Spotlights on new publications

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New drug targets - XIV

***Plasmodium* spp.**: Control of malaria depends mainly on three strategies: effective chemotherapy, vaccination, and measurements taken against mosquitoes to prevent transmission. In the last two decades, there has been several publications that reported clinical trials on effective vaccine candidates. Also, molecular evolutionary progress enabled investigators to propagate transgenic mosquitoes' strains incapable of transmission of *Plasmodium* spp. In spite of that, development of a new effective drug with novel mechanism of action remains the main obstacle in malaria control. It is worth mentioning that drugs resistance due to parasite gene mutations is well documented in highly endemic African countries. Parasite gene mutations to all available anti-malarial drugs result in emergence of several resistant strains. Therefore, there will be continuous urgent need for identification of new parasite targets. Surprisingly, *P. falciparum* genomic analysis identified more than 2600 genes, essential for only the asexual erythrocytic stages growth and survival, leading to identification of a relevant number of cellular processes with thousands of potential drug targets. The ideal novel anti-malarial drug should be characterized by three important points: single dose with rapid action, broad therapeutic potential against all *Plasmodium* spp., as well as against asexual and sexual erythrocytic stages, and with a novel mechanism of action on molecular basis. Besides, it should have potent efficacy to clear hepatic hypnozoites (anti-relapse agent) as well as chemo-prophylactic efficacy. Moreover, it should be safe for administration to children and pregnant women. The main objective of the present compilation, reviewed by an Ethiopian researcher, **Tafere Mulaw Belete**, is to discuss the recent progress and technological molecular advances achieved in identification of novel drug targets and subsequent development of novel inhibitors.

First, Belete TM reviewed the available drugs that could be grouped in major three derivatives: quinoline, antifolate, and artemisinin. Most of them target the asexual erythrocytic stages, with relative limited or no efficacy against hepatic and sexual erythrocytic stages, responsible for occurrence of relapse and transmission, respectively. He supplemented his review with a diagram showing *Plasmodium* life cycle stages with their effective anti-malarial drugs.

Second, he reviewed the major metabolic pathways that were reported in the last two decades for identification of new targets to develop novel drugs. These include heme detoxification, synthesis of fatty and nucleic acids, and signaling system, targeting hemoglobin degradation, membrane transport required in egress and *de novo* invasion cascade, and molecules required for oxidative stress, respectively.

Third, he discussed several compounds inhibiting the novel parasite targets. *Plasmodium* **proteases** have essential roles in hemoglobin degradation and egress and *de novo* invasion cascade. Hence, several cysteine, serine, metallo, aspartyl, and threonine inhibitors were investigated and showed only *in vitro* significant results. However, fluoromethyl ketone, a cysteine protease inhibitor, cured 80% of *P. vinckei*-infected mice. Also, he mentioned that subsequent publications identified several compounds inhibiting falcipains, the major cysteine protease in *P. falciparum*, including chalcones and phenothiazines. It is worth mentioning that cysteine protease inhibitors were previously reviewed in details (PUJ 2019; 12(2): 74-93). *Plasmodium* spp. express two classes of **protein kinase**; phosphoinositide-3-kinase and phosphatidylinositol 4-kinase. Both are essential in several cellular processes including proliferation, survival, trafficking, and intracellular signaling. Similar to proteases, several compounds were investigated as inhibitors such as aminopyridine, UCT943, and imidazopyrazines (KAF156). The latter is currently in phase II clinical trial. Similarly, due to its satisfactory prophylactic activity *in vivo* against *P. cynomolgi*, MMV048, another protein kinase inhibitor, is currently in phase IIa clinical trials in Ethiopia.

