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In vivo biodistribution and oxygenation potential of a new generation of oxygen carrier

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Natural giant extracellular hemoglobins (Hbs) from polychaete annelids are currently actively investigated as promising oxygen carriers. Their powerful oxygenating ability and their safety have been demonstrated in preclinical studies, motivating their development for therapeutic and industrial applications. HEMARINA-M101 (M101) is derived from the marine invertebrate *Arenicola marina*. It is formulated as a manufactured product designated HEMOXYCarrier® (HEMARINA SA, France). The aim of the present study was to unveil the fate of M101 after a single intravenous (i.v.) injection in mice. For this purpose, M101 was tagged with a far-red fluorescent dye. Repeated non-invasive fluorescent imaging revealed a rapid diffusion of M101 in the whole body of animals, reaching all the examined organs such as brain, liver, lungs and ovaries. Functional M101 was circulating in bloodstream for several hours, without inducing any obvious side-effects. Last, a single i.v. injection of M101 in mice bearing human-derived subcutaneous tumors demonstrated the ability of this Hb to reduce hypoxia in poorly vascularized tissues, thus supporting the biological relevance of M101 oxygen release to vertebrate tissues. Altogether, these results further encourage the development of M101 as an oxygen carrying therapeutic.

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1. Introduction

Over the last years, several studies have focused on the Hb found in the marine invertebrate *Arenicola marina* (Rousselot et al., 2006a; Tsai et al., 2012). This natural extracellular respiratory pigment, named M101, is a high molecular weight protein (~3,600 kDa). It displays a series of structural and functional properties that are mostly compatible with the specifications of an ideal oxygen carrying therapeutic: (i) M101 is a hexagonal-bilayer Hb (HBL-Hb) composed of 156 globins and 44 non-globin linker chains that has a large oxygen binding capacity, carrying up to 156 O_2 molecules when saturated (Royer et al., 2007; Zal et al., 1997); (ii) M101 releases O_2 according to a simple gradient without requiring any allosteric effector, providing the environment with the right amount of O_2 (Ochiai and Weber, 2002; Rousselot et al., 2006a, 2006b; Smani et al., 2007; van Bruggen and Weber, 1974; Winslow, 2003); (iii) M101 exhibits an intrinsic superoxide dismutase-like activity linked to Cu/Zn metals preventing both the occurrence of potentially harmful heme-protein-associated free radical species and the release of Hb degradation products that could induce endothelial and surrounding tissue damages (D'Agnillo and Chang, 1998; Rousselot et al., 2006a); (iv) M101 is a non-glycosylated protein that does not induce immunogenic and allergenic responses upon i.v. injection in mice (Rousselot et al., 2006a); (v) Neither

Abbreviations: AUC, area under curve; FPLC, fast purification liquid chromatography; Hb, hemoglobin; i.v., intravenous.

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significant vasoconstriction effect nor impact on heart rate are induced after M101 top load i.v. injection in rodents (Tsai et al., 2012).

In the present study, M101 was tagged with a fluorescent dye in order to be tracked *in vivo*. Analytical assays were performed to verify that this labeling did not impact on any M101 property. Next, biofluorescence imaging was conducted to visualize the real-time biodistribution of M101 following a single i.v. injection in mice. Finally, the ability of M101 to deliver oxygen to highly hypoxic tissue was investigated in mice bearing HT29 human colorectal adenocarcinoma subcutaneous tumors.

2. Materials and methods

2.1. M101 production

M101 (HEMARINA SA, France) is manufactured using GMP ("good manufacturing practices") standards governing medicinal products (Tsai et al., 2012). Briefly, extracellular Hb is extracted from *Arenicola marina* frozen lugworms under gentle agitation at 4 °C, then purified and conditioned in 20 mL bags. It is developed as an oxygen carrying therapeutic, formulated in a commercial product designated HEMOXYCarrier®. It was conserved at −80 °C then defrosted at 4 °C before conducting experiments.

2.2. Labeling reaction

The far-red fluorescent dye DY676 (Dyomics) was used to label M101. This dye displays bright solid state emission and it is negatively-charged. Its maleimide group can react with one sulfhydryl group of the Hb, leading to a covalent linkage between these two molecules. M101 and DY676 were mixed at 1:48 molar ratio in order to expect labeling 4 cysteines per dodecamer (Zal et al., 1997). Reactions were performed during several hours, protected from light, at 4 or 20 °C, and at pH 6.5–7.0 (Fig. 1A). Next, free (un-reacted) dye was separated from labeled protein using an Amicon Ultra 100 kDa column (Millipore) and centrifugation at 4,000 × g, 4 °C for 10 min. Purified labeled M101 was sterilized using a 0.22 μm filter; it was stored at −80 °C until subsequent use. The free dye portion was determined by spectrometry measurements at 670 nm.

