Spotlights on new publications

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New drug targets - XX

***Plasmodium* spp.**

Protein kinases (PKs), wether eukaryotic (ePKs) or atypical (aPKs), are enzymes essentially involved in protein phosphorylation required in several signaling pathways that transmit cellular signals in all eukaryotes. In signaling pathways, PKs transfer the phosphate groups from adenosine triphosphate (ATP) to the protein substrate. Hence, they are vital components in regulating protein functions involved in parasite growth, differentiation, stress response, and apoptosis, i.e., suitable as potential drug targets. In contrast to aPKs, ePK have a highly conserved catalytic domain, and motif with essential roles in their functions and stability. The ePKs are classified into eight groups: 1) tyrosine kinase (TK); 2) calcium/calmodulin-regulated kinase (CAMK); 3) casein kinase I (CK1); 4) TK-like (TKL); 5) serine-threonine kinase (STE); 6) receptor guanylate cyclases (RGC); 7) (ACG group) that includes cyclic adenosine (PKA), guanine (PKG), and cytosine (PKC) monophosphate-dependent PKs; and 8) CMGC group including four PKs, cyclin-dependent kinase (CDK), mitogen activated PK (MAPK), glycogen synthase kinase (GSK3), and CDC-like kinase (CLK). Notably, PKB that is related to both PKA and PKC, is occasionally termed AKT. The ninth classified group is termed "others" and includes other PKs not fitting in the previously annotated groups. In an attempt to update PKs in *Plasmodium* spp., **Joyce Villa Verde Bastos Borba** and her Brazilian colleagues conducted kinomics studies complemented with bioinformatics pipeline analyses of eight species: *P. falciparum, P. vivax, P. malariae, P. ovale, P. berghei, P. chabaudi, P. yoelii,* and *P. knowlesi*.

To achieve their objective, the investigators searched for PKs motif in each *Plasmodium* proteome utilizing KinBase database (https://www.kinbase.com). The predicted kinome for each species was compared with the assigned PKs groups and related PKs identified in *T. gondii* using PlasmoDB (https://plasmodb.org/plasmo). For the unclassified PKs, the present compilation used InterproScan database (https://www.ebi.ac.uk/interpro/search/sequence-search). To compare identified PKs among *Plasmodium* spp., phylogenetic trees for each kinase group were constructed. Utilizing drug target prioritization approach, the investigators created a subset of the top-ranked PKs that were selected from the protein network interactions of *P. vivax* PKs. The present compilation included a chemogenomics study to investigate a list of drugs or compounds targeting *P. vivax* PKs, being the most distributed and endemic species in Brazil. Sequences of the top-ranked PKs were *in silico* tested for known drug targets in DrugBank (https://go.drugbank.com/) and TDR (https://tdr.who.int/) database. The investigators selected this approach because drug therapy used for *falciparum* malaria failed to control relapses in *vivax* malaria. The investigators assessed the inhibitory efficacy of these investigated drugs and compounds on the growth of *in vitro* cultivated erythrocytic stages of two *P. falciparum* laboratory strains namely chloroquine-sensitive (3D7) and multidrug-resistant (Dd2) strains. Their cytotoxicity effects on two mammalian cell lines were also evaluated. The investigators hypothesized that drugs or compounds, with potential inhibitory activity against *P. vivax* PKs, and inhibition of *P. falciparum* growth and survival would be a single therapy for the most common *Plasmodium* spp. causing human malaria, i.e., *P. vivax* and *P. falciparum*.

The investigators succeeded to identify 76-97 PKs among all *Plasmodium* spp., where the highest number was identified in *P. falciparum*, and the lowest number in *P. berghei* and *P. yoelii*. Around 90% of the ePKs were assigned to seven assigned groups CAMK, CMGC, CK1, TKL, STE, AGC and "others". It is worth mentioning that CAMK was the largest group represented in all species followed by CMGC, while no STE PKs were identified in *P. berghei*, *P. chabaudi* and *P. yoelii*, species causing rodent malaria. Regarding aPKs, *P. falciparum* expressed 21 kinases belonging to the FIKK group, while other *Plasmodium* spp. expressed only one FIKK kinase. Interestingly, bioinformatics analyses allowed the investigators to suggest 33 classified ePKs expressed in *P. vivax* and *P. falciparum* including 13, 8, 6, 4, 1, and 1 belonging to CMGC, CAMK, "others", AGC, TKL, CK1 groups, respectively. Additionally, two aPKs group (RIO, PI4K), one orphan kinase (PK7), and unclassified PK were also proposed. Construction of the phylogenetic trees for each group confirmed the usefulness of kinomics in classifying PKs.

