RP column, Cd/Zn metallothionein from rabbit liver (Sigma, Taufkirchen, Germany) and copper-containing methanobactin prepared according to Choi et al. [12] were used.

Results and discussion

Characterization of copper–methanobactin

Copper in liver cytosol from animals with high hepatic copper levels is almost exclusively bound to metallothionein [8,9]. Incubation of the cytosol with methanobactin led to the appear-