Extracellular Vesicles Derived from *Plasmodium*-infected and Non-infected Red Blood Cells as Targeted Drug Delivery Vehicles

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Abstract

~~The main~~Among several factors behind drug resistance evolution in malaria is~~are~~ the ~~difficulty~~ challenge~~to~~ of administering ~~appropriate~~ overall doses that are not toxic for the patient but that, locally, are sufficiently high to rapidly kill the parasites. Thus, a crucial antimalarial strategy is the development of drug delivery systems capable of targeting antimalarial compounds to *Plasmodium* with high specificity. ~~as a result of the complex life cycle of *Plasmodium*, the short half-lives of most antimalarial drugs, and the blood fluidic conditions affecting the interaction of molecules with target cells. Extracellular vesicles (EVs) have been widely explored as delivery vectors of nucleic acids and proteins.~~ In the present study, extracellular vesicles (EVs) ~~were~~ have been evaluated as a drug delivery system for the treatment of malaria. EVs derived from naive red blood cells (RBCs) and from *Plasmodium falciparum*-infected RBCs (pRBCs) were isolated by ultrafiltration followed by size exclusion chromatography. Lipidomic characterization showed that there we~~a~~re no significant qualitative differences between the lipidomic profiles of pRBC-derived EVs (pRBC-EVs) and RBC-derived EVs (RBC-EVs). Both EVs were taken up by RBCs and pRBCs, although pRBC-EVs were more efficiently internalized than RBC-EVs, which suggested their potential use as drug delivery vehicles for these cells. When loaded into pRBC-EVs, the antimalarial drugs atovaquone~~, lumefantrine~~ and tafenoquine inhibited *in vitro P. falciparum* growth more efficiently than their free drug counterparts, indicating that pRBC-EVs can potentially increase the efficacy of several small hydrophobic drugs used for the treatment of malaria.

Keywords: *Plasmodium falciparum*; malaria; extracellular vesicles; drug delivery; antimalarial drugs.

1. Introduction

Malaria is a ~~serious~~ disease of global health importance with nearly half of the world's population at risk of developing it. In 2018~~,~~ occurred 228 million cases of malaria~~, which led to~~ and an estimated 405,000 deaths, of which 67% (272,000) were children under 5 years of age (World M~~m~~alaria R~~r~~eport 2019). One of the main difficulties in controlling malaria resides in the occurrence of drug resistance partially due to the exposure of the parasite to low therapeutic

doses. There are several factors behind this problem, including (i) the formidable barrier imposed by the *Plasmodium* life cycle with its intracellular localization in hepatocytes and red blood cells (RBCs) (Balducci et al., 2016; Ofir-Birin et al., 2017), (ii) the physical environment of the circulatory system with strong flow drag and shear forces, affecting the interaction of drugs with target RBCs (Moles et al., 2015) and, (iii) the unfavorable physicochemical characteristics of antimalarial drugs, most of which are amphiphilic and widely distributed throughout the body tissues after administration. This leads to quick drug metabolism in the liver, resulting in short half-lives from a few hours to less than 1 hour (World Health Organization, 2015).

In this scenario, the development of more efficient treatments for malaria is urgent. Frequently, the administration of antimalarial drugs occurs in a narrow therapeutic window between too high amounts inducing toxic side effects and too low local concentrations generating resistance (Balducci et al., 2016; Moles et al., 2015; World Health Organization, 2015). The administration of combinations of two or more drugs having different mechanisms of action and/or different biochemical targets in the parasite is the current recommended treatment to minimize resistance development (Balducci et al., 2016; Biosca et al., 2019; World Health Organization, 2015). However, rather than concentrating all efforts on discovering new drugs whose efficacy is quickly decreased by the parasite's capacity to develop resistance (Aditya et al., 2013; Fernàndez-Busquets, 2016), a crucial strategy is the development of targeted drug delivery systems capable of specifically delivering the antimalarial compound to *Plasmodium*-infected RBCs (pRBCs), thus increasing the exposure of the parasite to doses sufficiently high to be lethal and minimizing the risk of drug resistance evolution (Fernàndez-Busquets, 2016; Moles et al., 2015).

Extracellular vesicles (EVs) have emerged as potential drug delivery systems to target specific organs or cells (Johnsen et al., 2014; Liao et al., 2019; Pinheiro et al., 2018; Stremersch et al., 2016; Tominaga et al., 2015; Vader et al., 2016). EVs have a size ranging from 40 to 1000 nm (El Andaloussi et al., 2013; Mantel and Marti, 2014; Raposo and Stoorvogel, 2013) and are released into the extracellular space by virtually all cells, thus being found in a large variety of body fluids including blood plasma, saliva, tears, urine, milk, and semen, among others (Liao et al., 2019; Raposo and Stoorvogel, 2013). EVs are heterogeneous in terms of size and cargo (Mardahl et al., 2019), containing cell-type-specific

