Spotlights on new publications

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New drug targets - XVI

Protein kinases (PKs): They are enzymes required to catalyze proteins phosphorylation involved in several signaling pathways that transmit intracellular and extracellular signals within eukaryotes. Through such a process, PKs transfer the phosphate groups from adenosine triphosphate (ATP) to the protein substrate. They are potential drug targets in therapeutic trials in several parasitic diseases. By proteins phosphorylation, PKs regulate proteins functions involved in several essential cellular processes such as growth, differentiation, development, and stress response, as well as cell apoptosis. Structural analysis of PKs revealed two types; eukaryotic (ePKs) and atypical (aPKs). The main difference is the lack of conserved eukaryotic kinase motifs in all ePKs members, and the presence of two motifs in aPKs. It is worth mentioning that ePKs are classified into several groups, however, the most commonly reported include tyrosine kinases (TKs), cyclin-dependent kinases (CMGC), calcium/calmodulin-regulated kinases (CAMK), cAMP-dependent PKs, cell kinase I (CK1), receptor guanylate cyclases (RGC), TK-like (TKL), and mitogen activated PK (MAPK). Other groups include PK C (AGC), NimA-related kinase (NEK), and conventional serine-threonine kinase (STE). It was reported that MAPK is a conserved cellular regulatory PK responsible for transmitting extracellular stimuli to intracellular responses. This means that MAPKs, activated by inflammatory cytokines will be present in the infected tissues causing inflammatory diseases.

On the other hand, TKs activity is essential in several cellular processes including cell adhesion, migration, proliferation, differentiation as well as initiation and maintenance of inflammatory events. They include receptor TKs and non-receptor or cytosolic TKs. The first is mainly localized as transmembrane receptors and expressed to regulate parasite survival, proliferation, and cell differentiation. The second TKs is involved mainly in signal transduction from extracellular receptors; *i.e.* cytokine and immune receptors.

In the present spotlights, four articles will be compiled focusing on utilization of PKs as potential novel drugs against schistosomiasis, toxoplasmosis, malignant malaria, and leishmaniasis.

Schistosomiasis: Several studies documented the essential roles of PKs in the growth, maturation, survival and development of *S. mansoni*. Besides, their association with oxidative stress, collaboration to maintain muscle-specific messenger RNA transcription in the tegument was recently reported. Due to the evolution in computational high throughput screening, several targets in *S. mansoni*, and novel drugs were identified, plenty of which are PKs inhibitors. Till now, 252, and 222 PKs were identified in the kinomic analysis of *S. mansoni* and *S. japonicum*, respectively. In spite of minimal difference in PKs number between both species, there is a scarcity of studies focusing on PKs as potential drug targets in *S. japonicum*. Accordingly, **Kaijuan Wu** and his Chinese colleagues published a review discussing PKs classification, their functions as well as relevant inhibitors. The reviewers claimed that their ultimate objective was to encourage interested investigators to invent novel anti-schistosomiasis drugs as well as vaccine candidates against *S. japonicum*.

During the last decade, several studies were conducted to identify *Sj*PKs distribution in *S. japonicum* different life cycle stages. Few *Sj*PKs were identified such as PK10, thymidylate kinase, and cAMP-dependent PK type II regulatory subunit. However, technical limitation regarding preparation of specific antibodies disabled identification of phosphorylation sites in *S. japonicum* life cycle stages. In spite of that, these studies pointed to the fact that *Sj*PKs have essential regulatory roles, but their identity and specific function are still unknown. The reviewers tabulated ten *Sj*PKs with their localization and expression in different life cycle stages, and proposed functions. They included five TKs, three CMGCs, and one for each CAMK and aPK. While tegument, reproductive organs and eggs are the main localization sites, advances in the parasite survival, growth and development, and participation in sexual maturation are the most assigned functions.

Among the mentioned TKs, epidermal growth factor receptor (EGFR), a transmembrane glycoprotein, is a member of receptor TKs. Its transcript was expressed in the tegument of different life cycle stages. Besides, knocking down its encoding gene resulted in growth suppression with alterations in

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immature spermatocytes and oocytes. However, no significant alterations were observed on adult worms. Two homologs of the insulin receptor (IR1 and IR2), another member of receptor TKs, were identified in essential metabolic pathways such as glucose metabolism, and phosphoinositide-3-kinase pathway. They showed different expression sites; whereas *Sj*IR-1 was in the tegument basal membrane, muscles and adult' intestinal epithelium, *Sj*IR-2 was expressed in female vitelline tissue and male parenchyma. Another two cytosolic TKs (TK3 and TK4) were characterized with similar assigned function of advanced growth and development. The first had variable expression levels in life cycle stages, with the highest in eggs, then gradually decreased in cercariae and juveniles, to be followed by increased levels in adults. Its expression in females proved to be significantly higher than that in males. In contrast, *Sj*TK4 showed significant higher transcripts in males than females, suggesting its link to the development of germ cells. The reviewers claimed that amino acid sequences of the genes encoding *Sj*EGFR, *Sj*TK3 and *Sj*TK4 showed high homologous similarity with those identified in *S. mansoni*. Therefore, it will be a feasible task to investigate these *Sj*TKs as potential drug targets with already characterized data in *S. mansoni*.