Fourth, the reviewer discussed other potential drug targets: *Plasmodium* **transporters** and **enzymes**. This part includes statements reported

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and investigated in few publications. Before *de novo* invasion and for survival and rapid growth, *Plasmodium* intra-erythrocytic stages express essential molecules to transport required electrolytes as well as nutrients, and to remove metabolites. Due to possession of *Plasmodium* transporters, glucose uptake by infected erythrocytes is 100 faster than that of non-infected RBCs. There are several *Plasmodium* transporters: 1) Hexose transporter (*Pf*HT) for glucose and fructose transport (inhibited by a long chain O-3-hexose derivatives); 2) Lactate:H^+ for lactate transport (inhibited by several compounds such as MMV007839 and MMV000972); 3) P Type Na^+-ATPase (*Pf*ATP4) for regulation of Na^+ influx essential to maintain intracellular Na^+ concentration (inhibited by cipargamin, and KAE609); 4) V Type H^+–ATPase for importing H^+ essential to maintain the neutral intracellular pH (inhibited by MMV253); and 5) Choline is a precursor utilized by *P. falciparum* to synthesize phosphatidylcholine *de novo*. The latter is the major lipid component of the cell membrane. Choline transport was inhibited by Albitiazolium, with a single curative injection dose at high levels of parasitemia. This drug reached phase II clinical trials. Interestingly, host aquaporin-3 (AQP3), an aquaglyceroporin facilitating water and glycerol transport to mammalian cells, was considered in some studies as a potential drug target. Although host AQP3 was expressed in infected hepatocytes, significant increase in *Plasmodium* replication was reported. It was suggested that host AQP3 facilitates glycerol entry into *P. berghei* contributing in parasite replication. Therefore, treatment with auphen (AQP3 inhibitor) reduced *P. berghei* in hepatocytes and *P. falciparum* in RBCs. It is worth mentioning that AQP3 genetic depletion is not lethal in mice. On the other hand, other transporter inhibitors include also phlorizin, dantrolene, furosemide, and niflumate as effective anion blockers, and drugs such as glibenclamide, meglitinide, and tolbutamide for inhibition of choline influx.

The reviewer specified three enzymes possessed by *Plasmodium* spp. as potential drug targets. They are (1) Dihydroorotate dehydrogenase (DHODH) essential for *de novo* pyrimidine synthesis (inhibited by selective inhibitors such as DSM265, P218, and KAF156). All inhibitors are currently in either phase 1 or 2 clinical trials. (2) *P. falciparum* 1-deoxy-dxylulose-5-phosphate reductoisomerase (*Pf*Dxr) involved in mevalonate pathway of isoprenoid biosynthesis (inhibited by Fosmidomycin, MMV019313, and MMV008138). (3) Farnesyl-transferase required to catalyze farnesyl pyrophosphate to the C-terminus of proteins with CaaX motif. This step has essential roles in vesicular trafficking, signal transduction, DNA replication, and subsequently cell division. The reviewer discussed several parasite gene mutations occurred in using selective farnesyl-transferase inhibitors: BMS-388,891, BMS-339,941, and MMV019066. Additionally, *P. falciparum* translational elongation factor 2 (*Pf*EF2) is a ribosomal component working as a translational machine for protein synthesis between *Plasmodium* genomes: nuclear, mitochondrial, and apicoplast. Only one selective inhibitor (M5717) was reported, and is currently in phase 1 clinical trials. Also, the reviewer claimed that *P. falciparum* expresses heparan sulfate causing sequestration of infected RBCs to facilitate attachment to vascular endothelium and microvascular obstruction. He suggested utilizing compounds with anti-adhesive polysaccharide such as sevuparin.

Finally, the reviewer tabulated 15 novel anti-malarial drugs with their mechanisms of action and responsible sponsor, that are currently in progress under clinical trials. Compiled from **"Recent progress in the development of new antimalarial drugs with novel targets. Drug Des Devel Ther 2020; 14: 3875–3889."**

Toxoplasmosis: Aminopeptidase N is a metallo-peptidase that belongs to clan MA, family M1. It has a wide range of cellular functions including maintenance, growth, development, and contribution in the defense mechanism against host immune response(s). It has been investigated as drug target and vaccine candidate in several species of protozoa: *Plasmodium*, *Trypanosoma, Cryptosporidium* and *Eimeria*. It was reported that *T. gondii* (ME49 strain) possesses three aminopeptidases. The first, *Tg*APN1, showed immunogenic character, suggesting its essential role against host immune response(s). The second, *Tg*APN2, is localized within the cytosol, and expressed all through different life cycle stages. Bioinformatics analysis of ME49 genome suggested that *Tg*APN2 is the only typical intra-cellular aminopeptidase with 38% sequence identity to *Pf*A-M1, and 55% similarity in its catalytic domain. The last, *Tg*APN3, is localized within the GRA protein in the parasitophorous vacuole, and expressed only in tachyzoites.