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Besides, search in DrugBank and TDR online websites identified 71 predicted drugs and compounds with inhibitory activity against *P. vivax* PKs. However, literature search identified only six compounds that were not investigated as antimalarial drugs for experimental evaluation against *P. falciparum* strains. They included compounds against NIMA-related kinase 1 (two), PIK4, PKA, CK1, and GSK3 (one for each). In a concentration of 5 μM, results revealed success of only three compounds to inhibit more than 30% of erythrocytic stages growth with neglected cytotoxicity on both mammalian line cells. The investigators concluded that use of kinomics, chemogenomics, and bioinformatics analyses was a useful integrated approach to develop novel antimalarial drugs. Compiled from **"Update and elucidation of *Plasmodium* kinomes: Prioritization of kinases as potential drug targets for malaria. Comput Struct Biotechnol J 2022 Jul 8; 20:3708-3717."**

Pathogenic trypanosomatids

Three years ago, Barrett *et al*., published a review article attributing treatment failure to existence of persister cells in *Plasmodium* spp., *T. gondii*, *Leishmania* spp., and *T. cruzi* (Protozoan persister-like cells and drug treatment failure. Nat Rev Microbiol 2019; 17:607-620). Existence of transient dormant stages is incriminated as an important obstacle in novel drug development with complete cure outcomes. In the present compilation, **Manu De Rycker** and his British colleagues reviewed the challenges facing control and elimination of the endemic tropical diseases caused by *Leishmania* spp. and *T. cruzi*. These include: 1) growing evidence suggesting existence of persister forms in *Leishmania* spp., and *T. cruzi*; 2) several animals acting as reservoir hosts; 4) difficulty of accurate diagnosis of early asymptomatic patients; and 5) insufficient or poor progress in drug development. The review is divided into six sections.

1. **Persister forms:** It was reported that persister forms exist due to either metabolic or physiologic factors that render them less susceptible to drugs. This simply means that these forms differ from the normal actively dividing and growing forms, either by having sluggish metabolic activity or physiological changes, e.g., altered transporters. In both conditions respectively, the administered drug does not affect its metabolic target or its effect is reduced. To confirm existence of persister forms, drugs targeting DNA replication or transporters do not affect sluggish metabolic cells due to absence of drug target, or cells with altered physiological changes due to reduced drug uptake or efflux. Besides, existence of persister forms leads to treatment failure, not real drug resistance due to gene mutations. In mice experimentally infected with *T. cruzi*, population-level replication rates in chronic infection are fewer than in animals with acute infections. Yet it is not completely understood whether these persister forms represent a new term of life stage or slowing replicating amastigotes. Whatever they are, lower drug efficacy was observed in treatment of chronic Chagas' disease. Therefore, the reviewers suggested higher doses (3-5 times the conventional dose) with extended durations (up to 30 w). However, this approach relies on persister forms dynamics if they become susceptible to the administered drug by time. To reduce adverse side effects from long treatment, an intermittent treatment with higher doses was suggested. However, this approach faced the golden rule for treatment "shorter is better". Accordingly, the reviewers concluded that understanding of persister forms biology and large-scale phenotypic screening against several compounds would be a successful approach. Similarly, persister forms exist in ~15% of patients with post-kala-azar dermal leishmaniasis (PKDL) in Indian subcontinent, *in vitro* cell cultures and *in vivo* laboratory animal models.

2. **Progress in drug discovery and development:** In African trypanosomiasis, Fexinidazole that directly acts against trypomastigotes in both peripheral and central nervous systems, was registered for treatment against *T. b. gambiense* and is now in an advanced clinical trial against *T. b. rhodesiense*. Acoziborole that targets polyadenylation specificity factor 3 (CPSF3), an endonuclease involved in mRNA maturation, is also in phase IIb/III clinical trials. In visceral leishmaniasis and PKDL, the majority of progress in clinical development of novel drug regimen was achieved by combined therapy of available drugs. This approach reduces risk of drug resistance and improves drug efficacy and safety. The reviewers suggested several drugs that were evaluated *via* phenotypic screening and identification of their targets. These included two proteasome inhibitors (GSK3494245/DDD01305143, and LXE408), Oxaborole targeting CPSF3, GSK3186899/DDD853651 targeting cdc2-related kinase 12 (CRK12), and Nitroimidazole. The drug target and mechanism of action of the latter was not yet identified. In contrast, there is a great challenge to control and achieve complete cure of Chagas' disease, as previously reviewed. However, Fexinidazole and Oxaborole are nowadays in phase I clinical trials.