2.3. Labeling efficiency

Fast Purification Liquid Chromatography (FPLC) was conducted using a TSK Gel G6,000 PWXL column associated with an Ultimate 3,000 device (Dionex). This allowed separating molecules from 1 to 10,000 kDa. Data were acquired at 280, 414, and 670 nm. The areas under the curve (AUC) were determined every 4 h during 20 h of labeling reaction.

2.4. Structure and functionality

Size and zeta-potential were determined by dynamic light scattering at 25 °C using a 3,000 Zetasizer device (Malvern Instruments). FPLC allowed determining M101 integrity, the detection of a peak at the retention time characteristic of M101 (19 min) demonstrating the integrity of its structure. UV–visible spectrometry was used to monitor M101 functionality (Fig. S1).

2.5. Effect toward eukaryotic cells

Native (non-fluorescent) Hb and tagged (fluorescent) Hb were compared regarding their effects toward four eukaryotic cell lines: HeLa, epithelial cells derived from a human epithelioid cervical carcinoma (ATCC, CCL-2); C2C12, mouse myoblast cells (ATCC, CRL-1772); SKMEL28, human melanoma cells (ATCC, HTB-72); 16HBE, human bronchial epithelial cells (Cozens et al., 1994). HeLa, SKMEL28 and C2C12 were grown in Dulbecco's modified Eagle's medium (DMEM) and 16HBE in Eagle's minimal essential medium (EMEM), both media being supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated fetal bovine serum. These cells were routinely maintained at 37 °C in a humidified 5% CO_2/95% air containing atmosphere. Briefly, each cell line was seeded in a 96-well plate (Costar) at a density of 10^4 cells/well. They were incubated with native or tagged M101 at a dose ranging from 0.05 to 3 mg/mL. Cells cultured in absence of M101 were used as control. After 36 h at 37 °C, cells were lysed and their protein content evaluated using the BCA assay kit (Interchim). Viability was assessed using the Vialight assay kit (Lonza).

2.6. Treatment

Six- to nine-weeks old female nude NMRI mice (Janvier laboratories) were housed and maintained at the university animal housing facility (Brest). They were processed in accordance with the Laboratory Animal Care Guidelines (NIH publication #85–23 revised 1985), the ARRIVE guidelines (Kilkenny et al., 2010), and with the agreement of the regional veterinary services. Animals were fed with a chlorophyll-free diet (Special Diet Service, US) at least 24 h before starting biofluorescence imaging. Mice were i.v. injected on the first day *via* their tail vein within 5–10 s. Fourteen mice were used as follows: 3 mice were injected with M101 buffer (group #1); 3 mice were injected with native non-labeled M101 (group #2); 8 mice were injected with labeled M101 (group #3). In all groups, the injected volume was ∼200 μL and solutions were isotonic to plasma (*i.e.* 300 mosm/kg). In groups #2 and #3, M101 was injected at the dose of 200 mg/kg.

2.7. Biofluorescence imaging

Acquisitions were performed 15 min after i.v. injection then regularly, up to 5 h post-administration. They were thereafter repeated 24, 48, 72, and 96 h post-injection. Mice were imaged individually. Before an acquisition, the animal was anesthetized by either ketamin/xylazine intraperitoneal (i.p.) injection (40 mg/kg) or isofluoran inhalation (2% air-isofluran blend). Then, it was placed inside the acquisition chamber of an *in vivo* imaging system equipped with a cooled slow-scan CCD camera and driven with dedicated software (WinLight 32, Berthold Technology). Images were captured at 1 × 1 binning with exposure times ranging from 0.1 to 1.0 s, depending on the fluorescence intensity. Signals quantitated within the regions of interest were expressed as Relative Fluorescent Units (RFU).

2.8. Blood sampling

Blood was collected from the saphenous vein using Microvette 300 anticoagulant-treated tubes (Sarstedt). After sampling, 3 volumes of 0.9% NaCl were i.p. injected. Collection tubes were centrifuged at 10,000 × g for 2 min in order to separate plasma (supernatant) from cells (pellet).

2.9. Functionality in blood

Eight mice were i.v. injected with native (non-fluorescent) M101 at the dose of 200 mg/kg. Spectrometry measurements were done using plasma collected 30 min and then 2, 4, 6, and 24 h after injection ($n = 2$ blood samplings/time point; replicates were considered close enough so that no more mice were included in that experiment). Plasma from two non-injected mice was used as control.

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