Utilizing genome wide CRISPR screen, *Tg*APN2 (ME49 strain) showed its contribution in tachyzoite growth but is apparently not essential. However, the transcript was up-regulated in bradyzoites, which is the most critical stage for drug development. For this reason and due to its similarity, more or less, to that of *P. falciparum* (*Pf*A-M1), **Emilia M Marijanovic** and her colleagues from Australia and Poland hypothesized that *Tg*APN2 is a potential drug target in treatment of toxoplasmosis. The present compilation described how the investigators examined *Tg*APN2' structure and function and compared it with those of *P. falciparum* (*Pf*A-M1). Recombinant *Tg*APN2 ecto-domain was prepared and showed a metal-dependent aminopeptidase activity with optimal

activity at neutral pH. Its structure resolution was hampered by unexplained density coordinated to the catalytic metal (zinc). To clear its resolution, the investigators added a second zinc ion to the active catalytic site. Utilizing structure activity relationship (SAR) data, the investigators tested the efficacy of *Pf*A-M1 selective inhibitors on *Tg*APN2. Interestingly, efficient ability of coordinating a second metal ion into the active catalytic site was explained when the investigators observed that some of the tested inhibitors succeeded to inhibit *Tg*APN2 in the presence of excess zinc.

Overall results revealed that *Tg*APN2 showed unique substrate specificity and inhibition profile. It is worth mentioning that M1 family members showed diverse variety of substrate preferences. Investigating its substrate specificity with that of *Pf*A-M1 showed a significant difference in the substrate preference of P1 residue. On the other hand, its substrate preference is, more or less, similar to human aminopeptidase and that of *E. tenalla*. The investigators observed that *Tg*APN2 prefers basic branched residues (arginine > lysine) with reduced activity toward leucine and alanine. Then, the results obtained from characterization of *Tg*APN2 crystal structure showed significant changes in the inhibitory kinetics of the investigated *Pf*A-M1 inhibitors. These changes were attributed to the S1 substrate specificity pocket of *Tg*APN2. It was concluded that SAR is a useful tool that provides facility to modify already investigated inhibitors, and consequently develop *Tg*APN2 suitable potent inhibitors. Furthermore, the investigators recommended further studies investigating *Tg*APN2' biological role(s) that may help in understanding its capacity as a potential drug target for treatment of toxoplasmosis. Additionally, the investigators recommended future design of novel *T. gondii* aminopeptidase selective inhibitors that could be captured into hydrogen bonds with multiple residues within the S1 catalytic pocket. Compiled from **"X-ray crystal structure and specificity of the *Toxoplasma gondii* ME49 *Tg*APN2. Biochem J 2020 Oct 16;477(19):3819-3832."**

The next three compilations will discuss new approaches for identification of parasitic targets and development of novel drugs.

Schistosomiasis: In 2016, **Alessandra Guidi** and her colleagues from Italy concluded that the anti-anginal drug, perhexiline maleate (PHX), represents a promising topic for novel anti-schistosomal drug discovery projects (PLoS Negl Trop Dis 2016 Aug 12; 10(8):e0004928). It was reported that PHX showed *in vitro* significant inhibitory activity against several *S. mansoni* developmental stages; schistosomula, juvenile, and adults. However, its mode of action was not identified. In the present compilation, the same group of investigators utilized 1H nuclear magnetic resonance (NMR) spectroscopy to clarify metabolic response of *S. mansoni* adult males in response to PHX administration, and subsequently its mechanism of action could be identified. The 1H NMR spectroscopy is one of the main tools used to study metabolomics; simultaneous detection and measurement of metabolites in the whole organism at baseline and after treatment. Utilizing metabolomics in parasitology research enables investigators to understand host-parasite interaction, and to identify essential metabolic pathways that could be considered as potential drug targets. On the other hand, male stages were selected because the investigators hypothesized that males coupling females in the gynecophoric canal is an essential process for the sexual maturation of the female reproductive organs, and consequently egg production.