lipids, proteins, microRNAs and messenger RNAs that are essential for their function (Coakley et al., 2015; Mantel and Marti, 2014; Mardahl et al., 2019). Interestingly, many of these molecules are functional and can trigger phenotypic changes in recipient cells (Coakley et al., 2015; Mantel and Marti, 2014; Pinheiro et al., 2018). They are therefore believed to participate in intercellular communication and in both physiological and pathological processes (Raposo and Stoorvogel, 2013; Yáñez-Mó et al., 2015). Several studies have demonstrated that EVs play an essential role in parasite-host and parasite-parasite communication (Mantel and Marti, 2014; Marcilla et al., 2014; Mardahl et al., 2019; Ofir-Birin et al., 2017). During malaria infection, EV quantities in plasma were increased in patients when compared with healthy individuals, with the highest EV concentrations being observed in patients with severe *Plasmodium falciparum* malaria (Nantakomol et al., 2011; Pankoui Mfonkeu et al., 2010). Although these studies did not show a direct connection between higher EV levels and disease severity (Mantel and Marti, 2014), it was demonstrated in a mouse model that EVs derived from pRBCs induced systemic inflammation (Couper et al., 2010). *In vitro* studies also revealed that pRBCs release EVs which contain parasite and host cell proteins (Mantel et al., 2013; Regev-Rudzki et al., 2013), RNA and DNA (Mantel et al., 2016; Regev-Rudzki et al., 2013; Sisquella et al., 2017). These molecular cargos have potential roles in intercellular communication (Regev-Rudzki et al., 2013), modulation of immune response (Saxena et al., 2019; Sisquella et al., 2017), and parasite survival and malaria pathogenesis (Mantel and Marti, 2014; Mantel et al., 2013; Marcilla et al., 2014; Ofir-Birin et al., 2017; Regev-Rudzki et al., 2013). It was also shown that EVs derived from pRBCs can be internalized and transmit genetic material to other pRBCs (Mantel et al., 2013; Regev-Rudzki et al., 2013), monocytes (Sisquella et al., 2017) and endothelial cells (Saxena et al., 2019).

EVs present several advantages over synthetic delivery systems including (i) greater stability in the blood because of their natural surface composition (Kooijmans et al., 2012; Stremersch et al., 2016), (ii) possibly better protection of the encapsulated cargo due to a proteo-lipid architecture (Stremersch et al., 2016), (iii) endogenous cell and tissue targeting features afforded by their adhesion molecules and surface ligands (Kooijmans et al., 2012; Stremersch et al., 2016), (iv) higher biocompatibility allowing improved permeability through biological barriers, including the blood-brain barrier (Qu et al., 2018; Usman et al., 2018), and

(v) almost nonimmunogenic character when used from autologous sources (Johnsen et al., 2014; Kao and Papoutsakis, 2019; Kooijmans et al., 2012; Liao et al., 2019; Lu et al., 2018; Pinheiro et al., 2018; Stremersch et al., 2016; Usman et al., 2018).

EVs from ~~various~~ diverse cell types have been used as drug delivery systems for a variety of therapeutic agents (Vader et al., 2016), including both macromolecules (DNA, RNA (Alvarez-Erviti et al., 2011; Skog et al., 2008; Viñas et al., 2016) and proteins (Cho et al., 2018)) and small molecules (e.g. curcumin (Sun et al., 2010; Zhuang et al., 2011), doxorubicin (Lee et al., 2018; Tian et al., 2014), paclitaxel (Pascucci et al., 2014; Saari et al., 2015), methotrexate and cisplatin (Tang et al., 2012), photosensitizers (Silva et al., 2013), porphyrins (Fuhrmann et al., 2015) and dopamine (Qu et al., 2018)). Most of these studies focused on cancer therapy (Cho et al., 2018; Lee et al., 2018; Pascucci et al., 2014; Saari et al., 2015; Skog et al., 2008; Tang et al., 2012; Tian et al., 2014; Zhuang et al., 2011), but it was also shown that EVs can be used to treat other pathologies such as cerebral occlusion (Xin et al., 2013), Alzheimer´s (Alvarez-Erviti et al., 2011) and Parkinson´s disease (Qu et al., 2018), and ischemic kidney injury (Viñas et al., 2016). In the present study, EVs have been evaluated for the first time as drug delivery systems for a parasitic disease. EVs derived from pRBCs and non-infected RBCs were isolated and their size and composition were determined by bead-based flow cytometry assay, nanoparticle tracking analysis, cryogenic transmission electron microscopy, and lipidomic approaches. The targeting behavior of EVs towards RBCs and pRBCs was evaluated by flow cytometry. Finally, antimalarial drugs were loaded into EVs and their efficacy in inhibiting parasite growth was determined~~, showing that EVs have potential to treat parasitic diseases~~.

2. Materials and Methods

2.1. Materials

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich® (Saint Louis, USA).

