# Spotlights on new publications

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## New drug targets V

### • Malignant malaria

During the last years (2010-2016), several reports were puplished for the potential efficacy of benzoxaboroles compunds as new drug targets against several protozoan diseases; African trypanosomiasis, malignant malaria, cryptosporidiosis and toxoplasmosis. Last year, it was reported that two of these compunds (AN6426 and AN8432) showed potent efficacy against *in vitro* multi-drug resistant *P. falciparum* strains, Ugandan field isolates, and *in vivo P. berghei* murine infections (**Sonoiki *et al*., 2016).** Both compounds were found to target leucyl-tRNA synthetase, and some *P. falciparum* strains showed resistanc *in vitro*, with point mutation in *cpsf* gene. The gene controls multi-protein complex, essential for transformation of pre-mRNA to mRNA through cleavage and polyadenylation process; i.e. addition of a poly (A) tail to a mRNA or in other words, it is a step for mRNA maturation. In the present compilation **(Ebere Sonoiki *et al*., 2017)**, the same team of American investigators with a Spanish investigator working in GlaxoSmithKline (a pharmaceutical company) tried to understand the role of *cpsf* gene in the mechanism of action of another benzoxaboroles compound (AN3661).

Selected multi-drug resistant *P. falciparum* strains and field isolates from Uganda were cultured and *in vitro*-treated with AN3661 in different concentrations. Drug efficacy was also evaluated in murine models of malaria (experimentally infected with *P. berghei*). Stage-specific parasite reduction ratios showed that late ring stage never develops to trophozoite form. This was followed by selection of *Plasmodium* strains which showed resistance to AN3661, they were subjected to whole-genome sequencing studies compared to wild type (AN3661-sensituive isolates). Results showed loss of three trophozoites proteins in the wild type, and the investigators attributed AN3661 resistance to alteration in the encoding messages of these trophozoite proteins. A point mutation in *P. falciparum cpcf*3 gene encoding *Pf*CPCF3 was also identified. This result was confirmed by inducion of gene mutation in CPSF3 in wild type *Plasmodium* isolates and the investigators observed that these isolates became resistant to AN3661. Therefore, two observations supported that the primary target of AN3661 in *P. falciparum* is *Pf*CPSF3; 1) *Pf*CPSF3 homology models placed these mutations in the active site at amino acids interacting with AN3661, and 2) loss of stability of trophozoite proteins in sensitive isolates, while AN3661-resistant strain didn't show this loss. In conclusion, CPSF3 is the protein responsible for polyadenylation process in *P. falciparum*, and the use of CPSF3 inhibitors might be a promising anti-malarial drug target. Finally, combination of *Pf*CPSF3 inhibitors with other anti-malaria regimens was recommended. **Compiled from: "A potent antimalarial benzoxaborole targets a *Plasmodium falciparum* cleavage and polyadenylation specificity factor homologue." Nat Commun 2017; 8: 14574.**

In another study conducted by **Paviga Limudomporn** *et al*. from Thailand and Canada, DNA helicases inhibitors were proposed as attractive anti-malarial drug targets. Since the first publication describing the whole genomic sequencing of *P. falciparum* in 2002, several drug targets were suggested. Among them are helicases, a highly conserved group of enzymes, that play an essential role in all aspects of DNA and RNA metabolism; i.e. replication, repair and transcription. Meanwhile, RuvB, an ATP-dependent DNA helicase, was reported to be associated with several cellular activities, e.g. prevention of DNA replication defects, maintainance of normal levels of cellular resistance to stress-induced mutagenesis, regulation of transcriptions, and promotion of growth, as well as an essential factor for organism' viability. Whole gene sequencing of *P. falciparum* genome showed only two RuvB homologues similar to RuvB like proteins detected in yeast and human. Ten years later, Indian investigators published the amino acid sequence analysis of a third RuvB (*Pf*RuvB3), and reported that it is essential for intraerythrocytic schizogony. In addition, they mentioned that *Pf*RuvB3 showed ATPase activity, but lacks DNA helicase activity. In the present compilation, the investigators hypothesized that methodology for *Pf*RuvB3 cloning and expression were not optimal to show its DNA helicase activity, therefore they re-evaluated *Pf*RuvB3 activities.

