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Review

Applying chemical genetic tools to the study of phospho-signalling pathways in malaria parasites☆

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ABSTRACT

Until very recently there has been very little information about the phospho-signalling pathways in apicomplexan parasites including the most virulent species of human malaria parasite, *Plasmodium falciparum*. With the advancement of mass spectrometry-based phosphoproteomics and the development of chemical genetic approaches to target specific parasite protein kinases, the complexity of the essential role played by phosphorylation in maintaining the viability of apicomplexan parasites is now being revealed. This review will describe these recent advances and will discuss how these approaches can be used to validate parasite protein kinases as drug targets and to determine the on- and off-target action of protein kinase inhibitors. This article is part of a Special Issue entitled: Inhibitors of Protein Kinases.

1. Introduction

Despite the efforts to control malaria there are still approximately 600 thousand malaria-related deaths annually [1]. Human malaria is caused by five species of *Plasmodium* namely — *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*. Of these *P. falciparum* is responsible for almost all human malaria-related deaths, most of which are children under five years of age. All *Plasmodium* spp. which infect humans have a similar complex life cycle involving two hosts — humans and the female *Anopheles* mosquito. The bite of an infected mosquito transfers sporozoites to the human body where they invade liver cells [2]. Inside the hepatocyte, the parasite goes through multiple rounds of cell division generating many thousands of merozoites which are released into the blood stream via parasite filled vesicles called merosomes [3]. The released merozoites invade red blood cells (RBCs) in a multi-step process that involves attachment and reorientation so that the apical end of the merozoite is in contact with the RBC. This is followed by the release of apical organelle proteins that mediate receptor–ligand interactions between the receptors on the RBCs and ligands on the surface of merozoites [4]. This process promotes formation of a tight junction and drives the invasion of the parasite into the RBC where the parasite multiplies to generate up to 32 merozoites which are released into the blood and invade further RBCs in a cycle that causes the clinical symptoms of malaria. During this process a small number of infected RBCs form either male or female gametocytes that are taken up in the blood meal of a mosquito. The combination of low temperature with either, an increase in pH and/or the presence of xanthurenic acid within the mosquito mid-gut triggers the formation of gametes from the gametocytes [5]. Male gametocytes release eight gametes from each gametocyte as it goes through three rounds of replication. In contrast, each female gametocyte only forms one gamete. The female and male gametes fuse to form a diploid zygote from which haploid motile ookinetes develop that cross through the mosquito mid-gut to form oocysts. Multiple rounds of replication in oocysts result in sporozoites that are released and migrate to the mosquito salivary glands, ready for release into another human host during a blood meal (Fig. 1).

Artemisinin-based combination therapies (ACTs) are the current frontline therapy for *P. falciparum* infection. Although highly effective, the emergence of resistance to artemisinin means that it is urgent that we find new drugs to treat malaria. In other human diseases, in particular cancer, protein kinases have been successfully targeted; however targeting protein kinases in malaria drug discovery is in its infancy [6]. This is despite the fact that 36 of the 65 eukaryotic protein kinases in *P. falciparum* have been shown to be essential for the survival of the blood stage parasite. Furthermore, extensive studies of parasite kinases

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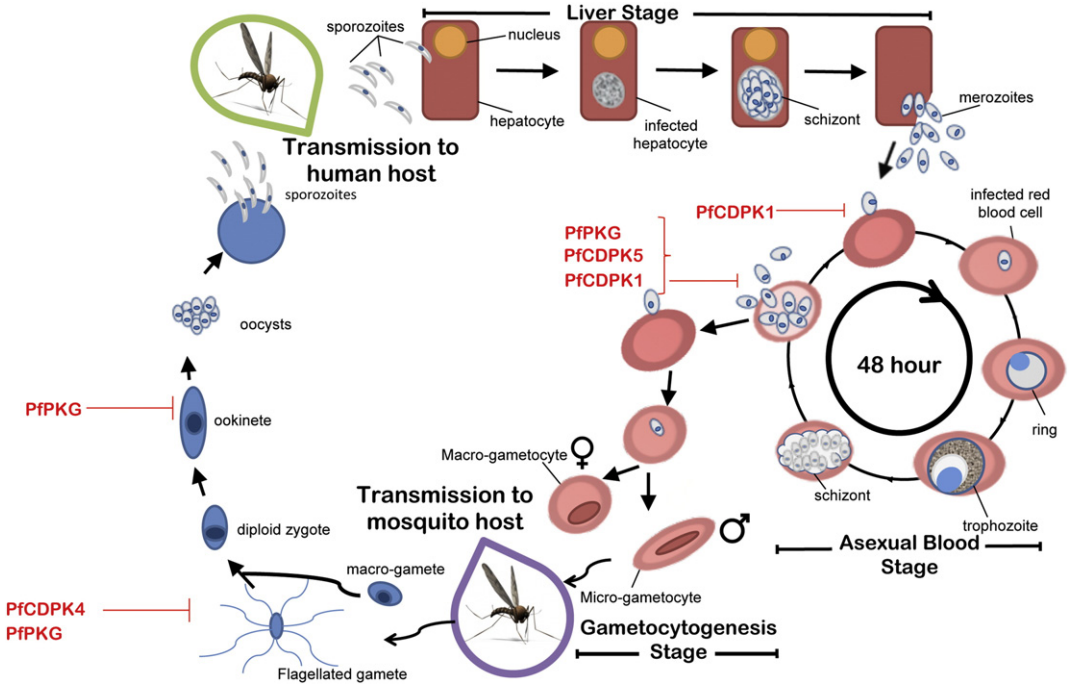