2.2. *P. falciparum* culture

The *P. falciparum* 3D7 strain was grown *in vitro* in group B human erythrocytes as described previously (Cranmer et al., 1997). Briefly, parasites were cultured at 37 °C in T-75 flasks with

RBCs at 3% hematocrit in Roswell Park Memorial Institute (RPMI) complete medium containing 5 mg/mL Albumax II (RPMI-A, Life Technology, Auckland, New Zealand), supplemented with 2 mM L-glutamine (RPMI-AG), under a gas mixture of 92.5% N_2, 5.5% CO_2, and 2% O_2. Parasitemia was determined by microscopic counting of blood smears fixed briefly with methanol and stained with Giemsa (Merck Chemicals, Darmstadt, Germany) diluted 1:10 in Sorenson's buffer, pH 7.2, for 10 min. To obtain cultures synchronized in early ring stages (0-24 h post-invasion), a 5% sorbitol lysis was performed (Lambros and Vanderberg, 1979). Late-form trophozoite and schizont stages (24-36 h and 36-48 h post-invasion, respectively) were selected in 70% Percoll (GE Healthcare, Uppsala, Sweden) (Lambros and Vanderberg, 1979). For culture maintenance, parasitemia was kept at 3-5% by dilution with freshly washed RBCs, and the medium was changed every 1-2 days.

2.3. Isolation of EVs derived from RBCs and pRBCs

EVs were isolated by centrifugation and ultrafiltration followed by size-exclusion chromatography (SEC). A previously described protocol (Díaz-Varela et al., 2018) was adapted for EV~~s~~ isolation from either non-infected RBCs at 3% hematocrit in RPMI-AG (48 h incubation) or *P. falciparum* cultured in RPMI-AG medium synchronized in ring stages and grown for 48 h at 2% ~~of~~ initial parasitemia (RBC-EVs and pRBC-EVs, respectively). Briefly, the culture was centrifuged at 400×g for 10 min to remove cells, and the resulting EV-containing supernatant was centrifuged twice at 2,000×g for 10 min to remove cell debris. To further concentrate EVs, the supernatant was passed through an Amicon Ultra-15 c~~C~~entrifugal f~~F~~ilter (100 kDa molecular weight cut off; Millipore-Merck, Cork, Ireland). Finally, 1 mL of the resulting concentrate was loaded onto a Sepharose CL4B200 column (10 mL), which was pre-equilibrated with phosphate-buffered saline (PBS), and the purification was performed by gravity flow at room temperature (RT). 0.5-mL fractions were collected, and fractions 8 and 9 were used for the following experiments, after being concentrated using an Amicon Ultra-4 c~~C~~entrifugal f~~F~~ilter (100 kDa molecular weight cut off; Millipore-Merck). Two different Amicon Ultra c~~C~~entrifugal f~~F~~ilters with the same cut-off were used based on the initial volume of the samples and the desired volume of recovery. The protein concentration of EV suspensions in PBS was

determined using a Pierce™ BCA Protein Assay Kit (ThermoScientific, Rockford, USA) according to the manufacturer's instructions.

2.4. EV characterization

2.4.1. Bead-based flow cytometry

Following a previously published protocol (de Menezes-Neto et al., 2015; Díaz-Varela et al., 2018), SEC fractions were analyzed by bead-based flow cytometry for the presence of the proteins glycophorin A (GPA) and histidine-rich protein 2 (HRP II), present in all RBCs and only in pRBCs, respectively. Briefly, SEC fractions were coupled to 4 µm-aldehyde/sulfate latex beads (Invitrogen, Carlsbad, USA) for 15 min at RT. Beads were then resuspended in 1 mL of bead-coupling buffer (BCB: PBS containing 0.1% BSA and 0.01% NaN_3) and incubated overnight (RT, on rotation). EV-coated beads were then centrifuged (2,000×g, 10 min, RT) and the supernatant was removed, leaving the required sample volume to evaluate the different markers, before incubation with mouse monoclonal IgG anti-human GPA (Acris Antibodies, Herford, Germany) or mouse monoclonal anti-HRP II (Acris Antibodies) at 1:500 dilution for 30 min at 4 °C. After washing with BCB, EV-coated beads were incubated for 30 min at 4 °C with Alexa Fluor 660®-labeled goat anti-mouse secondary antibody (Invitrogen) at 1:500 dilution. Coated beads incubated with secondary antibodies only were used as a control. Labeled samples were analyzed by flow cytometry using a BD LSRFortessa flow cytometer (Becton, Dickinson and Company, New Jersey, USA), detected by excitation through a 640 nm laser and emission collection with a 710/50 nm bandpass filter; 20,000 beads per sample were examined and mean fluorescence intensity was determined using Flowing Software 2.5.1.

2.4.2. Nanoparticle tracking analysis (NTA)

The size and concentration of particles were determined at RT with a NanoSight LM10 system (Malvern Instruments Ltd) (de Menezes-Neto et al., 2015). Before analysis, EVs were diluted 2 to 100 times in PBS. For each measurement, three 60-sec videos were recorded with the camera level set at 16. Data were analyzed with NTA software (version 3.1).

