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Development of drug-loaded immunoliposomes for the selective targeting and elimination of rosetting *Plasmodium falciparum*-infected red blood cells

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ABSTRACT

Parasite proteins exported to the surface of *Plasmodium falciparum*-parasitized red blood cells (pRBCs) have a major role in severe malaria clinical manifestation, where pRBC cytoadhesion and rosetting processes have been strongly linked with microvascular sequestration while avoiding both spleen filtration and immune surveillance. The parasite-derived and pRBC surface-exposed PfEMP1 protein has been identified as one of the responsible elements for rosetting and, therefore, considered as a promising vaccine candidate for the generation of rosette-disrupting antibodies against severe malaria. However, the potential role of anti-rosetting antibodies as targeting molecules for the functionalization of antimalarial drug-loaded nanovectors has never been studied. Our manuscript presents a proof-of-concept study where the activity of an immunoliposomal vehicle with a dual performance capable of specifically recognizing and disrupting rosettes while simultaneously eliminating those pRBCs forming them has been assayed *in vitro*. A polyclonal antibody against the NTS-DBL1α N-terminal domain of a rosetting PfEMP1 variant has been selected as targeting molecule and lumefantrine as the antimalarial payload. After 30 min incubation with 2 μM encapsulated drug, a 70% growth inhibition for all parasitic forms in culture (IC50: 414 nM) and a reduction in ca. 60% of those pRBCs with a rosetting phenotype (IC50: 747 nM) were achieved. This immunoliposomal approach represents an innovative combination therapy for the improvement of severe malaria therapeutics having a broader spectrum of activity than either anti-rosetting antibodies or free drugs on their own.

1. Introduction

Malaria infection by the blood-borne parasites of the genus *Plasmodium* remains one of the most life-threatening diseases worldwide with an estimated 214 million cases and 438,000 deaths occurring only in 2015, of which 70% were children under 5 years [1]. Red blood cells (RBCs) infected with mature stages (trophozoites and schizonts) of the malaria parasite bind to endothelial cells in the capillaries of tissues through cytoadhesion events, which allows *Plasmodium* to replicate while evading splenic clearance [2]. Parasitized RBCs (pRBCs) can also adhere to non-infected erythrocytes giving rise to rosettes [3], and they can form clumps through platelet-mediated binding to other pRBCs. These events, which may lead to occlusion of the microvasculature, are thought to play a major role in the fatal outcome of severe malaria. Because the blood-stage infection is responsible for all symptoms and pathologies of malaria, pRBCs have traditionally been a main chemotherapeutic target [4].

The extensively studied and described relation between rosetting processes and clinical severe malaria [5–9] has been highlighted by the association found between host erythrocyte polymorphisms interfering with rosetting and an increased protection against this form of the disease [10,11]. The *var* gene-encoded, pRBC surface-exposed *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) contains a tandem repetition of Duffy binding-like domains (DBL) and cysteine-rich interdomain regions. PfEMP1 has been identified as one of the key molecules responsible for cytoadhesion and rosetting [12,13] and one of the most important proteins promoting antigenic variation at the pRBC

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surface [14,15]. PfEMP1 is encoded by around 60 *var* genes widely distributed throughout the parasite genome [16], among which those encoding the N-terminal segment (NTS)-DBL1α domain have been found directly related to rosetting [17]. Some examples of rosette-inducing transcripts include IT4*var*60 [13], IT4*var*9 [18] and PA*var*O [19] from, respectively, the FCR3S1.2, R29 and PAvarO *P. falciparum* strains. Antibodies raised against the NTS-DBL1α domain of rosette-forming PfEMP1 variants have been shown to prevent the rosetting phenotype [13,20,21] and to promote the opsonization of antibody-targeted pRBCs [22]. The subdomain 3 (SD3) loop of DBL1α has been identified as a target in anti-rosetting activity [20] and consistently predicted as a B-cell-recognized epitope [21]. However, despite the potential role of anti-PfEMP1 antibodies as rosette disrupting tools, no direct effect against pathogen viability has been observed, thus requiring immune system stimulation [22] or antimalarial drug supplementation therapies in order to successfully remove malaria parasites.

An underestimated application of rosetting-associated PfEMP1 variants is their potential role as targets for drug-loaded nanovectors [23,24]. The capability of these vehicles to carry a diverse range of molecules, including fluorescent tracers, antimalarial drugs and proteins such as antigenic determinants or phagocytic markers [25], makes them interesting polyvalent tools for the targeting, disruption and elimination of both rosettes and those parasites causing them, as part of future new clinical strategies against severe malaria. Liposomes (LPs) bearing cell-specific ligands have been widely considered as efficient drug carriers in targeted drug delivery therapies because of their organic-aqueous biphasic character and their *in vivo* biodegradability and low toxicity [26–28]. Antibody-conjugated liposomes (immunoliposomes, iLPs) against pRBCs were first assayed for the treatment of *Plasmodium berghei* infections in mice [29,30] and more recently in targeting assays against the pRBC antigens membrane-associated histidine-rich protein 1 and glycophorin A [31–33] from *Plasmodium* and human origin, respectively. Other studies include the use of pRBC-binding glycosaminoglycans such as heparin [34,35] as targeting molecules of drug-loaded liposomes, and of polymeric nanoparticles as antimalarial agent carriers with self-targeting capacity towards pRBCs [36,37]. In these works the efficacy of antimalarial drugs was significantly improved when specifically targeted against pRBCs. Nevertheless, pRBCs adhered to microvasculature capillaries or hidden within erythrocyte clumps could remain undetected by the aforementioned nanovectors during severe malaria complications.