Using 1H NMR approach, the investigators compared the metabolic profile and pathways induced by PHX on male *S. mansoni*, with that induced by gambogic acid (GA). The latter is the principal pigment of *Garcinia cambogia*, a tropical fruit that looks like a pumpkin, but is green in color. To standardize their metabolic data, the investigators performed the comparison between PHX *versus* DMSO, GA *versus* DMSO, and PHX *versus* GA. These comparisons allowed the investigators to analyze their metabolic data, and to validate utilizing 1H NMR approach in detecting variable metabolic profiles induced by different therapeutic compounds. Furthermore, this approach allowed the investigators to identify 43 soluble metabolites of male *S. mansoni* in response to PHX treatment. Accordingly, the investigators searched for metabolic pathways yielding these metabolites. Among the pathways enriched in PHX-treated samples was glycerophospholipids pathway. In *Schistosoma* spp., this is the main pathway in lipid and choline metabolism that is essential for tegmental development and its turnover in all *Schistosoma* stages, as well as eggs production. Meanwhile, metabolites yielded from this pathway were decreased in PHX-treated samples and increased in GA-treated ones. On the other hand, it was previously reported that male *S. mansoni* exports choline from the host to initiate *de novo* synthesis of phosphatidylcholine *via* Kennedy pathway. Besides, PHX-treated samples showed that glutamine was among the dysregulated metabolites suggesting disability of male *S. mansoni* to utilize it. Glutamine was reported as a precursor of glycerol suggesting occurrence of glyceroneogenesis in *S. mansoni* sporocysts. Interestingly, Kennedy pathway was suggested as potential drug target in *P. falciparum* and *T. brucei*. Further studies were recommended to investigate the potentiality of Kennedy metabolic

pathway in *S. mansoni* as drug target. Compiled from **"Drug effects on metabolic profiles of *Schistosoma mansoni* adult male parasites detected by 1H-NMR spectroscopy. PLoS Negl Trop Dis 2020 Oct 12;14(10): e0008767."**

Filariasis: The major obstacle in treatment of lymphatic filariasis, a disease caused by *B. malayi, B. timori*, and *W. bancrofti*, is the continued life span for up to 15 years of the causative adult worms. Additionally, the major available drugs; diethylcarbamazine and ivermectin, are contraindicated during mass administration programs in endemic foci where lymphatic filariasis is associated with onchocerciasis (*O. volvulus*) and/or loiasis (*L. loa*). Furthermore, although using long courses of antibiotics targeting the endosymbiotic bacteria (*Wolbachia*) significantly sterilized adult worms and accordingly reduced symptoms, they are contraindicated in children and pregnant women. During the last two decades, several drug targets were identified and investigated in treatment of diseases caused by unicellular protozoa. The majority of these reports utilized genome scale metabolic networks. Because **David M Curran** and his colleagues from Canada and USA observed that few publications dealt with bioinformatics focusing on multicellular parasites, they utilized flux balance analysis (FBA) for the first time to analyze the metabolic model for *B. malayi* (*i*DC625) on the genomic scale. The aim of the present compilation was to identify essential genes in the parasite' metabolic pathways that result in identification of potential therapeutic targets. The presented model allowed the investigators to predict reactions essential for adult worms' adaptation against different environmental situations, and subsequently maintenance for its survival for a long time in the host. It is worth mentioning that FBA used in solving knowledge gaps in Parkinson's disease, and some pathogens such as *L. major, Pseudomonas aeruginosa*, and *Desulfovibrio vulgaris*.

Utilizing *B. malayi* genome sequence, the investigators first generated a network representation with the previously published stage-specific transcriptomic data for both *B. malayi* and its *Wolbachia* endosymbiont. Second, the investigators obtained the results of FBA that hypothesized generating novel biological metabolic pathways. Results revealed 1266 total reactions, of these 1011 were enzymatic reactions; 575 and 166 are associated with *B. malayi* cytosolic compartment, and mitochondria, respectively, and the remaining (270) are assigned to *Wolbachia*. Also some of these enzymatic reactions (No. 226) are involved in transport across compartments; 37, 80 and 109 representing metabolite transport between mitochondria ↔ cytosol, *Wolbachia* → cytosol, and cytosol → extracellular matrix. Utilizing KEGG reaction database (www.genome.jp/kegg/reaction/), a knowledge database for predicting biodegradation and biosynthesis, the investigators filtered the enzymatic reactions and found them representing 761 KEGG reactions unique to *B. malayi*. The investigators conducted several *in silico* knockouts to predict only 102 reactions involved in cellular metabolic processes essential for parasite survival. The investigators validated the performance of their described *i*DC625 *B. malayi* model using two approaches. They observed that ~80% of the predicted reactions are encoded with one or more genes. Second, they selected a subset of the predicted reactions to be inhibited using known drugs; Fosmidomycin, MDL-29951 and Tenofovir to inhibit isoprenoid precursor biosynthesis, gluconeogenesis, and purine metabolism, respectively.