2.4.3. Transmission electron microscopy (TEM)

~~Knob-bearing erythrocytes infected with mature blood-stage *P. falciparum* parasites were purified from *in vitro* cultures in 70% Percoll (Dluzewski et al., 1984) and fixed at 4 °C for 2 h with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Ultrathin cryosections were then obtained and processed as described elsewhere (Urbán et al., 2014), placed on Formvar®-coated 200-hexagonal mesh nickel grids, and finally spiked, for focusing aid purposes, with an IgG coupled to 12 nm colloidal gold particles (Jackson ImmunoResearch). The observations were done in a Tecnai Spirit electron microscope.~~

For cryogenic TEM (cryo-TEM), a thin film of pRBC-EVs and RBC-EVs was formed on a holey carbon grid and vitrified by plunging into ethane maintained at its melting point, using a Vitrobot (FEI Company, Eindhoven, The Netherlands). The vitreous films were transferred to a Tecnai F20 TEM (FEI Company), and the samples were observed in a low dose mode. Images were acquired with a CCD Eagle camera (FEI Company) at 200 kV at a temperature of –173 °C, using low-dose imaging conditions.

2.4.4. Lipidomic analysis

Lipid extraction was performed mixing 150 µL of fraction 8 or 9 of SEC-purified RBC-EVs or pRBC-EVs in PBS with 150 µL of chloroform:methanol (2:1), vigorously stirring for 2 min with a vortex. The resulting emulsion was centrifuged for 1 min at 16,000×g, and the lower phase containing the lipids was collected using a glass Pasteur pipette and stored at 4 °C until mass spectrometry analysis. Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectra were recorded on a Sciex TOF/TOF 4800 MALDI mass spectrometer equipped with an Nd:YAG laser (355 nm). Time-of-flight mass analyses were acquired in reflectron and positive ion modes (low response was obtained in negative ion mode). One-µL aliquots of the extracted lipid solutions were mixed with 1 µL of the matrix solution (2,5-dihydroxybenzoic acid saturated solution in acetonitrile), and 1 µL of these mixtures was spotted on the sample plate and left to air dry before the analysis. Spectra were recorded from 500 to 1500 m/z values and the dominant species were observed in the range of 650-850 m/z values. Chemical identification of the most abundant lipids in the sample was done based on the m/z values detected: $[M+H]^+$ and $[M+Na]^+$ for every compound, according to (Käkelä et al., 2003; Löhmann et al., 2010). The lipidic identification was confirmed based on major fragment ions of the

MS/MS spectrum after performing a manual monoisotopic mass selection for every individual molecular species (H^+ and Na^+ adducts).

2.5. Cell targeting analysis

For pRBC targeting assays, pRBC-EVs and RBC-EVs were labeled with the amine-reactive fluorescent tag NHS-rhodamine (Thermo Scientific, Rockford, USA). Lyophilized dye was dissolved at a concentration of 10 mg/mL in dimethyl sulfoxide (DMSO), and 900 µL of combined EV fraction 8 and 9 were mixed with 100 µL of 100 mM sodium bicarbonate (pH 8) containing 0.1 mg/mL NHS-rhodamine and incubated for 1 h at RT (Kooijmans et al., 2018). The unbound label was finally removed by SEC as described above. Fractions 8 and 9 were concentrated in Amicon Ultra-4 cCentrifugal fFilters (100 kDa cut off).

Labeled RBC-EVs (Rho-RBC-EVs) and pRBC-EVs (Rho-pRBC-EVs) were analyzed in a BD LSRFortessa flow cytometer. Forward- and side-scatter on a logarithmic scale and a mix of fluorescent beads of varied diameters (Megamix-Plus SSC, BioCytex, Marseille, France) were used to gate EVs. NHS-rhodamine fluorescence was detected by excitation through a 561 nm laser at 50 mW power and emission collection with a 582/15 nm bandpass filter. The acquisition was configured to stop after recording 20,000 events within the EV population.

Rho-pRBC-EVs or Rho-RBC-EVs in PBS (protein content: 290 µg/mL, ca. 9.7×10^8 ±0.2 particles/mL) were added to desynchronized living *P. falciparum* 3D7 cultures and incubated for 24 h. The cells were then stained for 10 min at RT with 4 µg/mL of the DNA dye Hoechst 33342, washed once (500×g, 5 min) with RPMI-A, and resuspended in RPMI-AG. Samples were analyzed at 0.06 % hematocrit in PBS in a BD LSRFortessa flow cytometer and also in an imaging flow cytometer.

Forward- and side-scatter in a logarithmic scale were used to gate the RBC population. NHS-rhodamine and Hoechst 33342 fluorescence were detected by excitation through 561 and 405 nm lasers at 50 mW power and emission collection with 582/15 and 450/50 nm bandpass filters, respectively. The acquisition was configured to stop after recording 50,000 events within the RBC population. For imaging flow cytometry (Amnis® ImageStream®X, Luminex). The acquisition was configured to stop after recording 10,000 events within the RBC population. Double events,

debris, and out of focus events were excluded, gating the population of interest. Thereby, the population evaluated consisted of best focus single events.