Fig. 1. Life Cycle of *Plasmodium falciparum*. Illustrated is the life cycle of *P. falciparum*. The life cycle stages where inhibition or knockout of PKG and the CDPKs, kinases that are discussed in this review, results in parasites unable to progress through that part of the life cycle are shown.

such as; *Pf*PKG — involved in gametogenesis [7], ookinete motility [8] and egress of merozoites from RBCs [9]; CDPK4 — involved in gametogenesis; CDPK1 — which has an essential role in egress of merozoites from infected RBCs [10,11]; CDPK5 — involved in egress of merozoites from RBCs [12] and the NEK kinase family — involved in progression through the asexual stages and meiosis [13,14] point to malaria protein kinases as excellent targets in the treatment of malaria.

In order to pursue malaria protein kinases as drug targets it will be important to understand the essential role played by these enzymes in maintaining the viability of the malaria parasite and define the effects of targeting specific parasite kinases. Mass spectrometry-based global phosphoproteomic techniques have made a significant impact on our understanding of the role played by protein phosphorylation in malaria [15]. Furthermore, there have been a number of genetic and chemical genetic techniques that have been employed in the study of specific malaria protein kinases. By combining these two approaches it is likely that we will not only validate specific malaria protein kinases as targets in drug discovery, but also provide much-needed basic biological information regarding the essential role played by key malaria protein kinases.

Here we review the advances made in understanding the malaria phosphoproteome and the genetic and chemical genetic approaches that have been employed in the study of malaria protein kinases, as well as the development of selective pharmacological tool inhibitors. We discuss how these advances can be used to inform malaria drug discovery. While the focus of this review is on malaria, we also make reference to relevant work in other apicomplexans.

2. Defining the phosphoproteome of the malaria parasite

To date there have been five mass spectrometry malaria parasite phosphoproteomes published that have established that protein phosphorylation plays a wide regulatory role in malaria, controlling key processes such as metabolism and cell growth, as well as malaria-specific processes such as egress, invasion and cytoadherence [16–20]. The basic work flow for a global phosphoproteome study is as follows: 1) extraction of most of the proteins in the parasite infected cell; 2) digestion of the proteins to small peptides with, for example, trypsin (an enzyme which cuts at lysine or arginine residues); 3) chromatographic fractionation of the peptides to simplify the complexity of the sample; 4) enrichment of phosphopeptides using an enrichment technique such as IMAC (immobilized metal affinity chromatography) or TiO_2 and 5) identification of the peptides and the phosphorylation sites on a mass spectrometer.

Treeck and colleagues [20] employed mass spectrometry to identify the global phosphoproteome of late blood stage of *P. falciparum* and invasive stage of *Toxoplasma gondii* (tachyzoites). Using enrichment of the phosphopeptides by IMAC and setting the peptide false discovery rate of <1%, they identified 8463 phosphorylation sites on 1673 proteins in the schizont stage of *P. falciparum*. This study also identified 12,793 phosphorylation sites on 2793 proteins in the tachyzoite stage of *T. gondii*. Interestingly, they observed significantly more phosphorylation sites (24,298) in free tachyzoites, which the authors suggested was due to a technical detail, namely that the presence of host-cell proteins reducing the sensitivity of the mass spectrometry-based determination of parasite proteins once the tachyzoites had invaded the host cells. Alternatively, the large number of phosphorylation events may indicate that free parasites (tachyzoites) are more active and that the increase in phosphorylated proteins correlates with parasites preparing to invade host cells. A recently published phosphoproteome of free *P. falciparum* merozoites has identified 1765 phosphorylation sites with almost half of sites being merozoite-specific, supporting the notion that the invasive stage of the parasite prepares for invasion by regulating biochemical pathways in a timely manner [21].

The phosphoproteome of the schizont stage in *P. falciparum* has also been identified by Solyakov et al. [19]. They performed six independent mass spectrometry analyses and reported only phosphopeptides that were verified by manual inspection and that appeared in at least two out of six biological replicates. Using these criteria they reported 1177 phosphorylation sites on 650 parasite proteins in the schizont stage of the parasite [19]. Importantly, this phosphoproteomic study was linked with a reverse genetic analysis of the kinome of *P. falciparum* where 36 parasite protein kinases were identified as being essential for parasite survival in the blood stage.

Lasonder et al. [17] also identified the phosphoproteome of schizont stage of the parasite where they reported phosphopeptides with a mascot score of at least 15, 1% false discovery rate and 75% probability of correct localization of phosphorylation sites. Applying these criteria