European Journal of Pharmaceutical Sciences 62 (2014) 1–7

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Chelating polymeric beads as potential therapeutics for Wilson's disease

Jana Mattová [a], Pavla Poučková [a], Jan Kučka [b], Michaela Škodová [b], Miroslav Vetrík [b], Petr Štěpánek [b], Petr Urbánek [a], Miloš Petřík [c], Zbyněk Nový [c], Martin Hrubý [b,*]

[a] Institute of Biophysics and Informatics, First Faculty of Medicine, Charles University in Prague, Salmovska 1, 120 00 Prague 2, Czech Republic
[b] Institute of Macromolecular Chemistry AS CR, v.v.i., Heyrovsky Sq. 2, 162 06 Prague 6, Czech Republic
[c] Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University, Hnevotinska 5, 77900 Olomouc, Czech Republic

ARTICLE INFO

Article history:
Received 23 December 2013
Received in revised form 1 May 2014
Accepted 3 May 2014
Available online 9 May 2014

Keywords:
Wilson's disease
Polymer beads
Chelators
Copper chelation

ABSTRACT

Wilson's disease is a genetic disorder caused by a malfunction of ATPase 7B that leads to high accumulation of copper in the organism and consequent toxic effects. We propose a gentle therapy to eliminate the excessive copper content with oral administration of insoluble non-resorbable polymer sorbents containing selective chelating groups for copper(II). Polymeric beads with the chelating agents triethylenetetramine, *N,N*-di(2-pyridylmethyl)amine, and 8-hydroxyquinoline (8HQB) were investigated. In a preliminary copper uptake experiment, we found that 8HQB significantly reduced copper uptake (using copper-64 as a radiotracer) after oral administration in Wistar rats. Furthermore, we measured organ radioactivity in rats to demonstrate that 8HQB radiolabelled with iodine-125 is not absorbed from the gastrointestinal tract after oral administration. Non-resorbability and the blockade of copper uptake were also confirmed with small animal imaging (PET/CT) in mice. In a long-term experiment with Wistar rats fed a diet containing the polymers, we have found that there were no signs of polymer toxicity and the addition of polymers to the diet led to a significant reduction in the copper contents in the kidneys, brains, and livers of the rats. We have shown that polymers containing specific ligands could potentially be novel therapeutics for Wilson's disease.

1. Introduction

Wilson's disease is a genetic disorder of copper metabolism resulting in hepatic cirrhosis and basal ganglia degeneration (Ala et al., 2007; Butterworth, 2010). Inheritance is autosomal recessive, and the disease is caused by the malfunction of ATPase 7B (Bruha et al., 2010); more than 400 mutations of this gene have been described to date (Merle et al., 2007). Worldwide prevalence of this disease is 1:30,000 (Ala et al., 2007). The impaired function of the ATPase causes an inability to excrete copper into the bile, which is the main pathway for copper elimination in humans. This leads to high copper accumulation in the organism, especially in the liver and central nervous system. High concentrations of copper lead to numerous symptoms due to toxic oxidative-stress-related damage to the liver tissue, brain, and other parenchymal organs (Ala et al., 2007; Butterworth, 2010; Merle et al., 2007). The three typical clinical presentations are liver disease, neurological disease, and psychiatric disease (Ala et al., 2007; Bruha et al., 2010; Huster, 2010). Liver disease is the most common presentation, and in the later course of untreated Wilson's disease, it may be fatal in all presentations. Patients with liver disease typically develop all possible complications, including liver cirrhosis, portal hypertension, hepatic encephalopathy, and massive bleeding from esophageal varices (Ala et al., 2007;Uno et al., 1997).

The currently recommended first-line therapy is based on lowering copper concentrations in organs through the administration of copper-chelating agents (penicillamine, triethylenetetraammine, or tetrathiomolybdate) (Ala et al., 2007; Roberts and Schilsky, 2003), which leads to decreased uptake and increased elimination of copper into the urine. In addition, high doses of zinc salts are administered as a maintenance therapy because zinc blocks copper uptake from the gastrointestinal tract (Ala et al., 2007; Huster, 2010).

