Spotlights on new publications

Sherif M Abaza

Medical Parasitology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

Corresponding Author: Sherif M Abaza, **Tel.:** +20 1005243428, **E-mail:** smabaza@hotmail.com

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New drug targets - XV

***Falciparum* malaria**

Compilation No. (1): Pharmacological evidence proved that protein kinases (PKs) are potential drug targets in clinical trials for several human diseases, due to possible inhibition by small molecules or prepared antibodies. Several publications documented that PKs, by their phosphorylating roles, regulate proteins functions involved in several essential cellular processes such as growth, differentiation, development, and stress response. On the other hand, severity of *falciparum* malaria is attributed, partially, to its ability to export a number of proteins into infected erythrocytes causing major structural and functional changes. These include increased membrane rigidity, decreased deformability, and subsequent adherence to vascular endothelial cells. Among exported proteins a family of highly atypical kinases (FIKKs) including 21 distinct members was identified in *P. falciparum* genome. It is worth mentioning that FIKKs family is unique to apicomplexans that possess apical secretory organelles specialized for host cell invasion and egress. Additionally, *P. falciparum* FIKKs have no orthologues in humans, therefore they are highly potential anti-malarial drug targets. Bioinformatics analysis proved that three fikk genes (*fikk7.2, fikk9.2, fikk14*) either encode internal stop codons or do not contain Plasmodium export element (PEXEL) motif required to export protein. Among the remaining 18 FIKKs, previous studies utilized recombinant or purified native proteins, and gene knocking out techniques to identify only seven FIKKs members (4.1, 4.2, 7.1, 8, 9.3, 9.6, and 12) to date. The studies reported that these FIKKs were exported into infected RBCs. Among them, roles of FIKKs 4.2, 7.1 and 12 in altering infected RBC membrane rigidity were identified. Besides, FIKK4.2 revealed its role in RBC adhesive properties and knob morphology, i.e., development of severe clinical complications associated with *falciparum* malaria. Interestingly, the role of exported FIKK8 in growth and survival was recognized only in *P. berghei* hepatic stages.

The main objective of the present compilation (**Ghizal Siddiqui** and her colleagues) is to localize and characterize the remaining FIKK members. Unfortunately, the investigators failed to knock out five genes encoding FIKKs 3, 9.1, 9.5, 10.1 and 10.2. Instead, they succeeded to genetically manipulate them by addition of gene sequence encoding hemagglutinin tag, i.e., modifying genes action instead of deletion (transgenic parasite lines). Due to inability to delete these genes, the investigators suggested their essential role in parasite growth and survival. Interestingly, only three (FIKK9.1, FIKK10.1 and FIKK10.2) were exported into infected RBCs. The first two FIKKs were exported through Maurer's clefts, derived structures involved in parasite' protein trafficking and pathogenesis in *falciparum* malaria. Moreover, both FIKKs were co-localized with MCs marker which are parasite-derived membranous structures with similar function to that of Golgi apparatus, i.e., trafficking of virulence factors. Based on the obtained results, the investigators hypothesized their roles in signaling processes required to regulate export of essential proteins and virulence factors. The last (FIKK10.2) was expressed only in young ring stages and partially co-localized with MCs marker. In late ring stages, FIKK10.2 became diffuse, suggesting that it transits MCs marker on route to its final destination within the infected RBCs. Accordingly, it was suggested that FIKK10.2 expression allows continuous re-modelling of the infected erythrocytes for the subsequent stages.

In contrast, FIKK3 and FIKK9.5 were not exported, instead they were localized in rhoptry organelle, and nucleus, respectively. The investigators hypothesized that FIKK3 rhoptry bulb localization leads to the significant release of bulb molecules involved during de novo RBCs invasion. However, localization of FIKK9.5 in close proximity to the nucleus suggested its role as a mitotic kinase involved in regulating nuclei division. Unexpectedly, both non-exported FIKKs appeared to possess a PEXEL motif; an attained result that raised two questions for further studies. The first is related to the presence of PEXEL motif as an accurate prediction for the exported protein, while the second concerns additional signals required, in addition to the PEXEL motif, for protein export in *P. falciparum*.

In conclusion, *P. falciparum* possesses eight FIKK members as potential drug target; six exported and two non-exported. The six exported FIKKs include three (9.1, 10.1, and 10.2) involved in parasite growth and survival in infected RBCs, and other three (4.2, 7.1 and 12) involved in merozoites egress. The non-exported FIKKs (3 and 9.5) are involved in *de novo* RBC invasion and mitotic nuclear division, respectively. Both are critically important for parasite growth and

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survival. Compiled from **"Identification of essential exported *Plasmodium falciparum* protein kinases in malaria-infected red blood cells." Br J Haematol 2020; 188(5):774-783.**