2.6. Growth inhibition assays

pRBC-EVs that had been isolated by centrifugation and ultrafiltration followed by SEC from a 3-day-old *P. falciparum* 3D7 culture (0.5% initial parasitemia, late stages) were incubated in RPMI-AG for 24 h at RT without ~~agitation~~ stirring with two different concentrations of lumefantrine, tafenoquine, or atovaquone. A fresh *P. falciparum* 3D7 culture (6% hematocrit and 1.2% parasitemia, ring stages) was seeded in 96-well plates and incubated for 48 h in the presence of the mixture of EVs (15.25 or 6.10 µg protein/mL) and drugs (atovaquone at 0.085 and 0.17 nM, lumefantrine at 1.89 and 23.62 nM, and tafenoquine at 937.5 and 1875 nM final concentrations). Parasite growth was determined in a BD FACSCalibur flow cytometer (Becton, Dickinson and Company, New Jersey, USA); samples were analyzed at 0.024 % hematocrit in PBS, and the nuclei of pRBCs (the only nucleated cells present in the culture) were stained by addition of 0.1 µM Syto11 (Invitrogen), added 10 min before analysis (Marques et al., 2016; Moles et al., 2015). Forward- and side-scatter on a logarithmic scale were used to gate the RBC population. Syto 11 green fluorescence was detected by excitation ~~through a~~at 488 nm ~~at~~ (50 mW power) and emission collection with a 530/30 nm bandpass filter in the logarithmic scale. The acquisition was configured to stop after recording 50,000 events within the RBC population. Growth inhibition was determined by comparison of the parasite growth between treated and non-treated cultures.

A drug-free EV concentration range from 3 to 200 µg protein/mL was also evaluated for potential *in vitro* antimalarial activity. Cell-free RPMI-AG was processed identically as pRBC and RBC cultures for EV isolation, and the resulting EV-free fractions were used as control.

2.7. Statistical analysis

Differences between two data groups were analyzed using t-tests. Comparisons between more than two groups were made using one-way ANOVA with a Tukey post hoc test. Statistical differences were considered significant when $p < 0.05$.

3. Results and Discussion:

3.1. EV isolation and characterization

~~TEM images of *P. falciparum* 3D7 *in vitro* cultures revealed the presence of regions with abundant EVs in the close vicinity of pRBCs (Fig. 1). The concentration gradient observed, with a higher abundance of EVs near the plasma membrane and a thinning out of their numbers as the distance from the cell increased, was an indicator of the pRBC that was producing them.~~

To ~~further~~ characterize RBC- and pRBC-derived EVs, vesicles were isolated by combining ultrafiltration with SEC, a method which led to higher EV yield compared to ultracentrifugation (Nordin et al., 2015). SEC also offered a separation of free proteins in solution, increasing the purity of EVs (Vader et al., 2016). Moreover, the mild conditions of this method should better preserve the biophysical properties of EVs and allow them to retain functionality (Nordin et al., 2015; Stremersch et al., 2016; Vader et al., 2016). Since EVs have surface proteins of the parental cell (Mardahl et al., 2019), EV-containing SEC fractions were identified by the presence of HRP II and of GPA. HRP II is a parasite protein expressed during most of the *P. falciparum* life cycle (Howard et al., 1986) and GPA is an abundant membrane protein present in all RBCs (Siebert and Fukuda, 1986) and both were previously identified in microvesicles derived from pRBCs (Mantel et al., 2013). According to bead-based flow cytometry assays, HRP II was best detected in SEC fractions 8 and 9 (Fig. ~~2~~1A), whereas GPA showed a broader elution profile, and was most abundant in fractions 7, 8 and 9 for RBC-EVs, and in fractions 6, 7, 8 and 9 for pRBC-EVs (Fig. ~~2~~1B). Based on these results, fractions 8 and 9 were chosen for further experiments.

NTA revealed that RBC-EVs (fraction 8~~: mean size~~ 211.0 ±8.1 nm; fraction 9~~: mean size~~ 202.2 ±5.6 nm) were slightly larger than pRBC-EVs (fraction 8~~: mean size~~ 179.7 ±6.8 nm; fraction 9~~: mean size~~ 175.2 ±1.0 nm) (Fig. 1~~2~~C,~~-~~D, respectively). This was in contrast with the results reported by Mantel el at. (Mantel et al., 2013), where the majority of EVs derived from pRBCs and RBCs were sized between 100 and 150 nm, a discrepancy that may be attributed to the use of a different EV isolation method (sucrose cushion ultracentrifugation). In 3% ~~of~~ hematocrit cultures incubated for 48 h, pRBCs produced more EVs than RBCs (Fig. ~~2~~1C,~~-~~D), in agreement with data from previous studies (Mantel et al., 2013). The determined protein content was 103 ±41 and 147 ±2 µg protein/mL for RBC-EVs and pRBC-EVs, respectively.