Unfortunately, suitable zinc formulations are not available in all countries. The use of these drugs is limited by multiple and occasionally severe side effects, e.g., myelosupression, lupus, and myasthenia for penicillamine (Huster, 2010; Das and Ray, 2006), which originate from the general recomplexation of essential elements within the body after uptake from the gastrointestinal tract. Significant gastrointestinal disorders have been reported for zinc therapy (in fact, the doses applied cause mild zinc poisoning)

* Corresponding author. Tel.: +420 296809230.
E-mail address: mhruby@centrum.cz (M. Hrubý).

http://dx.doi.org/10.1016/j.ejps.2014.05.002
0928-0987/

(Huster, 2010; Shimizu et al., 2010). The typical dietary intake of copper is 0.6–1.6 mg per day, and thus, a low-copper diet is recommended as an adjuvant measure at the beginning of therapy. However, copper is omnipresent in almost all consumables, making a copper-free diet is nearly impossible (van den Berghe and Klomp, 2009). Thus, the diet typically consists of avoiding only the foods with the highest copper content (e.g., liver, mushrooms, and nuts) (Ala et al., 2007).

We propose the use of macroporous polymeric microbeads containing groups that selectively chelate copper as a dietary supplement for the treatment of Wilsońs disease. The macroporous nature of the microbeads assures full accessibility of the chelating groups to the copper(II) ions. The polymer would pass along with the gastrointestinal tract contents and scavenge all copper released from food during digestion. Moreover, it should also adsorb copper that is secreted into gastrointestinal tract, i.e., ca. 5.3 mg copper per day, of which 7% is secreted into saliva, 20% into gastric fluid, 50% into bile, 18% into pancreatic fluid, and 5% by duodenal secretion (Linder et al., 1998), further shifting the copper balance toward elimination. Because polymer microbeads are generally not resorbable and are insoluble (Mattiasson et al., 2009), they are completely nontoxic and are fully eliminated along with the feces. In our previous paper (Skodova et al., 2013), we described the chemistry and *in vitro* copper scavenging ability of the synthesized macroporous microbeads in detail. In this study, we evaluate the biological effectiveness of this new therapeutic approach in vivo.

2. Materials and methods

2.1. Synthesis of the polymers

Macroporous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate)-based beads (particle size 20–40 μm) containing the ligands *N*,*N*-di(2-pyridylmethly)amine (DPAB), triethylenetetraammine (TTAB), and 8-hydroxyquinoline (8HQB) were synthesized according to the procedure described in our previous paper (Skodova et al., 2013). The ligand contents of the particular polymers were 1.15 mmol/g of *N*,*N*-di(2-pyridylmethly)amine in DPAB and 0.48 mmol/g of 8-hydroxyquinoline in 8HQB.

2.2. Experiment A – Preliminary copper uptake experiment using ^{64}Cu in rats after the administration of polymeric microbeads

All animal experiments described hereinafter were performed in accordance with the corresponding legislation in the Act on Experimental Work with Animals (Decrees No. 311/97; 117/87 and Act No. 246/96 of the Czech Republic), which is fully compatible with the corresponding European Union directives.

To measure copper uptake from the gastrointestinal tract, we used healthy female Wistar rats (average weight 220 g, 11 weeks old, obtained from AnLab, Ltd., Prague, Czech Republic) that received a stock solution of radioactive copper with or without polymer and tracked the fate of copper inside the body.

A stock solution of radioactive copper was prepared using 200 μl of $^{64}CuCl_2$ (containing 170.6 MBq ^{64}Cu, ca. 100 MBq ^{64}Cu/mg Cu, Nuclear Physics Institute of the ASCR, v.v.i.) in 0.05 M HCl and 2.300 μl of physiological solution.