Compilation No. (2): Hundreds of publications documented that drug resistance for the commonly available anti-malaria drugs is developed due to parasite gene mutation of the drug' target. This issue encouraged a team of investigators from USA, and Italy to develop novel anti-malarial drug utilizing host component. Human RBCs are known to express tyrosine kinase (TK), while genomic studies of *P. falciparum* showed no genes encoding TK. Previous studies showed that host TK is involved in phosphorylation of the cytoplasmic domain of band 3 (cdb3), a RBC' transmembrane protein. Intramolecular association of the phosphorylated cdb3 within RBC membrane cytoskeleton accelerates separation of ankyrin and spectin-based membrane cytoskeleton from the lipid bilayer, resulting in membrane breakdown. Additionally, dramatic increase of cdb3 phosphorylation was reported to be associated with the maturation of *P. falciparum* erythrocyte merozoites. Accordingly, **Kristina Kesely** and her colleagues hypothesized that *P. falciparum* might trigger host TK to increase cdb3 phosphorylation that helps in membrane breakdown and merozites egress. Based on this hypothesis, activation of host TK is required for merozites egress and *vice versa*. The investigators screened a blinded library of kinase inhibitors from Eli Lilly and Co. searching for TK inhibitors with potential anti-malarial activity.

Although the majority of investigated TK inhibitors exhibited negative effects on parasite survival, only one subset of TK inhibitors (Syk) showed blockage of certain erythrocytes' TK activity. Furthermore, the investigators observed that other TK inhibitors with no effect on cdb3 phosphorylation, succeeded to inhibit parasite proliferation at stages other than merozoites. Based on these results, the investigators confirmed the significant increase in cdb3 phosphorylation during merozites egress. Besides, Syk is an essential erythrocyte TK required for cdb3 phosphorylation; and it is activated and significantly recruited to RBC membrane cytoskeleton. With non-significant results, the investigators observed that all investigated Syk inhibitors exhibited anti-malarial activity in vitro. However, it was suggested that selective Syk inhibitors might be potential novel anti-malarial drugs without future drug resistance due to absence of parasite gene mutations.

Finally, the investigators proposed that the mechanism of action of the selective Syk inhibitor in cessation of *P. falciparum* parasitemia is similar to that of diamide, i.e., hemoglobin consumption. The subsequent heme release results in establishment of an oxidizing environment that inhibits the active sites of erythrocytes TK, stabilizing cdb3 phosphorylation. Currently, the hypothesis utilizing host target for development of novel anti-malarial drug is undergoing in a clinical trial in Vietnam and Laos. Compiled from **"Identification of tyrosine kinase inhibitors that halt *Plasmodium falciparum* parasitemia" PLoS One 2020; 15(11): e0242372.**

***Cryptosporidium* spp.:** They are intracellular opportunistic apicomplexans affecting human, causing severe gastrointestinal manifestations in children and immunocompromised patients. Several studies evaluating efficacy of Nitazoxanide, the sole FDA-approved drug, documented limited efficacy in children and AIDS patients. In those high risky groups, autoinfection or recurrent infections are common due to rapid intracellular parasite proliferation followed by egress and re-invasion of new epithelial host cells. A group of American scientists (**Samantha Nava** and her colleagues) attributed the continuous prolonged spread of *Cryptosporidium* infection in children and AIDS patients to the essential roles played by molecules contributing in merozoites egress.

Several studies confirmed the functions of cyclic GMP-dependent protein kinase G (PKG) in similar intracellular apicomplexan protozoa, i.e., *T. gondii* and *P. falciparum*. Among these functions, its vital role as a key mediator of the signal transduction cascade was proved in micronemes secretion, the first step in egress cascade. Identification of a PKG homologue in the genomic analysis of *C. parvum* encouraged the investigators to silence PKG encoding gene using antisense RNA sequences. In a short communication, the investigators conducted an *in vitro* case control study where human colonic epithelial cells (HCT-8) were infected with silenced PKG and wild-type *Cryptosporidium* oocysts that were incubated in PRMI culture media supplemented with 10% fetal bovine serum. In both experiments, with wild type and silenced PKG parasite infection of HCT-8, the investigators were able to determine the time course of PKG expression.

Briefly, culture monolayers were subjected to lysis at 1, 2, and every 2 h through 26 h post-infection. The extracted total RNA from each lysate was measured using quantitative real time PCR (qRT-PCR). Besides, the latter was used to determine the number of egressed merozoites by amplifying RNA lactate dehydrogenase. Fluorescent microscopy was used to confirm sporozoites viability following excystation, while confocal microscopy and flow cytometry were used to visualize *Cryptosporidium* merozoites trapped in or egressed from HCT-8, respectively. Moreover, western blot was utilized to evaluate PKG levels in both experiments. It is worth mentioning that the investigators designed antisense sequences (silencing complex) to block PGK expression. To confirm PGK silencing, total RNA was measured using qRT-PCR.