Cryo-TEM analysis confirmed for both RBC-EVs and pRBC-EVs a mean diameter around 200 nm (Fig. ~~2~~1E,~~-~~F), and the presence of mostly unilamellar smaller and larger EVs spanning a size range between ca. 100 and 400 nm. Other structures not enclosed by a lipid bilayer, especially abundant in pRBC-EV preparations, were also revealed by cryo-TEM (Fig. ~~2~~1F).

3.2. Lipidomic analysis of EVs

Lipids are involved in the biogenesis and function of EVs, and some lipids are more abundant in EVs when compared to their parent cells. For example, sphingomyelins, cholesterol, and phosphatidylserine, which are the main components of lipid rafts, are especially abundant in EVs (Brzozowski et al., 2018).

Proteomic analyses of RBC-EVs and pRBC-EVs are well described by Mantel et al. (Mantel et al., 2013). However, there is a lack of information regarding the lipid composition of pRBC-EVs and its potential alteration in pathological conditions (Brzozowski et al., 2018). Lipidomic analysis did not reveal significant qualitative differences between the lipid composition of EVs derived from RBCs and pRBCs (Fig. ~~3~~2). ~~For both EVs t~~The most abundant lipids identified in both EVs were phosphatidylcholines (m/z 734.6, 756.6, 758.6, 760.6, 762.6, 780.6, 782.6, 784.6, 786.6, 788.6, 804.6, 806.6, 808.6, 810.6), and~~, S~~ small amounts of sphingomyelins were also detected (701.4, 703.6, 725.6, 835.7, 837.7). The nature of these compounds was confirmed in MS/MS analysis by the presence of the characteristic m/z fragment of 184.1 Da derived from the phosphocholine headgroup (Löhmann et al., 2010). MS/MS ion fragmentation spectra of two phosphatidylcholines and two sphingomyelins are shown in the Supplementary Material~~Information~~ (Figs. S1 and S2, respectively). These results are consistent with the lipid composition of the RBC plasma membrane, where phosphatidylcholine and sphingomyelin are two of the main components (Quinn et al., 2009).

3.3. Cellular uptake of EVs

EV uptake is still a vexing question in the EV field because some studies demonstrated that the uptake of fluorescently labeled EVs can be done by practically every cell type evaluated (Svensson et al., 2013; Zech et al., 2012), whereas other reports proposed that EV take up only occurs by a very specific process if cell and EV share the right combination of ligand and

receptor (Mulcahy et al., 2014). Also, ~~I~~it has been proposed that EVs can interact with target cells through several mechanisms such as direct fusion with the plasma membrane, endocytosis, binding to cell surface receptors and docking at the cell surface (Armstrong and Stevens, 2018; Kao and Papoutsakis, 2019; Mardahl et al., 2019; Pinheiro et al., 2018). Notably, the mechanism of EV interaction with cells, which depends in part on the recipient cell type (Kao and Papoutsakis, 2019), will influence their biodistribution and therapeutic potential (Pinheiro et al., 2018).

To determine the targeting behavior towards RBCs and pRBCs of EVs, these were labeled with NHS-rhodamine. EVs from RBC and pRBC were labeled with similar efficiency (70.1 ±0.5% and 74.1 ±3.5%, respectively).

~~To investigate EV targeting to RBCs and pRBCs, r~~Rhodamine-labeled EVs were incubated for 24 h with an *in vitro P. falciparum* culture. Flow cytometry analysis revealed that both RBC- and pRBC-EVs bound RBCs and pRBCs (Fig. ~~4A~~3A, ~~-~~B); pRBC-derived EVs were incorporated by both cell types with significantly higher avidity, and both vesicle types bound pRBCs significantly better than non-infected erythrocytes. In addition, EV targeting to RBCs and pRBCs using rhodamine-labeled EVs was also evaluated incubating pRBC-EVs for 15 min with an *in vitro P. falciparum* culture. EV uptake already occurred within this timeframe (Fig. S3).

The interactions of EVs with both RBCs and pRBCs could benefit their function as carriers of antimalarial drugs in drug delivery strategies. As shown in previous studies, delivering antimalarial drugs to non-infected RBCs could be an interesting strategy because *Plasmodium* would encounter a hostile environment immediately after invading the RBC, which would compromise its survival capacity (Moles et al., 2015). This approach resulted in a significantly improved efficacy of drugs encapsulated inside liposomes targeted to both pRBCs and RBCs (Moles et al., 2017, 2015). pRBC-EVs and RBC-EVs were incorporated by 100% of RBCs infected by *Plasmodium* late forms, whereas they were internalized by 87.1 ±1.4% and 76.9 ±1.9% of RBCs infected by *Plasmodium* ring forms, respectively (Fig. ~~4A~~3A). The internalization of EVs by pRBCs containing early blood stages of *Plasmodium* is an attractive feature for the potential development of EVs as drug carriers, since delivering antimalarial compounds to parasite cells early in their intraerythrocytic cycle will contribute to reducing the viability of the pathogen (Moles et al., 2015). Using monocytes as targeting cells, Sisquella et al.

demonstrated that pRBC-EVs showed a higher level of uptake by monocytes as compared to RBC-EVs (Sisquella et al., 2017). This suggests that target cells prefer uptake of EVs derived from infected cells over those from non-infected cells, possibly through the upregulation of specific surface markers on ~~the~~ pRBC-EVs ~~which~~ favourings ~~s~~ interactions with the plasma membrane of target cells (Mardahl et al., 2019).