3. **Role of evolutional technology in development of novel drugs:** The reviewers concluded several issues. First, functional genomics using RNA interference accompanied by bioinformatics analyses is a mandatory strategy to identify novel potential drug targets. Second, structural genomics, i.e., prediction or determination of a protein molecular structure is fundamental for drug target discovery. For example, using high-resolution cryogenic electron microscopy enabled the investigators to identify proteasome inhibitors against *Leishmania* spp. In other words,

using structural information facilitated compounds' optimization according to *L. donovani* ribosome structure. Third, *in silico* phenotypic screening against online libraries remains a vital step for identification of novel drugs with confirmed mechanism of action and molecular target. Fourth, genome editing using CRISPR–Cas9-based technology enabled the investigators to target specific chromosomal loci. Notably, CRISPR–Cas9 facilitates generating drug-resistant strains to provide increased knowledge of structure–activity relationships (SAR) that is essentially utilized in evaluating drug pharmacokinetics and pharmacodynamics.

4. **Common issues limiting drug discovery studies:** The reviewers listed three common obstacles challenging researchers interest in drug discovery. First, there are several methods to evaluate drug cytotoxicity such as experimental animal models and *in vitro* assays against mammalian cell lines. Besides, drug discovery studies should be aware of other assays that determine potential genotoxicity and mitochondrial toxicity. Second, selective animal model is a key challenge for drug discovery progress to human clinical trials. Although Syrian golden hamsters were highly susceptible to visceral leishmaniasis, mice models were preferred for drug discovery studies due to differences in both models in parasite clearance. It is worth mentioning that drug efficacy requires precise integrated assessment of drug intake and influx. On the other hand, several animal models were developed with similar preference in Chagas' disease. However, due to existence of potential persister forms, the challenge is focused on host immune response that influences disease outcome, i.e., acute, or chronic. Therefore, genetically modified, or humanized mouse model became an important tool to justify host immune response simulating infections in humans. Third, assessment of drug pharmacokinetic, and pharmacodynamics properties in animal models is a complicated challenge due to the potential differences in disease pathology, and infection course. Mice are ideal models for both assessments in drugs discovery against visceral leishmaniasis, but neither cutaneous leishmaniasis, nor Chagas' disease. The reviewers recommended further studies investigating skin pharmacokinetics and pharmacokinetics in animal models. Future studies were also suggested to investigate drug levels in blood and infected tissues in relation to its *in vitro* activity.

5. **Approaches other than drug discovery for control and elimination:** The reviewers discussed the progress in identification of antigen candidates for vaccines development, as well as host-directed therapy. The reviewers claimed that drugs commonly used to target host-parasite interactions are efficient therapy through modulating host immune response. Although they are expensive and outside drug discovery interest, the reviewers suggested immunomodulators combined with chemotherapy in cutaneous leishmaniasis.

6. **Concluding diagram:** Finally, the reviewers summarized and drew up a chart demonstrating steps for the ideal approach in drug discovery in visceral leishmaniasis and Chagas' disease. It included: 1) high-throughput phenotypic screening of axenically cultured amastigotes; 2) compounds, with no cytotoxic effects on mammalian cell lines, are investigated to identify its target and mechanism of action; 3) these identified compounds are validated for structure and purity; 4) validated hit compounds progress to investigation in suitable animal model; and 5) assessment of drug efficacy should be determined against different life cycle stages, several strains (sensitive and resistant) and its potential use in future combination therapy. Compiled from **"Anti-trypanosomatid drug discovery: Progress and challenges. Nat Rev Microbiol 2022 Aug 22; 1-16."**