Gene encoding *Pf*RuvB3 of *P. falciparum* isolate (resistant to chloriquie and pyrimethamine) was cloned and the recombinant plasmid was tested by BioDesign to confirm presence of *Pf*RuvB3 and verify nucleotide sequence. Moreover, its identity was again verified by western blotting coupled with tandem mass spectrometry analysis. The recombinant protein was purified and subjcted to DNA helicase activity assay. To optimize the conditions favorable for testing *Pf*RuvB3 DNA helicase activity, the investigators evaluated the required co-factors, i.e. ATP concentrations, divalent cation requirement, and

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NaCl concentration. Meanwhile, they conducted ATPase activity assay of *Pf*RuvB3. Finally, they evaluated the effects of known DNA helicase inhibitors on *Pf*RuvB3 activities using either DNA intercalators (daunorubicin and doxorubicin), or DNA minor groove binder (netropsin) or others (aphidicolin genistein and mitoxantrone). Results showed that recombinant *Pf*RuvB3 posseses both DNA helicase and ATPase activities, its activites are dependent on divalent cations ($Cu^{2+}$, $Mg^{2+}$, $Ni^{2+}$ or $Zn^{2+}$) for ATP hydrolysis. However, DNA helicase activity was inactivated in high NaCl concentrations. *Pf*RuvB3 was inhibited by DNA intercalators, less sensitive to netropsin, and not affected by other inhibitors. The investigators recommeneded subjecting recombinant *Pf*RuvB3 for further studies to design a novel specific inhibitor as a new weapon in anti-malarial arsenal. **Compiled from: "Characterization of *Plasmodium falciparum* ATP-dependent DNA helicase RuvB3." Malar J 2016; 15: 526.**

## • Leishmaniasis

Histones, by using their N-terminal tails, play a central role in protein acetylation for several regulatory mechanisms that occur within and outside the nucleus, while ocurrance of reversible acetylation of proteins in the cytoplasm denotes post-translational modification. In the last decade, family sirtunis was investigated in yeasts, plants and animals, and its members proved to be highly conserved proteins from archaea to higher eukaryotes. Moreover, parasitic sirtunis possess conserved and unique functions regulating several and different cellular processes. Therefore, they are considered suitable drug targets for parasitic diseases. The name is aquired from the phrase "silent information regulator (SIR)", with SIR2 as the major member. It is responsible for regulation of transcriptional silencing of ribosomal DNA which is an essential event for survival. Sirtunis, also known as SIR2-like genes, possess $NAD^+$-dependent deacetylase activity, with strong association with deacetylation of cytoplasmic proteins. SIR2 proteins were described in different cellular compartments in several protozoa; *T. brucei*, *T. cruzi*, *L. infantum* and *P. falciparum* and are involved in several functions including DNA repair (i.e., cellular responses to DNA-damaging agents), and parasite proliferation with differentiation among its cycle stages. Overexpression of mitochondrial SIR2RP3 in *T. cruzi* resulted in increased proliferation, movement, and differentiation due to increased deacetylation of mitochondrial targets within the parasite. Therefore, it was suggested as a drug target for treatment of Chagas' disease. With this in mind, the development of a new drug for visceral leishmaniasis (VL) is the research point in the research conducted by Indian investigators **(Nimisha Mittal *et al*.)**. *In silico* analysis of *Leishmania* genome showed three SIR2 homologs; cytosolic SIR2RP1, and two mitochondrial SIR2RP2 and SIR2RP3. The cytosolic sirtunis was previously investigated in *L. major* and *L. infantum* and found to be essential for parasite' survival and infectivity, so it was considered as a new drug target for leishmaniasis. On the other hand, the other two mitochondrial sirtuins were not yet characterised.