The internalization of pRBC-EVs by other pRBCs ~~(Mantel et al., 2013; Regev-Rudzki et al., 2013)~~ and their role in intercellular communication affecting malaria pathogenesis have been described in the literature (Mantel et al., 2013; Regev-Rudzki et al., 2013; Saxena et al., 2019; Sisquella et al., 2017). However, to date, of our knowledge, there is no description of the role of EVs in physiological communication between RBCs. It has been claimed that EVs derived from group O RBCs can be used for large-scale EV production since they are readily available in blood banks and they are devoid of DNA (Usman et al., 2018), but EVs derived from RBCs have been associated to activation of coagulation (Biró et al., 2003; Burger et al., 2013; Rubin et al., 2013), endothelial activation (Straat et al., 2016b) and immunomodulation (Danesh et al., 2014; Straat et al., 2016a), and thus could be involved in side effects that occur after blood transfusion (Danesh et al., 2014; van Manen et al., 2019). In contrast with EVs produced by circulating RBCs, EVs produced during RBC storage do not have detectable levels of membrane markers associated with clearance, except for phosphatidylserine which was exposed in low amount by a small fraction of EVs (van Manen et al., 2019).

Imaging flow cytometry revealed an intracellular localization of EV-derived molecular components. Fig. ~~4C~~ 3C shows representative images of RBCs and pRBCs after uptake of rhodamine-labeled EVs, where a distribution of rhodamine-labeled EV molecular components throughout the cells can be observed. In parasitized erythrocytes, both RBC-EV- and pRBC-EV-derived components colocalized with the nuclei of the parasites (Figs.3C and S~~4~~3).

Although this study shows the uptake of RBC-EVs and pRBC-EVs by RBCs and pRBCs, the mechanism by which this phenomenon occurs remains unclear. One could hypothesize that, since RBCs lack a typical endocytosis machinery, EV uptake occurs through fusion with the RBC membrane. Mechanistic studies of EV interaction with RBCs are still lacking, but assays done with liposomes, which share a phospholipid bilayer structure with EVs, may provide important clues. A proof of concept study evaluating liposomes containing quantum

dots observed that liposomal contents entered the pRBCs through a process that likely involved fusion of the liposome lipid bilayer with the cell plasma membrane (Pujol et al., 2014; Urbán et al., 2011). In addition, phosphatidylcholine liposomes were shown to have better fusion/interaction with pRBC membranes compared with sphingomyelin liposomes (Hasan et al., 2011). As observed in the lipidomics study presented here, EVs derived from RBCs and pRBCs are rich in phosphatidylcholine, which could favor similar fusion processes, although it could also be speculated that structural and functional changes induced in RBCs by the parasite during its intraerythrocytic maturation (Hasan et al., 2011; Urbán et al., 2011) favor the higher uptake of EVs by pRBCs containing late forms of the parasite compared with pRBCs containing early blood stages of *Plasmodium*.

3.4. Growth inhibition assays

After evaluating the targeting behavior of EVs, which showed that pRBC-EVs had the most effective incorporation by RBCs and pRBCs, we next investigated the efficacy of pRBC-EVs as a drug delivery system. Therapeutic agents can be loaded into EVs in different manners, which can be divided into two major strategies (Kao and Papoutsakis, 2019; Liao et al., 2019; Vader et al., 2016). In the first approach, usually employed to encapsulate RNA and proteins, these are loaded into the parent cells by transfection or incubation and later the cells release EVs containing the therapeutic agent. A second method consists of loading drugs into EVs after their purification, following one of several protocols involving co-incubation, electroporation, saponin treatment, sonication, extrusion and freeze-thaw cycles (Liao et al., 2019; Vader et al., 2016). Co-incubation is a simple and usually efficient method to load EVs with lipophilic drugs (Johnsen et al., 2014; Liao et al., 2019), which was used to encapsulate e.g. curcumin (Sun et al., 2010; Zhuang et al., 2011), paclitaxel (Saari et al., 2015) and dopamine (Qu et al., 2018). However, the therapeutic agents can leak out of the EVs in biological fluids, which can limit their applicability (Stremersch et al., 2016).