Three members of CMGC group were identified: 1) glycogen synthase kinase 3 (GSK3), previously described as a tumor suppressor; 2) extracellular signal-regulated kinase (ERK); and 3) c-Jun amino-terminal kinase (JNK). The reviewers claimed that their signaling pathways and targets as well as proposed functions require further studies to be explored. On the other hand, increased transcription of *Sj*CAMK II after Praziquantel (PZQ) treatment suggested that this PK is involved in calcium pathway to up-regulate calcium levels, a known mechanism of PZQ action. Hence, utilizing CAMK inhibitors might decrease frequency of PZQ drug resistance observed in resistant *S. japonicum* strains. The last PK characterized in *S. japonicum* literature is called right open reading frame protein kinase 2 (Riok-2), an aPK member. The latter is involved in RNA biogenesis and cell cycle processes, and its transcription level was higher in females and eggs than in males. In addition, *Sj*Riok-2 was mainly localized in the vitellarium and ovary, suggesting its essential role in female sexual maturation. Bioinformatics analysis revealed that *Sj*Riok-2 possess glycosylation sites, suggesting its high potentiality for development of an efficient vaccine.

Finally, the reviewer discussed TKs inhibitors. Imatinib, an inhibitor of abelson (ABL) family kinases that belong to TKs. However, experimental studies showed variable results ranging from inefficiency regarding worm burden or egg production *in vivo*, to significant reduction of motility and pairing stability with 100% death after 3-5 days *in vitro*.

Three issues were recommended: 1) to develop a safe and effective drug or vaccine utilizing *Sj*IRs, it is important to explore their functions in relation with host IRs; 2) future studies to investigate other PKs inhibitors that are used as anti-cancer drugs in treatment of schistosomiasis *japonicum*, that include Genistein, Sorafenib, Bosutinib, Crizotinib, Nilotinib, and Dasatinib; and 3) combined application of CRISPR technology, that allows modification of genes of interest with virtual screening, will accelerate development of anti-schistosomiasis drugs. Compiled from **"Protein kinases: Potential drug targets against *Schistosoma japonicum*. Front Cell Infect Microbiol 2021; 11: 691757."**

Toxoplasmosis: Calcium dependent PKs (CDPKs) are essential effectors of calcium signaling, the major initiative stimulus for host cell invasion, intracellular replication, and egress cascade. All CDPKs contain a kinase domain, and a C-terminal motif containing calmodulin-like domain (CLD) with a regulatory junction connecting both domains. However, CDPK7 contains two N-terminal motifs connected by long linkers to a pleckstrin homology (PH) domain adjacent to the kinase domain at the C-terminus. It is worth mentioning that PH domain is a protein domain (~120 amino acids) observed in several proteins involved in intracellular signaling. Pleckstrin is the major substrate of PKC in platelets where PH domain was first described. On the other hand, interaction of *P. falciparum* (*Pf*CDPK7) with phosphoinositides (PIPs) *via* the PH domain was previously reported as an essential step for *Pf*CDPK7 cellular localization. Due to scarcity of studies conducted in *Tg*CDPK7, and its critical role in *T. gondii* survival, growth and differentiation, **Priyanka Bansal** with her Indian colleagues conducted a study with a French scientist, **Yamaryo-Botté Y**. To identify *Tg*CDPK7 parasitic target(s) in tachyzoites development, the investigators conducted a quantitative phosphoproteomic analysis of *T. gondii* with deletion of its encoding gene.

Interestingly, the investigators observed trafficking impairment of glycosylphosphatidylinositol (GPI)-anchor proteins, required for synthesis of phospholipids, an essential component in tachyzoites replication. It is known that SAG1 protein domain, located on tachyzoites surface, is the major GPI-linked protein. Besides, several putative *Tg*CDPK7 substrates involved in phospholipids synthesis and vesicular trafficking were identified. Phosphoproteomics analysis showed significant impaired phosphorylation of proteins potentially involved in lipid metabolism and lipid-protein trafficking.