To achieve their objective, the investigators identified the localization of *L. donovani Ld*SIR2RP1, and generated a mutant strain with complete loss of genetic SIR2RP1, using double targeted gene replacement technique. To show the effects of loss of *Ld*SIR2RP1 expression, they conducted several studies including proliferation (growth curve analysis for 12 days), virulence (infectivity assay to murine macrophages), defects of life cycle stages (cell cycle analysis to determine the growth phase of promastigotes), and mitochondrial functions (measurement of ATP levels). Their studies extended to include the sensitivity of a mutant strain to four known SIR2 inhibitors; sirtinol, nicotinamide, Ex-527 and cambinol. The investigators also studied the cyotoxic activities of these inhibitors on murine macrophage cell line and investigated their activities on recombinant *Ld*SIR2RP2.

Before summerizing the results of the present compilation, sufficient data should be clarified. Nicotinamide adenine dinucleotide (NAD) is an essential coenzyme with two main functions in all metabolic reactions. NAD is found in two forms; oxidized ($NAD^+$) and reduced (NADH), hence it is involved in redox reactions, i.e. carrying electrons from a reaction to another. The other function is its post-translational modifications which is important for drug discovery. This is done either by deacetylation or adenosine diphosphate (ADP)-ribosylation, i.e. it acts as a substrate for organism' DNA ligases to remove acetyl group from a protein or it is consumed by ADP-ribosyltransferases in ADP-ribose transfer reactions to add ADP-ribose to a protein. Therefore, enzymes that exploit $NAD^+$ for post-translational modification are important in developing new drug targets by designing enzyme inhibitors.

Results showed 1) *Ld*SIR2RP2 is localized in the mitochobdria of *Leishmania* promastigotes, 2) it exploits $NAD^+$ to add ADP-ribose, 3) its ability to be $NAD^+$-dependant to remove acetyl group was not detected, 4) mutant strain showed significant decrease in the growth rate and infectivity to murine macrophages compared to the wild type (WT) promastigotes, 5) mutant strain also showed increased (accumulated) G2/M population denoting its slow growth kinetics, 6) there was altered mitochondrial functions in mutant strain as observed by decreased ATP intracellular levels due to altered mitochondrial transmembrane potential. *Ld*SIR2RP2 gene is essential to maintain this potential required for ATP synthesis by mitochondria. Testing the effects of SIR2 inhibitors on the growth of promastigotes and amastigotes of mutant and wild strains, the results showed that 1) **Sirtinol** inhibited the growth of both stages in a concentration-dependent manner in both strains. However,

its $IC_{50}$ was not significantly different. 2) **Nicotinamide** (NAM) only inhibited growth of amastigotes in both strains, due to presence of thick lipophosphoglycan layer around promastigotes interfering with NAM entry. Its $IC_{50}$ was significantly lower in mutant strain than that of wild strain, but its $IC_{50}$ for murine macrophages was 3 fold higher than that observed for amastigotes. 3) **EX-527** gave satisfactory results for promastigotes and was the most effective inhibitor for amastigotes in both strains. Its $IC_{50}$ for mutants was ~2 fold lower than that of the wild in both stages. Moreover, it inhibited murine macrophages only at higher concentrations. 4) **Cambinol** inhibited growth of both stages in both strains and its $IC_{50}$ for mutant strain was 24-fold lower than that of the WT strains, but it was cytotoxic to murine macrophages. Finally, results of testing the effect of these inhibitors on the activity of recombinant *Ld*SIR2RP2 showed that cambinol was the most effective inhibitor of *Ld*SIR2RP2 activity (ADP-ribosyltransferase activity). Results of the present compilation pointed out the essential role of mitochondrial homoeostasis in *Leishmania* parasite's survival and infectivity which might be a promising stepforward towards the development of new anti-leishmanial chemotherapy. Finally, the investigators recommended further studies to test another specific inhibitor to *Ld*SIR2RP2. **Compiled from: "The mitochondrial SIR2 related protein 2 (SIR2RP2) impacts *Leishmania donovani* growth and infectivity." PLoS Negl Trop Dis 2017 May 11; 11 (5): e0005590.**

