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Chemical Genetics to Study *Plasmodium* Kinases

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Malaria is one of the major infectious diseases of the developing world that continues to spell havoc on mankind. There have been an estimated 584,000 deaths in 2013 due to malaria infection. Although the disease is completely curable with the available drugs, the recent reports on emergence of resistance against the front line drug, artemisinin combination therapies in Southeast Asia is gravely worrisome. There is an urgent need to validate new drugs that can be used to control malaria infection and also novel targets that can be used in drug discovery programs against malaria.

Protein kinases have been well documented to play critical roles in almost all important physiological processes of eukaryotes and prokaryotes. They have been extensively used as drug targets to treat various human ailments including cancer. Since protein kinases play indispensable roles in physiological processes inside a cell, studying their function using conventional gene knock-out approach may not be straight forward. Moreover, knock-down of protein levels using various post-transcriptional and post-translational approaches have their own limitations such as matching the exact timing of the knock-down in enzyme levels to the functional activity inside the cell, residual level of enzyme left and side-effects of the agent used for knock-down.

One of the elegant strategies to study the functional roles of human kinases has been devised by Kevan Shokat's group called the "sensitization strategy" or "chemical genetic approach" [1]. The strategy involves engineering a unique pocket in the active site of a target kinase at a position called the gate-keeper position. The gate-keeper residue is adjacent to the ATP binding site and is mostly constituted by a bulky amino acid residue such as methionine, isoleucine or leucine. Substitution of the bulky residue in the target kinase with a smaller residue such as alanine, glycine or serine renders the enzyme sensitive towards a particular group of inhibitors better known as Bumped Kinase Inhibitors (BKIs). Few notable studies utilizing the chemical genetics approach to decipher functional roles of eukaryotic target kinase include v-Src role in transformation of NIH3T3 fibroblasts [1], and Cla4 and Cdc28 in controlling cell cycle progression in *Saccharomyces cerevisiae* [2,3]. Additionally, the approach has been extensively used for identification of downstream targets of many protein kinases. For example, the substrates of JNK and v-Src were identified using chemical genetics combined with chemical synthesis of an ATP analog specific for the analog sensitive kinase [4,5]. A chemical genetic screen was used to identify downstream substrates of AMPKα2 [6]. Notably, Byron *et al* used chemical genetics to demonstrate that active Zap70 plays a critical role in the proliferative, effector and memory function of T cells [7]. In all the above studies the gate-keeper position of the target kinase was constituted by a bulky residue and hence substitution with a smaller residue rendered the enzyme sensitive towards BKIs, thereby facilitating identification of the function and substrates. Noteworthy, the strategy can be used to convert a sensitive kinase into a resistant one for the BKIs.

The chemical genetics approach has been recently employed to study the functional roles and substrates of *Toxoplasma gondii* and *Plasmodium falciparum* kinases. *T. gondii* Calcium Dependent Protein Kinase 1 (*Tg*CDPK1) has been shown to play an essential role in the secretion of an apical organelle called microneme, parasite motility, host cell invasion and egress using the chemical genetics approach [8]. In two independent studies, treatment with different BKIs such as 1NAPP1, 1NAPP2, 1NMPP1 and 3MBPP1 inhibited the invasion of fibroblast cells by the *T. gondii* tachyzoites [8,9]. Substitution of glycine gate-keeper residue with methionine (G128M) conferred remarkable resistance to the bumped kinase inhibitors. The approach was subsequently used to identify downstream targets of *Tg*CDPK1 using different ATP analogs [10].

Chemical genetics approach has also been used to decipher the functional roles of *P. falciparum* guanosine 3',5'-cyclic monophosphate (cGMP)-dependent protein kinase, *Pf*PKG. McRobert *et al* convincingly demonstrated the role of *Pf*PKG in gametogenesis of *P. falciparum* by using two different specific inhibitors, compound 1 and 2, that blocked the activity of wild type kinase but not T618Q gate-keeper mutant [11]. Although the compound 1 and 2 do not share similar structure as BKIs, they share same mode of action. Additionally, the same approach was used to show the central role of *Pf*PKG in the blood stage schizogony. *Pf*PKG has been shown to control the discharge of apical organelles such as micronemes and exonemes (12). Blocking the activity of *Pf*PKG using compound 1 or 2 blocked the discharge of *Pf*SUB1 to the parasitophorous vacuole and hence inhibited proteolytic processing of Merozoite Surface Protein 1 (MSP1) and Serine Repeat Antigen 5 (SERA5) and thereby leading to a block in egress [12]. Compound 2 treated wild type and the T618Q mutant parasites have been used to identify the substrates of *Pf*PKG in the late stage schizonts [13]. A total of 69 parasite proteins were identified to be the direct or indirect substrates of *Pf*PKG. Some of the targets of *Pf*PKG identified in this study include proteins implicated in the motility and invasion of red blood cells by merozoites such as calcium dependent protein kinase 1 (CDPK1), Myosin A (MyoA), and Glideosome-associated protein 40 (GAP40). Native CDPK1 phosphorylated at S64 has been shown to specifically localize at the apical pole of the developing merozoites in mature schizonts or free merozoites near the apical organelles and was shown to be associated with a high molecular weight complex [13].

Serine at the gate-keeper position in wild type *Pf*CDPK4 makes the enzyme susceptible to BKIs. Ojo *et al* inhibited the kinase activity of *Pf*CDPK4 with BKI-1 leading to block in exflagellation of male gametocytes and also subsequent stages of oocysts development and sporozoite formation [14]. Substitution of serine with methionine at

the gate-keeper position, S147M, did not affect exflagellation in the transgenic parasite strain upon treatment with BKIs [15].

*Pf*CDPK1 has been demonstrated to play critical role in egress of merozoites from mature schizonts using a pharmacological inhibitor, purfalcamine and conditional over-expression of the junction domain [16,17]. Interestingly, *Pf*CDPK1 has also been implicated in microneme secretion and invasion of red blood cells by *Plasmodium* merozoites [18]. However, molecular mechanisms involved in these critical processes of asexual proliferation of the parasite have remained elusive. Due to lack of genetic validation corroborating *Pf*CDPK1 function in the parasite and the fact that it's homolog in *P. berghei* could be knocked-out without affecting asexual proliferation of the parasites [19], the indispensability of *Pf*CDPK1 has been challenged. Chemical genetics should be a valuable tool to address this paradox. Substituting threonine, a small gatekeeper residue, in wild type *Pf*CDPK1 with methionine, a bulky residue, should make the enzyme resistant towards BKIs and may help in validating its function and also identification of the downstream substrates. It is important to note, the permeability of BKIs for parasitized RBCs in the asexual stages has not been evaluated and may become a limiting factor in using chemical genetics for studying kinases at this stage of the parasite.

In summary, chemical genetics is a valuable tool to study the functional roles of kinases and its potential have started being tapped to explore the function of *Plasmodium* kinases and validate them as potential drug targets. As evident, *Pf*PKG and *Pf*CDPK4 have been studied using this approach and are good drug targets for development of small molecule inhibitors for control of malaria.

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