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In vivo efficacy, toxicity and biodistribution of ultra-long circulating desferrioxamine based polymeric iron chelator

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ABSTRACT

Desferrioxamine (DFO) is currently in clinical use to remove iron from transfusion-dependent patients with β-thalassemia major, sickle-cell anemia and the myelodysplastic syndromes. However, its short half-life, burdensome, subcutaneous mode of administration and propensity to cause neurotoxicity at high doses greatly hinder its use. Thus, developing an optimized version of DFO with extended half-life, and reduced toxicity is a major goal. Using high molecular weight (MW), non-toxic, hyperbranched polyglycerol with high functionality, we demonstrate that the efficacy of DFO can be tuned with considerable reduction in toxicity. Using zebrafish embryos and mice, we tested toxicity, iron removal efficacy with low dosing and the biodistribution of ultra-long circulating DFO (ULC-DFO) conjugates. There was no significant difference in the mortality and development of zebrafish embryos upon exposure to ULC-DFO. Similarly, body weights and serum lactate dehydrogenase levels in mice treated with ULC-DFO remained within the normal range throughout the tolerance study. Moreover, ULC-DFO is significantly more effective than low MW DFO in promoting iron removal both from organs and via urine in iron overloaded mice despite using a moderate, once-weekly dosing schedule. This is probably due to the extended circulation half-life of ULC-DFO. The MW of ULC-DFO influences the accumulation and biodistribution, with highest MW (637 KDa) associated with up to 12% accumulation in the liver. In contrast, ULC-DFO with MWs of 75 KDa and lower were associated with relatively low organ accumulation, indicating that biodistribution of ULC-DFO can be tuned. Since ULC-DFO has improved iron removal properties, longer plasma retention time and possesses excellent biocompatibility, it represents a polymer conjugate with high clinical utility in comparison to DFO for the treatment of transfusion dependent iron overload. More importantly, ULC-DFO is anticipated to reduce the requirement for prolonged subcutaneous infusion of DFO.

1. Introduction

Polymer conjugation to low molecular weight (MW) drugs can play a significant role in mitigating toxicity, enhancing the biokinetics and redefining the biodistribution of drugs in the body [1–5]. This has been well demonstrated in the field of iron chelation therapy (ICT) [6]. ICT is used to treat transfusion dependent patients with sickle-cell anemia (SC), β-thalassemia (β-TM) and the myelodysplastic syndromes (MDS) [7–9]. It is also used to treat pediatric ingestion of iron [10]. Patients with β-TM, SC and MDS develop severe anemia due to ineffective red blood cell production and must be treated with transfused red blood cells (RBCs) to correct anemia [7]. However, due to the high iron content in RBCs and the absence of an iron excretion pathway in humans, repeated RBC transfusions lead to the accumulation of iron in cells of major

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organs and in the plasma [7,8]. This excess iron may catalyze the formation of free radicals through the Haber-Weiss reaction, ultimately causing severe organ damage and death if left untreated [11].

The most thoroughly characterized and oldest form of ICT is with desferrioxamine (DFO), whose primary mode of administration (MOA) is prolonged subcutaneous infusion for 8–12 h per day, 5–7 days per week [12,13]. This rigorous MOA is due to the short plasma half-life of DFO (20 min in humans) and is considered to be a hindrance to its use in ICT. Moreover, the MOA of DFO has been shown to result in non-compliance among large groups of patients, especially younger individuals [8,14]. Additional shortcomings of DFO include toxicity at higher doses which may result in hearing loss or blindness, and reactions at the infusion site [15–17]. Not surprisingly, one major aspiration of current research is a therapy with reduction in the arduous subcutaneous or intravenous administration of DFO.

Since DFO is considered as the gold standard in ICT, there has been significant interest in improving its properties to enhance ICT. This was achieved using high MW biocompatible polymers [5,6,18–21]. Different studies have shown that the properties of DFO can be tuned and enhanced to generate more suitable safety profiles, metal chelating efficacy and slower elimination. This was first described by Hallaway et al., who achieved half-life extension up to 80 min and reduced toxicity after conjugating DFO to hydroxyl ethyl starch and dextran [5]. Similarly, we have previously shown that hyperbranched polyglycerol (HPG) conjugates of DFO with circulation half-life of 16 h was capable of efficiently removing iron from iron overloaded mice when treated every other day, demonstrating the proof-of-concept [20].

In this study, we sought to further define the iron excretion efficiency using an ultra-long circulating (ULC) version of HPG-DFO conjugates. Although the properties offered by polymer-conjugated forms of DFO are desirable, there is currently limited information available in the literature on the use of ULC drug conjugates for ICT [6]. Therefore, the information obtained by paying special attention to toxicity, effect of extended half-life on organ accumulation, and the lowest effective doses for ICT are highly valuable in enabling optimization of ULC chelators. Since the toxicity of polymeric nanomaterials may influence tolerance and efficacy, and given the toxicities associated with high-dose DFO therapy; we sought to define the toxicological properties of these polymeric chelators in *in vivo* experimental models.