In another work, a team from Belgium and USA **(Géraldine De Muylder *et al.*)** thought of using host-directed therapy (HDT) in treatment of leishmaniasis. This new strategy to treat diseases caused by intracellular pathogens (bacterial, viral and parasitic) depends on targeting host cells to indirectly interfere with pathogen growth. In 2015, GlaxoSmithKline (GSK), a British pharmaceutical company, conducted a thorough phenotypic project to screen 1.8 million compounds associated with human proteins against already identified genomic proteins of three kinetoplastids; *L. donovani*, *T. cruzi* and *T. brucei*. To highlight compounds that may possibly target host-pathogen interactions, the company conducted secondary confirmatory studies, intracellular anti-parasitic assays, and cytotoxicity investigations to test for potentiality of causing non-specific cytotoxic effects. The compunds were chemically clustered according to their physical properties and chemical functions. Accordingly, the company listed ~ 200 compounds with potential mode of actions against kinetoplastid kinases, proteases and cytochromes as well as potential HDTs. Naloxonazine is one of these HDTs and it was previously identified to act against intracellular *L. donovani*, but when the investigators used it in axenic culture, it proved ineffective **(De Muylder *et al*., 2011)**. In leishmaniasis, and during phagocytosis, the phagosome matures through fusion with endosomes and lysosomes, creating an acidic cellular environment. However, virulent *Leishmania* parasites could accommodate and survive in such an acidic condition by arresting phagosomal maturation. In the present work, the investigators showed that naloxonazine interferes with host cell acidic components, making them unsuitable for promastigotes → amastigotes transformation inside host cell. The investigators validated their explaination of targeting phagosome acidification to act against *Leishmania* parasites, to a similar strategy of using similar compounds to acidify cellular components in tuberculosis and early chronic myeloid leukemia. The investigators inquired if these HDTs will induce resistance in the future? **Compiled from: "Naloxonazine, an amastigote-specific compound, affects *Leishmania* parasites through modulation of host-encoded functions." PLoS Negl Trop Dis 2016 Dec; 10 (12): e0005234.**

## • Dirofilariasis

In the last year, Canadian investigators **(Thangadurai Mani, Catherine Bourguinat, and Roger Prichard)** worked with macrocyclic lactones (MLs) resistance in dirofilariasis, a filarial nematode infecting dogs and cats, and occasionally man. It is caused by the heartworm, *D. immitis*, through bites of infected mosquitoes. In their previous three studies, the researchers investigated the mechanism of MLs resistance in dirofilariasis. Since P-glycoprotein (Pgp) genotypes were correlated with loss of MLs efficacy in treatment of heartworm, the investigators worked with ATP binding cassette (ABC) transporter genes because they found that these correlated Pgps belonged to ABC-B group of transporters. Using bioinformatics and PCR amplification of pooled *D. immitis* genomic DNA and cDNA, they succeeded to identify three Pgps genotypes (*Dim pgp* 3 10, and 11) and eight ABC transporter genes. Results of their study allowed the investigators to present all genes encoding ABC–B transporters and to generate an expected phylogenetic tree for *D. immitis* **(Bourguinat *et al*., 2016)**. They also pointed out the MLs binding sites on *Dim pgp* 11 **(Mani *et al.*, 2016a)**. Then, they worked with ion channels (ICs) receptors in the neuromuscular system of *D. immitis*, and they identified possible single nucleotide polymorphism (SNP) sites in its IC genes that could be used as new drug targets **(Mani *et al.,* 2016b, previously compiled in PUJ; 2016, 9 (2): 116).**

On the other hand, ABC transporters play essential roles in several helminth physiological functions such as excretion, reproduction and modulation of host immune responses. Therefore, development of their inhibitors could be successfully used as attractive drug targets. Meanwhile, ABC transporters were reported in resistance to MLs, triclabendazole and praziquantel in treatment of *O. volvulus*, *F. hepatica* and *S. mansoni* respectively. Therefore, the investigators attempted to utilize ABC transporter genes as new drug targets in the present compilation **(Mani *et al*., 2017)**. They hypothesized that these vital genes are involved in several critical functions; e.g. sterol transport, which could be a target for a new drug.