Finally, the investigators tested the efficacy of these drugs on worms *in vitro*. Results revealed 1) all the tested drugs reduced *Wolbachia* number/worm, 2) Fosmidomycin and Tenofovir reduced fecundity with significant decrease in microfilariae number/worm, and 3) Only Fosmidomycin decreased microfilariae motility. The investigators recommended future modifications of their model to yield more therapeutic targets. Also they recommended further studies to investigate more available inhibitors. Compiled from **"Modeling the metabolic interplay between a parasitic worm and its bacterial endosymbiont allows the identification of novel drug targets. Elife 2020 Aug 11; 9: e51850."**

Cryptosporidiosis: A group of American investigators **(Hadi H Choudhary *et al.*)** provided and validated a powerful conditional system to investigate and identify essential genes potentially highlighted as drug targets. To test their conditional system, they selected cryptosporidiosis due to limited or no efficacy of the only USA FDA-approved drug (nitazoxanide) especially in young children or immunocompromised patients, respectively. Additionally, there is no vaccine to prevent cryptosporidiosis to date. It is worth mentioning that cryptosporidiosis is the leading cause of diarrheal outbreaks linked to water and the third leading cause of diarrhea associated with animal contact in the United States. Calcium-dependent protein kinase-1 (CDPK1) is frequently reported in similar apicomplexan parasites; *T. gondii* and *Plasmodium* spp., to be involved in parasite' proliferation and survival. Previous results revealed an essential role of *cdpk1* gene for release of calcium-dependent proteins stored in the apical micronemes to control *T. gondii* motility, host cell invasion, as well as egress cascade. Whereas it has major roles in male gametogenesis and mosquito transmission in *P. falciparum* and *P. berghei*. On the other hand, its

role in *Cryptosporidium* spp. is not yet established. In spite of improved *in vitro* culture methods of *Cryptosporidium* spp. as well as available animal models for *in vivo* infection, there is critical shortage of genetic tools to investigate essential genes involved in parasite proliferation and survival. Development of a conditional strategy to control gene expression and its validation in *Cryptosporidium* spp. was the main objective of the present compilation.

The investigators hypothesized that conditional regulation at the post-translational level allows a rapid direct and tight regulation on the synthesized protein compared to other already used approaches. Therefore, they used *Escherichia coli* dihydrofolate reductase-based degradation domain (DDD) system to tag the gene of interest with DDD, which is unstable fusion protein. Its stability is retained by addition of a stabilizing compound, trimethoprim, to protect it from degradation by parasite proteasomes. This conditional strategy allowed the researchers to investigate the functions of gene of interest *in vivo*, *i.e.* during transformation of the life cycle stages.

First, utilizing direct knockout of *cdpk1* gene, the investigators confirmed its essentiality for the survival of *C. parvum*. Second, they *in vivo* generated transgenic *C. parvum* in immunosuppressed mouse model using CRISPR/Cas9 system with the conditional system (DDD and trimethoprim). *In vivo* measurements of CDPK1 levels in the stable transgenic parasite line confirmed the essential role of CDPK1 expression during *C. parvum* asexual proliferation. Lastly, the transgenic parasites showed increased sensitivity to kinase inhibitor upon conditional knockdown. Thus, the presented genetic tool conditionally regulates the levels of CDPK1 expression *in vivo* and *in vitro*, indicating its essential role for the parasite survival, Besides, its stabilization is vital for parasite growth. Compared with other genetic systems, the presented conditional system offers two advantages of using such a stabilizing trimethoprim compound: it is safe for prolonged use in animals, and is of low cost. Compiled from **"A conditional protein degradation system to study essential gene function in *Cryptosporidium parvum*. mBio 2020 Aug 25;11(4): e01231-20."**