The animals were randomly divided into three groups (two experimental groups and a control group, $n = 2$) and subsequently received a solution of $^{64}CuCl_2$ with a polymer suspension through a gastric probe in the following doses: 250 μl of $^{64}CuCl_2$ stock solution (standard sample – 250 μl, activity $A = 17.06$ MBq) with no polymer (*control group*); 250 μl of $^{64}CuCl_2$ stock solution with 250 μl of DPAB suspension (50 mg of polymer with *N*,*N*-di(2-pyridylmethyl)amine in 200 μl of physiological solution) (*DPAB group*); or 250 μl of $^{64}CuCl_2$ stock solution with 250 μl of 8HQB suspension (50 mg of polymer with 8-hydroxyquinoline in 200 μl of physiological solution) (*8HQB group*). After administration, the animals had free access to food and water. The rats were sacrificed 24 h later, and the radioactivity in the excised gastrointestinal tract, liver, kidneys, lungs, heart, spleen, and carcass and in excrements was determined using an ionization chamber (Bqmetr 4, Empos Ltd., Prague). All radioactivity data presented in this manuscript are decay-corrected (half-life of ^{64}Cu $T_{1/2} = 12.7$ h).

2.3. Experiment B – Determination of polymer uptake via the gastrointestinal tract

The polymeric microbeads should be not absorbable from the digestive tract according to our hypothesis. We chose the polymer 8HQB radiolabeled with ^{125}I to track its resorbability after oral administration in healthy Wistar rats because this polymer was the most efficient in inhibiting copper uptake and also because the 8-hydroxyquinoline moiety can be radiolabeled in high yield using a standard electrophilic radioiodination protocol.

The polymer 8HQB was radiolabeled with ^{125}I using the standard chloramine method according to Hruby et al. (2005) adapted for insoluble beads. Briefly, the polymer 8HQB (100 mg) was suspended in phosphate buffered saline (PBS, 1 ml); then, a solution of $Na^{125}I$ (Lacomed Ltd., Rez, Czech Republic, 185 MBq in 10 μl) and a freshly prepared solution of chloramine T (50 μL, 10 mg/ml) were added. After 60 min, the beads were washed via centrifugation and redispergation, twice with 1 ml of ascorbic acid solution (20 mg/ml in PBS), once with ascorbic acid solution (20 mg/ml in PBS) containing 2 mg of NaI (non-radioactive) with an overnight incubation and then twice with 1 ml of water. The net radiolabeling yield was 95% (174 MBq ^{125}I in 8 ml of aqueous suspension containing 100 mg of polymer). The pellet obtained after centrifugation was diluted with water to a total volume of 8 ml (stock suspension).

Healthy female Wistar rats (average weight 220 g, 11 weeks old, AnLab, Ltd., Prague, Czech Republic) were randomly divided into three groups ($n = 6$), and 0.45 ml of stock suspension of radiolabeled polymer 8HQB was administered to each animal. The animals then had free access to food and water until they were sacrificed at 2.5 h (group 1), 8 h (group 2), or 24 h (group 3) after polymer administration. The selected time intervals were sufficient to observe the passage of polymer through the digestive tract. Immediately after sacrifice, the internal organs, including the digestive tract, were excised, and radioactivity was determined using an ionization chamber (Bqmetr 4, Empos Ltd., Prague).

2.4. Animal imaging

Imaging techniques are useful in determining the localization of various processes. Therefore, we used PET/CT animal imaging with radionuclide ^{64}Cu to confirm the non-resorbability of the polymer (and also to compare it with Experiment B).

Carrier-free ^{64}Cu-chloride was obtained from the Helmholtz Centre (HZDR, Dresden, Germany). The 8HQB (5 mg per mouse) was labeled with ca. 15 MBq of $^{64}CuCl_2$-chloride using 0.15 M acetate buffer (300 μl) at room temperature. The second studied tracer contained only $^{64}CuCl_2$ (ca. 15 MBq) diluted with 0.15 M acetate buffer (300 μl).

The positron emission tomography (PET) and computed tomography (CT) images were acquired with an Albira PET/SPECT/CT small animal imaging system (Bruker Biospin Corporation, Woodbridge, CT, USA) (Sanchez et al., 2013). Female outbred NMRI mice (average weight 19 g, 4 weeks old, Charles River, Wilmington, MA, USA) were chosen for this experiment to compare the passage of