Meanwhile, the identified SNPs in these genes would be useful data in the process of drug design. In other words, known gene polymorphism would ensure that the developed drug is active against all the allelic forms of these targets. Three other observations encouraged the investigators to conduct this work. First, they found that *B. malayi* confers some sort of multi-drug resistances linked to Pgps which belong to ABC transporters (subfamily A and G). Second, some ABC transporters have vital roles in developing and maintaining drug resistance through protection of parasite neurons and other tissues from drug toxins. Third, in filarial nematodes such as *B. malayi*, use of inhibitors of ABC transporters increased its sensitivity to ivermectin. Therefore, their objective in the present compilation is to identify all possible SNPs in ABC transporter genes in *D. immitis.*

Since *D. immitis* genome is not completely generated, so to obtain all possible ABC transporter genes in *D. immitis*, they utilized the same methodology (complexed complementary approach) that was used in their previous work **(Mani *et al.*, 2016b)**. Briefly, they searched in the available databases (NCBI, Wormbase, Broad Institute and NEMBASE4) for nucleotide sequences that could encode ABC transporter genes or subunits from subfamilies A, B, C and G. They detected 169 nucleotide sequences; 13, 84, 29 and 43 belonged to ABC-A, -B, -C and -G encoding genes/subunits, respectively. On the basis of PCR amplification, sequencing and bioinformatics, they identified 15 unique ABC transporter genes, eight of them were already previously presented **(Bourguinat *et al.*, 2016)**. They also utilized their pooled samples previously used and classified as ML susceptible (SUS) and loss of efficacy (LOE) populations **(Mani *et al.*, 2016b)**. Briefly, four SUS obtained from 17 infected dogs from USA, Spain, Grenada and Italy, and another four LOE isolates obtained from infected dogs from different USA areas were their study samples. The investigators pooled worms from each country and microfilaria from each dog to form eight isolates which were applied for genomic sequencing. According to blasting of sequences of ABC transporter genes on genomic sequencing of the eight isolates, 231 single nucleotide polymorphic (SNP) sites were identified. Out of these SNPs, 67 were detected in all isolates, 89 and 75 were specific to SUS and LOE isolates, respectively.

The investigators discussed these SNPs and their mutant codon(s) in ABC transporter genes (either -B or -C or -G 'subfamily' once in view of their relation to either group of isolates (SUS or LOE), and another time in view of their relation to Pgps as members of ABC transporter superfamily; and each time they were linked with drug resistance in several helminths. The most relevant result was the detection of 59 SNPs in six ABC-B transporter genes; and the investigators found Dim-*pgp*-3 and Dim-*pgp*-11, as the most likely Pgps that could be utilized as potential drug targets. Multi-resistance proteins (MRPs), a member of ABC transporters with Pgps, were also discussed as new drug targets, alternative to MLs, due to their protective effects through reduction of the toxicity of heavy metals. The investigators discussed the role played by these MRPs in *C. elegans*, with special emphasis on MRP-1, MRP-5 and MRP-7. The investigators found that MRP-1 and MRP-5 are essential for regulation of larva formation and worm viability, respectively. However, renal tubular dysfunction was associated with multiple polymorphism in the gene encoding human MRP-7. In conclusion, studying polymorphism in SUS and LOE strain populations for *D. immitis* would help the investigators in understanding mechanism(s) of MLs resistance and thereby developing new drug targets. **Compiled from "Polymorphism in ABC transporter genes of *Dirofilaria immitis*" Int J Parasitol Drugs Drug Resist. 2017 Aug; 7 (2): 227–235.**