Here, we evaluated the loading of the antimalarial drugs lumefantrine, tafenoquine, and atovaquone in two different concentrations of pRBC-EVs (6.10 or 15.25 µg protein/mL) by simple incubation for 24 h at RT. These drugs were chosen due to their strong hydrophobic character (respective log P values of 8.7, 5.0, and 5.2), which predicted their efficient

incorporation in the lipid bilayer following incubation with EVs. We employed concentrations that, based on the IC50 of these compounds, would promote low inhibition of parasite growth (Table 1). Non-encapsulated free drug was not removed from the samples because the different methods assayed to eliminate the drug not ~~un~~incorporated ~~drug~~ in~~to~~ EVs (ultracentrifugation, desalting columns, and SEC) were not able to clear the free compounds down to undetectable levels in *in vitro P. falciparum* growth inhibition assays, especially for the most potent antimalarials with lowest IC50 (data not shown). Therefore, we evaluated whether the fraction of drug encapsulated in pRBC-EVs could improve the therapeutic effect of the same amount of drug 100% in free form. Thus, the mixture of drug-loaded-EVs and the non-encapsulated free drug was added to synchronized *P. falciparum* 3D7 cultures, and growth inhibition was determined after 48 h by flow cytometry. As an additional control, pRBC-EVs without drug were included in the assay.

At their higher doses assayed, atovaquone and tafenoquine were more efficient in reducing the parasite viability in *in vitro P. falciparum* cultures when encapsulated in the higher pRBC-EVs concentration used (15.25 µg protein/mL) (Fig. 4~~5~~A~~,~~-C). This result suggests that more drug could be encapsulated when more EVs were available and indicates that pRBC-EVs potentiate the effect of their drug cargo in inhibiting parasite growth, possibly through increased delivery to target cells. Saari et al. (Saari et al., 2015) also showed that EVs enhanced the cytotoxic effect of paclitaxel in a way directly proportional to the quantity of EVs and to the intravesicular drug load.

At the lower drug doses tested, both tafenoquine and atovaquone encapsulated in the lower pRBC-EV concentration assayed (6.10 µg protein/mL) were more efficient than equal free drug amounts in decreasing parasite growth in an *in vitro P. falciparum* culture (Fig. ~~5A~~4A-C). Nevertheless, a dose effect was not observed upon coincubation of lower drug doses with higher EV numbers, which suggests a likely saturation of pRBC-EV drug intake. At higher concentrations of EVs, however, these could still accommodate more drug in higher drug concentration assays. Lumefantrine offered the worst results at all drug and EV concentrations tested (Fig. ~~5B~~4B). It is conceivable that its high lipophilicity (log P = 8.7) confers it a strong affinity for lipid bilayers, which might stabilize its permanence in the pRBC plasma membrane, where its antimalarial activity would be minor.

It is also worth noting that tafenoquine is more potent inhibiting parasite growth at the lower drug dose encapsulated into pRBC-EVs than at the higher drug dose (Fig. ~~5C~~4C). Although there is no obvious explanation for this result, one can speculate a destabilizing effect on EV structure of tafenoquine when incorporated in the lipid bilayer of the vesicles. Such an effect has been recently described for the antimalarial drug domiphen bromide when inserted into liposomal membranes (Biosca et al., 2019). Tafenoquine has a relatively high molecular mass (ca. 582 Da), and it is likely that at high concentrations its bulk might destabilize the EV structure.

The same amounts of drug-free pRBC-EVs as those used in drug-containing samples had no effect on parasite growth (Fig. ~~5A~~4~~A~~ D). Drug-free RBC-EVs and pRBC-EVs started inducing significant inhibition of *P. falciparum in vitro* growth above 100 µg protein/mL ~~(Fig. 5D4D)~~. This result can be explained because large EV numbers will merge with the *Plasmodium*-infected RBC membrane and destabilize it, facilitating its rupture before the parasite cycle is complete; the resulting immature merozoites would likely have a decreased re-invasion capacity, which would result in a reduced parasite growth.

4. Conclusion

The current consensus is that a combination of different treatment strategies is required to eradicate malaria. In the drug therapy approach, it is important to have a drug delivery system that will efficiently encapsulate the drug and deliver it to the intended cell type. This will enable the use of overall reduced drug doses, with decreased adverse effects, but at the same time it will provide the capacity to deliver sufficiently high local amounts of drug to rapidly kill the parasite and thus limit drug resistance evolution. Here, we have isolated and characterized EVs from RBCs and pRBCs and explored them as antimalarial drug delivery vehicles. Both vesicle types showed potential as drug delivery carrier to RBCs and pRBCs because they were efficiently internalized by these cell types and the antimalarial drugs ~~lumefantrine,~~ atovaquone and tafenoquine, when loaded in pRBC-EVs, more efficiently inhibited parasite growth than the free drug counterparts. Hence, pRBC-EVs could be an interesting candidate drug delivery system for certain lipophilic antimalarial drugs.

Potential risks of using pRBC-derived EVs as drug delivery vehicles include accidental gene transfer. In the case of RBCs, since they are devoid of both nuclear and mitochondrial DNA, it is claimed that EVs derived from these cells are potentially safer than EVs derived from nucleated cells (Usman et al., 2018). However, although RBCs lack DNA, they contain a small set of miRNA species that are present in EVs derived from them and which have been described to modulate target gene expression in endothelial cells (Mantel et al., 2016). Taken together, these reports counsel further investigation of the safety of pRBC-EVs and RBC-EVs as drug delivery vehicles.

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Conflicts of interest

The authors declare no competing financial interest.

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