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ImmunoPEGliposomes for the targeted delivery of novel lipophilic drugs to red blood cells in a falciparum malaria murine model

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ABSTRACT

Most drugs currently entering the clinical pipeline for severe malaria therapeutics are of lipophilic nature, with a relatively poor solubility in plasma and large biodistribution volumes. Low amounts of these compounds do consequently accumulate in circulating *Plasmodium*-infected red blood cells, exhibiting limited antiparasitic activity. These drawbacks can in principle be satisfactorily dealt with by stably encapsulating drugs in targeted nanocarriers. Here this approach has been adapted for its use in immunocompetent mice infected by the *Plasmodium yoelii* 17XL lethal strain, selected as a model for human blood infections by *Plasmodium falciparum*. Using immunoliposomes targeted against a surface protein characteristic of the murine erythroid lineage, the protocol has been applied to two novel antimalarial lipophilic drug candidates, an aminoquinoline and an aminoalcohol. Large encapsulation yields of >90% were obtained using a citrate-buffered pH gradient method and the resulting immunoliposomes reached *in vivo* erythrocyte targeting and retention efficacies of >80%. In *P. yoelii*-infected mice, the immunoliposomized aminoquinoline succeeded in decreasing blood parasitemia from severe to uncomplicated malaria parasite densities (i.e. from ≥25% to ca. 5%), whereas the same amount of drug encapsulated in non-targeted liposomes had no significant effect on parasite growth. Pharmacokinetic analysis indicated that this good performance was obtained with a rapid clearance of immunoliposomes from the circulation (blood half-life of ca. 2 h), suggesting a potential for improvement of the proposed model.

1. Introduction

Malaria is one of the most devastating parasitic diseases affecting humans and a prominent global health concern, causing nearly half million deaths worldwide every year [1]. Most severe clinical manifestations, in which patients are frequently unable to take oral solid medications and high parasite densities are consistently found [2], are derived from the *Plasmodium falciparum* intraerythrocytic cycle [3,4]. For this reason, the blood stages of the pathogen have been widely considered for the development of several red blood cell (RBC) and/or parasitized RBC (pRBC)-targeted antimalarial approaches based on the use of liposomal and polymeric drug nanocarriers [5–11]. These strategies rely mostly on the cell-specific delivery of nanoparticle payloads in order to improve the shortcomings commonly associated with the pharmacokinetics

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and pharmacodynamics of current antimalarial compounds. These are mainly high biodistribution volumes and low effective local drug concentrations particularly in the case of easily metabolized and poorly water-soluble compounds [12–15], which incur the risk of generating resistant parasites [2,16,17]. As a consequence, the administration of high drug doses to the patient is required for a proper antimalarial effect, which results in an increased likelihood of causing toxic side effects.

RBCs offer opportunities in drug delivery systems, either functioning as long-circulating carriers for biologically active compounds [18] and/or providing numerous anchoring points for the attachment of nanoparticles [11,19,20]. Mature erythrocytes exhibit in this regard several attractive and exploitable features that include (i) minimal or absent MHC class I antigen presentation which masks this cell type from the immune system surveillance [21], (ii) absence of intracellular organelles along with minimal metabolic activity restricted to glycolysis maintenance through the Embden-Meyerhof pathway, generation of reductive potential for protection against oxidative stress and oxygen/carbon dioxide transport [22], (iii) a sialic acid-rich glycocalyx which together with the preferential localization of negatively charged lipids in the inner plasma membrane of the cell [23] provides a good retention of positively ionized, weakly basic compounds, and (iv) a large intracellular volume of ca. 90 fL [24,25]. Nevertheless, the use of RBCs as carriers requires tedious processes like a prior *ex vivo* cell manipulation step (e.g. intracellular drug loading or the covalent attachment of functional molecules to the RBC surface) for later reinfusion into compatible receptors [18].