Another important observation was reported in the present compilation that *Tg*CDPK7 regulates localization of *Tg*Rab11a by phosphorylating it.

Localization of *Tg*Rab11a in tachyzoites vesicular compartments is a critical step for lipid-protein trafficking required for their division and replication. The Rab family is the largest in the Ras superfamily of small G-proteins. Family members possess a GTPase fold that regulates the molecular switches in membrane trafficking such as vesicle formation and movement along actin and tubulin networks, and membrane fusion. In human, Rab family members are required for trafficking of cell surface proteins from Golgi apparatus to cell plasma membrane as well as recyclation. In infected cells, Rabs are the molecular effectors of lipid-protein trafficking from host cell membrane to the invaded tachyzoites. Additionally, because previous studies reported that *Tg*Rab11a was involved in SAG1 localization in tachyzoites surface membrane during division, the investigators hypothesized that *Tg*CDPK7 might regulate SAG1 trafficking to the surface membrane through *Tg*Rab11a phosphorylation. Similarly, the investigators hypothesized that *Tg*CDPK7 might regulate biogenesis of phosphatidylethanolamine (PE) that contributes in GPI-anchor proteins biogenesis. Finally, the investigators discussed possible mechanisms through which *Tg*CDPK7 modulates key enzymes involved in pathways of PE biosynthesis. Compiled from **"Protein kinase *Tg*CDPK7 regulates vesicular trafficking and phospholipid synthesis in *Toxoplasma gondii*. PLoS Pathog. 2021 Feb; 17(2): e1009325."**

Malignant malaria: It is well known that the main concept of signaling pathways is to transduce extracellular signals to specific intracellular process activation. This means that transduction of extracellular signals leads to increased levels of intracellular messengers such as increased calcium concentrations and cyclic nucleotides (cAMP and cGMP PKs). In *P. falciparum* merozoites, this will result in activation of calcium dependent PKs (*Pf*CDPKs), cAMP-dependent PK (*Pf*PKA), and cGMP-dependent PK (*Pf*PKG), respectively. A cyclic nucleotide is a single-phosphate nucleotide that contains three structures; sugar, nitrogen base, and a single phosphate group. Cyclic nucleotides are either cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP). Their main biological significance is involvement in protein-ligand interactions, *e.g.* as secondary messengers (SMs) in signaling pathways to facilitate communication within cells, *i.e.* transmembrane signal transduction. To achieve this essential process, they bind to receptors in the cellular membrane to transmit a certain signal that activates adenylyl cyclase, an enzyme in the cell membrane interior. This results in cAMP or cGMP release into the cell, where it stimulates a cyclic molecule either PKA or PKG, respectively. By phosphorylating proteins, PKs change and regulate target protein activity. The present compilation summarized a review published by **Edwin Lasonder** who contributed with several scientists from UK, France, Germany, India, and USA. In their review, they focused on the roles of signaling pathways, and SMs as key processes in merozoites egress and RBCs *de novo* invasion cascade in *P. falciparum*.

Because *Pf*CDPKs, *Pf*PKA, and *Pf*PKG phosphorylate several substrates during RBCs *de novo* invasion, the reviewers hypothesized that development of new specific inhibitors of these SMs will accelerate development of novel anti-malarial drugs. This is feasible with computational high throughput screening of compounds against purified recombinant SMs *in vitro*. The reviewers specified certain physical and biochemical characters in the new developed inhibitors: 1) they should be active at low concentration, and 2) they are able to cross the three membranes, namely the infected RBCs and merozoites plasma membranes, as well as parasitophorous vacuolar membrane. However, the reviewers claimed the technical difficulty of specific inhibitors development due to poor membrane permeability of cyclic nucleotides. An additional technical obstacle is the structural similarity between *P. falciparum* kinase domains and those of the host because they may be conserved across the eukaryotes. Accordingly, it will be a challenge in development of an inhibitor specific for parasite kinases over host kinases. Instead, the reviewers proposed three alternatives. First, exploiting evolutionary structural divergence to selectively target a *Plasmodium* kinase of interest. It was reported that some kinase domains are surrounded by additional domains regulating kinase catalytic function or mediating protein-protein interactions (PIPs) essential for kinase-mediated signaling pathways. These additional domains can be served as unique targets for selective inhibition of a kinase of interest, without targeting host kinases. Regarding this issue, the reviewers proposed compounds that can be utilized to target PIPs. Second, utilizing allosteric inhibitors that bind at a site other than the enzyme active site to change enzyme catalytic activity. However, this is feasible for *Pf*CDPKs, not for *Pf*PKA, and *Pf*PKG, due to its possession of two domains; a calmodulin-like domain, acting as a calcium sensor, and a junction-domain that undergoes significant conformational change with calcium binding. Third, utilizing genetic manipulation or CRIPR-Cas9 technology that allows researchers to alter gene DNA sequences modifying its function.