The use of combinations of antimalarials that do not share the same resistance mechanisms reduces the chance of selection [38], although *Plasmodium* strains evolving resistance to such combination therapies are likely to appear [39]. However, combination therapies involving a hybrid system carrying a chemotherapeutic agent along with a non-drug active element (e.g. a rosetting inhibitor mechanism) remain to be explored as a new concept within antimalarial combination therapies. This study reports the application as targeting agents for the functionalization of liposomal nanovectors of polyclonal antibodies raised against the rosetting-linked NTS-DBL1α domains of FCR3S1.2-^{IT4var60}, R29^{IT4var9} and PAvarO PfEMP1 protein variants [21] and the monoclonal antibody mAbV2–17.1 (M17.1) specific for a conformational epitope identified within the SD3-loop subdomain of NTSDBL1α-FCR3S1.2^{IT4var60} [20]. We have explored the capacity of this model for the detection, disruption and elimination of rosetting pRBCs using lumefantrine as antimalarial drug.

2. Materials and methods

2.1. Materials

Except where otherwise indicated, reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), and reactions were performed at room temperature (22 to 24 °C). The lipids (all ≥99% purity according to thin layer chromatography analysis) 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 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). Polyclonal FCR3S1.2, R29 and PAvarO and monoclonal M17.1 antibodies raised against the NTS-DBL1α domain of PfEMP1 variants were obtained in [20,21].

2.2. Preparation of liposomes and encapsulation of lumefantrine

Liposomes (LPs) with the lipid composition DSPC:cholesterol:DSPE-PEG2000-Mal:DOPE-Rho, 84.5:10:5:0.5, were prepared by the lipid film hydration method [40]. Briefly, stock lipids in chloroform were mixed and dissolved in chloroform:methanol (2:1 v/v) in a round-bottomed glass tube. Organic solvents were removed by evaporation (P-12 Multivapor, BÜCHI Labortechnik AG) under progressive reduced pressure and orbital agitation at 41 °C to yield a thin lipid film on the tube bottom. The dried lipids were then hydrated to 10 mM total lipid in 10 mM phosphate buffer (PB), pH 6.5, supplemented with 10 mM ethylenediaminetetraacetic acid (EDTA) and osmotically adjusted to 280 mOsm/Kg (isotonic with *P. falciparum* growth medium) by addition of 5 M NaCl. Multilamellar liposomes were formed by 3 cycles of constant vortexing followed by bath sonication (3 min each). For the generation of unilamellar vesicles, multilamellar liposomes were downsized by serial extrusion through 400 and 200 nm polycarbonate membranes in a Mini-extruder device equipped with a heating block (Avanti Polar Lipids, Inc.). Throughout the lipid film hydration and downsizing processes samples were maintained at 65 °C, above lipids' transition temperature. Liposome size and concentration (LP number/ml) was determined by nanoparticle tracking analysis (NanoSight NS500, Malvern Instruments Ltd) using samples at 1 μM lipid after dilution in phosphate-buffered saline (PBS) sterile-filtered through 0.22 μm pore size polyvinylidene difluoride (PVDF) filters (Millex-GV Syringe Filter Units, 4 mm, Millipore). For the preparation of LPs encapsulating lumefantrine (LMF), the drug stock diluted in chloroform (10 mM LMF) was incorporated in the initial lipid mixture at 1:40 drug/lipid molar ratio (250 μM drug in the final 10 mM lipid suspension). Alternatively, for tracking purposes, the green fluorescent dye pyranine was included in the lipid film hydration solution at a concentration of 30 mM and in a fluorescence quenched state at pH 6.5 for 450–480 nm excitation range. Pyranine experiences a shift in maximum excitation wavelength from 400 nm at pH ≤6.5 to 460 nm at pH ≥7.4 (Fig. S1) [41], leading to a significant increase in fluorescence emission at 488 nm excitation once released from liposomes in a neutral pH environment. Finally, unencapsulated material was removed by ultracentrifugation (150,000*g*, 1 h, 4 °C) and pelleted LPs were resuspended in isotonic PB. Drug release from LP samples was thereafter determined at different time points after removal of unencapsulated material through additional ultracentrifugation steps. For the characterization of LMF release in the presence of RBCs, these were removed first by mild centrifugation (420*g*, 5 min) and encapsulated drug concentration was subsequently determined from LPs after ultracentrifugation of supernatant fractions. Liposomal suspensions to be used in assays with live cells were sterile-filtered.

2.3. Generation of immunoliposomes (iLPs)

Following established protocols, freshly prepared maleimide-containing LPs were conjugated with previously thiolated antibodies [33,42] using the N-succinimidyl *S*-acetylthioacetate crosslinker (SATA, Thermo Fisher Scientific, Inc.), which contains a protected yet exposable sulfhydryl group [43]. This strategy, as described in [33], enables the covalent, efficient and stable conjugation onto liposome surfaces of antibodies through their primary amino groups. Briefly, antibodies in PBS were reacted for 30 min with 1× to 10× molar excess of the SATA