Turning *Plasmodium* survival strategies against itself

Livia Neves Borgheti-Cardoso & Xavier Fernàndez-Busquets

[1] Nanomalaria Group, Institute for Bioengineering of Catalonia (IBEC), The Barcelona Institute of Science and Technology, Baldiri Reixac 10-12, ES-08028 Barcelona, Spain.

[2] Barcelona Institute for Global Health (ISGlobal, Hospital Clínic-Universitat de Barcelona), Rosselló 149-153, ES-08036 Barcelona, Spain.

[3] Nanoscience and Nanotechnology Institute (IN2UB), University of Barcelona, Martí i Franquès 1, ES-08028 Barcelona, Spain.

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The fragile malaria parasite, *Plasmodium* spp., through millions of years of coevolution with its natural hosts, has developed exquisitely sophisticated strategies to survive hostile environments in the blood and in mosquito tissues. Some of its tricks are, among others, (i) storing in an iron-enriched crystalline structure the toxic heme group resulting from hemoglobin digestion; (ii) using as human host cell erythrocytes, terminally differentiated cells which do not emit any alarm signal when parasitized by the pathogen; (iii) adhering to vascular endothelia to remove itself from the circulation thus avoiding spleen clearance; and (iv) employing extracellular vesicle-mediated crosstalk to spread drug resistance throughout the parasite's population. In this Editorial we will discuss how we might profit from these evolutionary adaptations and turn them against the parasite, e.g. by (i) taking advantage of the iron-rich hemozoin crystal for applying magnetism to new antimalarial approaches, (ii) harnessing erythrocytes as antimalarial drug carriers, (iii) employing as targeting elements of drug-loaded nanovectors certain cell surface ligands used by *Plasmodium* for its sequestration in capillaries, or (iv) using *Plasmodium*-generated extracellular vesicles as specifically targeted drug nanocarriers.

Magnetic fields to treat and diagnose malaria?

During its growth within the red blood cell (RBC), *Plasmodium* feeds on hemoglobin, whose digestion releases its heme group that generates toxic reactive oxygen species as it converts to hematin, which can bind and disrupt biological membranes, leading to lysis of the host erythrocyte. To avoid this havoc, the malaria parasite detoxifies hematin by biocrystallization, storing it as insoluble and chemically inert, weakly magnetic β-hematin crystals called hemozoin [1]. Hemozoin is the only major magnetic compound that accumulates in *Plasmodium*-infected RBCs (pRBCs), and it cannot be found in uninfected RBCs in similar quantities. The magnetic properties of pRBCs might be exploited for the development of new malaria treatments where the rapid evolution of resistance is unlikely because this strategy would not act on individual gene products. Although a previous report described a possible inhibitory effect of magnetic fields on malaria parasite growth [2], there is no obvious magnetic characteristic identified in hemozoin which could explain this observation, and more research is needed to profit from the interesting properties of

hemozoin for the design of new antimalarial therapies. In the short term, however, magnetism seems more readily applicable to develop new diagnostic tools. A fraction of the malaria parasites found in the peripheral blood are gametocytes, the only stage capable of infecting mosquitoes. In *Plasmodium falciparum* infections, it is important to diagnose gametocytes at point of care because this stage is less prone to clearance by commonly used antimalarial drugs. Since malaria transmission is sustained to a large extent by submicroscopic gametocyte reservoirs, their detection is essential to provide adequate treatments. Magnetic fractionation has provided a limit of gametocyte detection below that corresponding to the lowest epidemiologically relevant densities [3], and an improved magneto-optical method was capable of detecting lower parasitemias than currently available rapid diagnostic tests [4].

Erythrocytes as antimalarial drug carriers

Erythrocytes are essentially hemoglobin-filled bags which lack most of the metabolism from other cells. The presence of such a poor molecular machinery suggested that this cell could be used as drug carrier [5] without significantly altering its oxygen- and carbon dioxide-transporting function. Although the original idea was to use RBCs as safe and innocuous transporters of therapeutic agents to diseased cells or tissues, this approach acquired a new and exciting application potential upon the realization that it was exquisitely suited to treat pathogens like *Plasmodium*, which have erythrocytes as their main residence in the human body [6]. RBCs can theoretically be loaded with most antimalarial drugs because the corresponding molecular targets are absent or not accessible (e.g.: chloroquine and its derivatives inhibit heme group detoxification; artemisinin is supposed to react with free heme and iron(II) oxide, resulting in the generation of toxic free radicals; fosmidomycin targets the methyl erythritol pathway of isoprenoid biosynthesis, which is not found in animals). RBC loading with drug concentrations sufficiently high to be lethal for *Plasmodium* can be done with drug-carrying liposomes targeted to ubiquitous RBC plasma membrane components such as glycophorin A [7]. Other attractive features of RBCs as drug carriers [8] are their biocompatibility, a long circulation half-life of about 120 days, and the existence of natural mechanisms for their safe elimination from the body. Last but not least, self RBCs do not elicit rejection reactions and can be used among blood-compatible individuals. Despite these benefits, erythrocytes have limitations as antimalarial drug carriers [6], such as the relatively expensive and technically demanding immunoliposomal technology required for drug loading. Potential foreseeable solutions to these obstacles are the substitution of antibodies by cheaper targeting elements like DNA aptamers or using the capacity of certain antimalarial drugs to become internalized and retained in RBCs for their direct liposome-free loading [9]. Indeed, many injected substances interact with erythrocytes, which partition and metabolize more than 50 known drugs [8].