Because low host cAMP levels reduced gametocytes V rigidity to facilitate clearing, the reviewers claimed that raising of host cAMP levels using inhibition of phosphodiesterases might be a new strategy to block malaria transmission. Finally, the reviewers presented several examples regarding roles of host cAMP signaling pathways in the pathogenesis of different apicomplexan parasites such as *T. gondii* and *Theileria* spp. Compiled from **"cAMP-dependent signaling pathways as potential targets for inhibition of *Plasmodium falciparum* blood stages. Front Microbiol 2021; 12: 684005."**

Leishmaniasis: With special emphasis on vector-borne transmitted protozoa; *Plasmodium* and *Leishmania* spp., life cycle stages differentiation is a fundamental process that requires specialized signaling pathways to adapt survival and growth in both mammalian and insect hosts. Concerning this issue, the essential roles played by the apical secretory organelles and their released proteins are repeatedly investigated and reviewed in several reports for *Plasmodium* spp. However, little is known regarding the molecular mechanisms undergoing for *Leishmania* survival, growth, replication, and differentiation of life cycle stages. This is an important step, *i.e.*, identification of essential targets, in development of novel drugs.

Several studies reported and confirmed the implications of PKs signaling pathways in life cycle stages differentiation in *Plasmodium* spp., *T. brucei*, and *T. gondii*. Meanwhile, 206 PKs were identified in *Leishmania* spp., 195 ePKs and 11 aPK. According to the catalytic domain conservation, *Leishmania* ePKs included six groups; CMGC, CAMK, CK1, STE, AGC, and NEK. Besides, Aurora, Polo and AMP kinases were reported as members of a 7th group and classified as "others". Whereas aPKs included 11 phosphatidylinositol 3′ kinase-related kinases (PIKK) with similar functions to lipid kinases. Interestingly, *Leishmania* genomic analysis revealed absence of genes encoding TK and TKL; instead, STE, and NEK groups are significantly abundant. The present compiled study was conducted because of three issues. First, TKs activity that is essential for parasite proliferation, differentiation, and maintenance of inflammatory events, is missed in *Leishmania* spp. Second, although several studies investigated the role of individual PK in promastigotes survival, and differentiation, as well as amastigotes replication, less than 10% of *Leishmania* kinome, mostly CMGC, and MAPK groups, were investigated utilizing genetic technology. Third, there is a gap in literature data regarding PKs role in amastigotes-promastigotes differentiation and survival in the vector host. Accordingly, **Nicola Baker** and her colleagues from UK and Czech Republic hypothesized that application of kinome-wide gene deletion and gene tagging technology in *Leishmania* promastigotes will certainly identify PKs genes essentially required for parasite survival, replication, and differentiation.

Kinome-wide gene deletion revealed only 43 genes refractory to deletion suggesting their essential roles in *Leishmania* survival, replication and differentiation. The investigators systematically tagged PKs of interest with mNeonGreen fluorescent protein for PKs localization. This was followed by conducting individual null mutant strains for those *Leishmania* PKs and PIKK using CRISPR–Cas9 technology. Lastly, they performed pooled *in vitro* and *in vivo* infections to assess and identify the importance of individual PK in survival, replication, and differentiation as well ultimate infection success in mammalian and vector hosts. The *in vitro* study was conducted in cultured macrophages to investigate amastigotes growth, survival and replication. Whereas *in vivo* studies were conducted to investigate metacyclic promastigote-amastigote differentiation in mice, and promastigotes colonization and motility in sand fly vector.

The investigators succeeded to identify and immunolocalized 29 PKs required for successful metacyclic promastigote-amastigote differentiation in mice, as well as amastigotes survival and replication in macrophages. Among them, only five MAPKs, and two CMCGs were previously investigated, while the remaining require further assessment and investigations as potential drug targets. Besides, 15 PKs, identified in the sand fly, have two functional roles. Eight and seven PKs were assigned for intrinsic and extrinsic roles, respectively. Accordingly, the investigators recommended further studies to opportunize the available databank of information individually assigned for each *Leishmania* PK. Investigating their signaling pathways will accelerate development of novel anti-leishmanial drugs. Compiled from **"Systematic functional analysis of *Leishmania* protein kinases identifies regulators of differentiation or survival. Nat Commun Feb 2021; 12: 1244."**