Erythroid-specific markers are abundant on the RBC surface and are widely conserved among humans [26–31] when compared to the highly polymorphic parasite proteins exported to the pRBC membrane [32–34], and their presence is not affected by *P. falciparum* infection [11]. These features allow for the design of novel combinatorial strategies relying on both antimalarial therapy and its prophylaxis, although such approach has been barely explored against bloodborne infectious diseases. Preliminary assays were conducted using passively-loaded chloroquine (CQ) into RBC-targeted immunoliposomes (iLPs), which resulted in significant *Plasmodium berghei* growth inhibition when compared to administration of the free compound [9]. However, only 20% of the injected iLPs bound circulating RBCs, possibly due to poor antibody recognition of target cells. Other common obstacles include limitations in the intracellular loading of drugs due to the lack of endocytic activity taking place at the surface of RBCs and pRBCs [35], and little evaluation of the LP encapsulation capacity of drugs exhibiting distinct physicochemical properties (e.g. charge and hydrophilic vs. lipophilic nature). A previously proposed iLP nanocarrier targeted against human glycophorin A (GPA), an erythroid lineage-specific protein, has displayed the best [RBC + pRBC] *in vitro* targeting efficacies and retention yields obtained so far (~100% after only 15 min exposure time to cells) and provided a significant improvement in CQ antimalarial activity *in vivo*, clearing *P. falciparum* parasitemia to below detectable levels (<0.01%) in a humanized mouse malaria model after a 4-day dosage regimen of 0.5 mg/kg [11]. Intracellular delivery of the encapsulated cargo was suggested to occur mainly by a sustained release process taking place at the targeted cell surface. The use of an active encapsulation strategy relying on the generation of a pH gradient [36–38] offered a highly efficient encapsulation and stable retention of the weakly basic antimalarial compounds CQ and primaquine.

Nevertheless, several weaknesses must be addressed before advancing this immunoliposomal approach into the clinical pipeline. The aforesaid outstanding performance of anti-GPA iLPs was evaluated in immunosuppressed mice engrafted with human erythrocytes in circulation, where the animals presented impaired innate and adaptive immune responses along with low blood parasite densities not exceeding 1–3% parasitemia (i.e. uncomplicated malaria clinical feature) [11,39–41]. Moreover, iLP binding to GPA resulted in considerable cell-agglutinating events when exceeding 10 μM lipid amounts *in vitro* [11], which limited the administration of large drug payloads *in vivo*. Exploring RBC-targeted therapeutics in a fully immunocompetent mouse model for falciparum malaria (i.e. mice infected with *P. yoelii* 17XL as murine-specific lethal strain effectively reproducing *P. falciparum* blood infection in humans [42]) is therefore a necessary step in order to properly validate antiparasitic efficacy in a severe malaria setting, and where the immune response of the host towards the delivered nanoparticles is not suppressed. Furthermore, the availability of a liposomal structure suitable for the encapsulation of lipophilic drugs is crucial in order to enlarge the number of antimalarial agents capable of being intravenously delivered. This route of administration is required when handling severe malaria clinical processes, and is mainly limited at present to hydrophilic/amphiphilic drug formulations [2]. Finally, in accordance with WHO guidelines updates, CQ is no longer considered as gold standard for malaria therapy due to the massive worldwide appearance of *Plasmodium* resistant strains [2,17]. These considerations have prompted us to research novel drug derivatives capable of overcoming parasite resistance mechanisms and to explore a targeted method for rapid delivery to the circulating parasitized cells before the carrier is cleared from blood.

2. Materials and methods

2.1. Materials

Except where otherwise indicated, reagents were purchased from Merck & Co., Inc. (Kenilworth, NJ, USA), and reactions were performed at room temperature (22–24 °C). The lipids (all ≥99% purity according to thin layer chromatography analysis) 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000-Mal), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[lissamine rhodamine B sulfonyl] (DOPE-Rho) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Mouse monoclonal IgG2b anti-human GPA (SM3141P) and rat monoclonal IgG2b anti-mouse TER-119 (AM31858PU-N) antibodies were purchased from Acris Antibodies GmbH (OriGene Company, Herford, Germany).

Lipophilic aminoquinolines 7c and 7d, formerly found to display potent *in vitro* activity against intraerythrocytic CQ-sensitive and CQ-resistant *P. falciparum* parasites [13], were synthesized as heterocyclic-cinnamic conjugates maintaining a chloroquine backbone structure and characterized following previously reported methods [13,43]. The aminoalcohols BCN-01 and BCN-02, showing high *in vitro* and *in vivo* antiplasmodial efficacy against multidrug-resistant strains of *P. falciparum* and a murine model of *P. berghei*, were synthesized and chemically characterized as formerly described [12]. 10 mM stocks for these weakly basic compounds were prepared in methanol; because of their rapid aggregative properties in water-based solutions, non-encapsulated 7c/d and BCN-01/02 for *in vivo* assays were formulated in 10% v/v chloroform/aqueous emulsion. Unless otherwise indicated, ionization relevant pKa values at physiological pH and drug partition coefficients (log *P*) were calculated *in silico* using the Chemicalize software developed by ChemAxon Ltd [44].