***Plasmodium* carbohydrate ligands as drug-loaded nanocarrier targets**

Some of the receptors that *Plasmodium* uses to invade RBCs, adhere to other cells or sequester in the capillaries and in some organs like the placenta are glycosaminoglycans such as heparin and chondroitin or heparan sulfates [10]. Having as receptor a polysaccharide whose structure does not change between the different individuals of a species seems *a priori* an advantage for the pathogen when compared to targeting

proteins, which can evolve with relative rapidity to new variants for which the parasite would have less affinity. However, nanotechnology provides the opportunity to quickly develop new efficient therapeutic strategies that could give us an edge in the fight against infectious disease. Because the malaria parasite relies so much on binding glycosaminoglycans for essential parts of its life cycle, these natural polymers could be easily adapted to direct drug-loaded nanocarriers to pRBCs with high efficacy. Unlike antibody-based targeting against the relatively few and often variable proteins exported by *Plasmodium* to the pRBC surface, heparin-mediated targeting is not expected to be rapidly inactivated through evolution. A strong argument that supports this prediction comes from research done on a useful characteristic of heparin, namely its activity as antimalarial drug itself. The concentrations where heparin is toxic for *Plasmodium* are evidently much higher than those naturally encountered by the parasite, and which it uses as binding ligand. However, not even a continued treatment of *in vitro Plasmodium* cultures with high heparin levels could induce the evolution of resistant pathogen strains [11].

Extracellular vesicles as targeted drug delivery vehicles

Extracellular vesicles (EVs) have been recently gaining increased attention due to their proven participation in numerous essential cellular processes [12], and their involvement in the pathology of numerous parasitic diseases has been undisputedly established [13]. In malaria, *Plasmodium*-derived EVs containing parasite proteins and nucleic acids are released from both early and late pathogen stages, and have been found to mediate cell-cell communication through their targeting to and internalization by pRBCs [14,15]. Host-derived vesicles from endothelial cells, platelets and erythrocytes have been also found to increase their numbers during infection and have been linked to enhanced disease pathogenesis [16], promoting the pRBC adhesion to brain capillaries characteristic of severe malaria [17,18]. EVs found in malaria parasite infections could be interesting as nanocarriers to deliver drugs due to intrinsic cell targeting properties imparted by their surface ligand and adhesion molecules, a natural ability to deliver cargo into cells, and their protection from degradation in the circulation [19]. Furthermore, they are able to cross biological filters such as the blood-brain barrier and, as the composition of their membrane is that of the donor cell, they are nearly non-immunogenic when used autologously. However, for therapeutic applications some issues have to be overcome in advance such as the development of methods for scalable EV isolation and efficient approaches for drug loading. Ultrafiltration- and size exclusion chromatography-based purification methods seem promising for large-scale clinical application, although further optimizations with regards to yield, reproducibility, and purity should be implemented [20]. An alternative method is preparing EVs from broken cells whose membranes have the structural and physical features of EV lipid bilayers, which can result in a 100-fold increased vesicle yield compared to isolation of naturally produced vesicles. The membrane of EVs, which is essential to protect their cargo from degradation in the bloodstream, makes loading of exogenous cargo into EVs challenging. Nevertheless, several methods to load therapeutic agents into EVs have already been described, among these the pre-incorporation of drugs into cells from which the EVs are later derived, and the loading of EVs after their isolation using methods such as incubation at room temperature, saponin-mediated permeabilization, sonication, freeze-thaw cycles, extrusion and electroporation [20]. Although

pRBC-derived EVs present surface ligands that favor interactions with other erythrocytes, their natural bioelimination in the spleen and liver challenges their efficient use as long-circulating nanocarriers; however, it is possible to engineer EVs in such a way that increased blood residence time and binding to the desired tissues or cellular targets are achieved [20].

Conclusion

After more than a century of failed attempts to eradicate malaria using drugs against which the parasite has always evolved resistance with astonishing rapidity, innovative out-of-the-beaten-path strategies are called for. Among them, those involving processes that do not depend on single gene products and which are at the core of the pathogen's pathophysiology will have higher chances of success. Of these, the hemozoin crystal, erythrocytes themselves, glycosaminoglycan interactions and EV trafficking are the result of sufficiently complex evolutionary tracks as to offer prospects for the development of new efficacious and lasting antimalarial approaches.

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