As expected for molecules contributing to egress cascade, results revealed that PKG mRNA peaked at 18–20 h post-infection, few hours prior to merozoites egress. Silenced PKG parasites showed the following results: successful excystation of viable sporozoites as examined by fluorescent microscopy with 98-99% reduction in PKG mRNA as measured by qRT-PCR. There was complete absence of PKG as measured by western blot with complete blockage of merozoites egress as measured by qRT-PCR and trapping of merozoites in HCT-8 as examined by confocal microscopy. Minimal number of egressed merozoites was observed in both silenced PKG and wild type parasites at 1 and 20 h post-infection. However, the latter showed a peak of egressed merozoites after 24 h post-infection, followed by significant decrease at 26 h due to subsequent HCT-8 reinvasion.

The investigators concluded that egress in *Cryptosporidium* spp. is a critical step for its proliferation and spread, and PKG plays an essential role in prolonged and/or recurrent cryptosporidiosis. Considering their conclusion, further studies to develop or design PKG blockers or inhibitors as novel drugs against cryptosporidiosis were recommended. Compiled from **"*Cryptosporidium parvum* cyclic GMP-dependent protein kinase (PKG): An essential mediator of merozoite egress. Mol Biochem Parasitol 2020 May; 237: 111277.**

Toxoplasmosis: In the present compilation, Chinese scientists (**Shuxian Liu** and her colleagues) claimed that in spite of the worldwide spread of toxoplasmosis, only few drugs are available for complete cure. This is attributed mainly to the host immune status because *T. gondii*, being an opportunistic pathogen, causes several neurological and ophthalmic complications in immunocompromised patients. Several publications reported potential novel drugs: calcium-dependent protein kinase inhibitors, artemisinin derivatives, and bumped kinase inhibitors. However, none proved to be ideal, safe, or effective therapeutically; due to variability in host' response and tolerance, occurrence of severe side effects such as bone marrow depression, and emergence of resistant strains. Because development of novel drugs is an expensive and long-term process, the investigators hypothesized that screening a library of well- established drugs would accelerate the development process. In such strategy, pharmacokinetic and pharmacodynamic issues for each drug, and its mechanism of action as well as its assumed parasitic target are established. A collection of 666 FDA approved drugs were screened for their significant inhibitory efficacy against *T. gondii* growth. Among the screened drugs, only 68 compounds showed more than 70% inhibitory efficacy against *T. gondii* growth. Out of these compounds, the investigators identified 18 drugs with robust inhibitory activities without apparent cytotoxicity. The investigators selected only two drugs; NVP-AEW541 and GSK-J4 HCl because they were not previously investigated.

Accordingly, the investigators conducted *in vitro* and *in vivo* studies to investigate the feasibility of these two as potential drugs in treatment of acute toxoplasmosis. *In vitro* studies included assessment of the inhibitory efficacy on parasite growth, and its ability to invade, proliferate and exit from host cells. Besides, viability of host cell or drugs' cytotoxicity was assessed. Results showed that both drugs had significant inhibitory efficacy with IC_{50} values 1.17 μM and 2.37 μM, respectively, with minimum cytotoxicity. Both drugs exhibited > 90% parasite growth inhibition and > 80% cell viability. It was observed that NVP-AEW541 inhibited parasite growth by blocking or preventing tachyzoite invasion. The investigators failed to determine its mechanism of action because the drug was used to induce cancer cell death *via* caspase-dependent and caspase-independent pathways. Both pathways were never reported in *T. gondii* invasion into host cells. Instead, host cell invasion utilizes formation of tight attachment between parasites and host cell membrane. Meanwhile, it is reported that this drug is a selective inhibitor of insulin-like growth factor-1 receptor (IGF-1R), a surface receptor. Being the drug target, the investigators recommended further studies to identify or deny the role of host IGF-1R in tachyzoites invasion. On the other hand, GSK-J4 HCl inhibited intracellular tachyzoite proliferation. This was attributed to the fact that GSK-J4 HCl is a selective inhibitor of histone demethylation used to suppress cellular differentiation. Treatment of cancer cells with GSK-J4 HCl induced cell death with minimal toxicity to normal cells. The investigators proposed similar mechanism of action in inhibition of intracellular tachyzoites proliferation.

In comparison with pyrimethamine, both drugs were tested *in vivo* utilizing murine model of acute toxoplasmosis infected with the highly virulent RH strain. Compared to infected non-treated mice, both drugs prolonged the survival time and significantly reduced parasite burden in liver, spleen, and brain tissues. On the other hand, while mice treated with pyrimethamine survived, the investigators noticed that they have to euthanize all mice treated by both compounds due to apparent signs of acute toxoplasmosis during the experimental observation period. Accordingly, modification in drug treatment conditions: dose, time and administration route, was suggested. Further studies were recommended to investigate the mechanism of action of both drugs, and to identify the definite target in *T. gondii*. Additionally, their pharmacokinetic parameters require more identification to evaluate using them whether as parasiticidal or only parasitostatic drugs or in combination with other available chemotherapeutic drugs. Compiled from **"Two old drugs, NVP-AEW541 and GSK-J4, repurposed against the *Toxoplasma gondii* RH strain." Parasit Vectors 2020; 13: 242.**