Zebrafish are a reliable model for measuring drug molecule toxicity and the results obtained in zebrafish are predictive of toxicity in more advanced mammalian models [22–27]. In addition, the zebrafish model has demonstrated utility in predicting the toxicity of diverse categories of biomaterials [23,28,29]. Therefore, the zebrafish model was selected for testing the potential and range of toxic effects of ULC-DFO. Effects on mortality, hatching success and zebrafish morphology during development were investigated. In addition to zebrafish studies, we investigated the toxicity, tolerance and biodistribution of HPG-DFO in mice. These data provide a comprehensive picture of the properties of ULC-DFO as well as some of the potential factors that mediate the observed effects.

As the current regimen of daily subcutaneous infusion of DFO is considered arduous and associated with low compliance, a long acting chelator that is significantly more effective with a less frequent dosing schedule is a major goal of ICT. Reducing the number of doses needed to promote iron mobilization should translate to reduced necessity for subcutaneous infusions and an enhancement in DFO therapy. Therefore, we also investigated the efficacy of an ultra-long circulating HPG-DFO with circulation half-life of 44 h in a weekly dosing schedule. These studies will help to define the clinical utility of DFO conjugates and improve our understanding of HPG-DFO.

2. Experimental

2.1. Materials and methods

Desferrioxamine (>99%), ferric ammonium sulfate dodecahydrate (FAS), sodium periodate ($NaIO_4$), sodium cyanoborohydride ($NaCNBH_3$), iron (II) sulfate heptahydrate (>99%), glycine, ethanolamine and nitric acid were purchased from Sigma-Aldrich Canada, Oakville, ON. Cellulose ester dialysis membranes were obtained from Spectra/PorBiotec (CA, USA).

2.2. Synthesis of polymer conjugates

2.2.1. Generation of ULC-DFO via covalent conjugation to DFO

Hyperbranched polyglycerols (HPGs) were synthesized using ring opening, multi-branching polymerization of glycidol as previously described [30,31]. ULC-DFO was obtained by conjugating DFO to HPG of different MW using reductive amination as previously reported [20]. In a typical reaction, 100–200 mg of HPG (25, 44 and 494 KDa) was dissolved in MilliQ water (2–4 mL). To oxidize the 1, 2-diol groups on the HPG, 84 μL of a 5 M solution of $NaIO_4$ was slowly added to the solution over a period of 1–3 min and the solution was left to stir for 24 h. The solution was then dialyzed against water to remove the unreacted $NaIO_4$. Dialysis was carried out for 24 h using a Spectra/Por dialysis membrane (MWCO 1000). DFO was then added in 1.2 M excess of the number of aldehydes generated. After 8 h, $NaCNBH_3$ was added at 1.2 M excess of the number of aldehydes generated and this solution was left to stir for 24 h. Excess, unreacted aldehyde groups were quenched using glycine. The remaining product was put to dialyze against water for 3 days with frequent changes of water. The conjugates were characterized for their hydrodynamic size, number of DFO/polymer and the molecular weight. Radio-labeled versions of ULC-DFO conjugates were used for biodistribution studies and were obtained by using tritiated sodium cyanoborohydride for reductive amination in the conjugation of DFO to HPG, as previously described [20]. The stability of the HPG-DFO in physiological buffer solutions at 37 °C was determined using gel permeation chromatography analysis and iron binding studies (see Supplementary Information for Details).

2.3. Cellular uptake of ULC-DFO in human umbilical vein endothelial cells (HUVECs)

5-(Aminoacetamido) Fluorescein (Fluoresceinyl Glycine Amide) (FGA) (Invitrogen) and Alexa 488-NHS ester (Invitrogen) were used to label HPG-DFO and DFO, respectively. In a typical reaction, 100–200 mg of HPG was dissolved into in MilliQ water (2–4 mL). Aldehyde groups were generated as previously described in section 2.2. Approximately 0.1 mol percent of FGA with respect to the aldehyde groups was added to the solution and stirred for 1 h at room temperature (22 °C). DFO was then added in 1.2 M excess of the number of aldehydes generated. After 8 h, $NaCNBH_3$ was added at 1.2 M excess of the number of aldehydes generated and the solution was left to stir for 24 h. Excess, unreacted aldehyde groups were quenched using glycine. The solution was put to dialyze against water for 3 days using a Spectra/Por dialysis membrane (MWCO 1000 in dark with frequent changes of water. Samples were acetone precipitated prior to taking absorbance readings to ensure that conjugation was successful and remove non-specifically bound fluorophore. DFO was labeled with Alexa 488-NHS ester in methanol in 1:10 ratio w/w (Alexa: DFO). All samples were dialyzed for 2