Chagas' disease

The objective of the present compilation was investigating Ivermectin (IVM) efficacy in treatment of Chagas' disease. Several issues encouraged **Laura Fraccaroli** and her Argentinian colleagues to conduct this study. First are the adverse side effects of the commercially available drugs (Benznidazole and nifurtimox). Second, besides its low cost, IVM broad-spectrum anti-parasitic properties showed minimal toxicity in human and veterinary medicine. Moreover, previous studies showed that IVM exhibited satisfactory efficacy in treatment of African sleeping sickness, and cutaneous leishmaniasis as well as showed *in vitro* and *in vivo* antimalarial effects through inhibition of survival, growth, and development of intra-erythrocytic stages. Two mechanisms of action were suggested, potentiation of the glutamate-gated chloride channels or inhibition of importin proteins (IMP-α, and IMP-β). This results in either hyperpolarization of parasite neurons and muscles (in helminths) or blocking protein transport to the nucleus across the nuclear complex pore (in protozoa). This simply means that IVM targets IMPs α and β in protozoa.

To achieve their objective, ascending IVM concentrations were investigated against cultured *T. cruzi* epimastigotes after 27 h either alone or in combination with Benznidazole/nifurtimox. In addition to assessment of mammalian cytotoxic activity on *Vero* cells, two parameters were assessed, viability and proliferation. The investigators also performed cell proliferation recovery assay, i.e., drug (50 and 100 μM) incubation for different time intervals (30 min to 3 h), followed by sterile PBS wash, and incubation in a drug free medium. A growth recovery curve was monitored after 4 and 8 d. The investigators extended their study

investigating IVM possible mechanism of action utilizing *in silico* approach to search for orthologous protein sequences of IMPs α and β in other trypanosomatids, pathogenic and non-pathogenic. Sequence alignments and analyses were conducted using several databases (ClustalOmega, GeneDoc 2.7, and Jalview 2.11.1.2).

With minimal cytotoxicity results, IVM exhibited, in a dose-dependent manner, significant efficacies against epimastigotes proliferation associated with inhibition of amastigotes and trypomastigotes survival. Based on the cell proliferation recovery assay, it was demonstrated that IVM concentrations (50 and 100 μM) acted as trypanostatic or trypanocidal, respectively. While synergetic effects were demonstrated on IVM combination assays, *in silico* studies confirmed that IMPs α and β are IVM potential targets in *T. cruzi*. Interestingly, genes encoding both IMPs showed ~76% similarity, while IMPα were with conserved Armadillo domains, IMPβ lacked the canonical binding sequence. Accordingly, the study strongly suggested IVM drug repurposing in treatment of Chagas disease. Compiled from **"Broadening the spectrum of Ivermectin: Its effect on *Trypanosoma cruzi* and related trypanosomatids. Front Cell Infect Microbiol 2022 Jul 28; 12:885268."**

Cryptosporidiosis

Since invasion and egress cascade in apicomplexans is stimulated by calcium ion flux, calcium-dependent protein kinases (CDPKs) were proposed potential drug targets. It was hypothesized that CDPKs, i.e., the effectors of calcium ion flux, act as secondary messengers for regulation of molecular expression of the apical organelles. Previous studies proved that *T. gondi* CDPK1, CDK3 had critical roles in invasion and egress through control of the micronemes exocytosis Due to absence of human CDPKs orthologues, apicomplexans CDPKs attracted much attention in development of novel drugs, especially *Cp*CDP1 due to its unique amino acid structure. On the other hand, treatment failures of cryptosporidiosis were frequently reported which encouraged **Jiayuan Su** and his Chinese colleagues to conduct the present compilation.

The investigators expressed recombinant *C. parvum* CDPK1 and CDPK9 in *Escherichia coli*, and performed comparative functional studies utilizing qPCR, immunofluorescence staining, and *in vitro* neutralization assay. The study aimed to determine quantitative gene expression, localization, and functions of both CDPKs during invasion and egress cascade, respectively. For immunolocalization, polyclonal antibodies against purified recombinant CDPKs were generated by rabbit' immunization. In addition, to prepare different developmental stages, intracellular parasites in HCT-8 cell cultures were harvested 24 and 48 h after infection. Utilizing molecular docking of genes encoding *Cp*CDPK1 and *Cp*CDPK9, the investigators extended their study to assess the inhibitory activity of CDPK inhibitors to identify novel drugs with anti-*Cryptosporidium* properties.

In vitro cultures showed the highest expression of both CDPKs at 12 h, and after 2-6 h, respectively. While only two bands (30 and 70 kDa) were recognized for *Cp*CDPK1, several bands were identified for *Cp*CDPK9 (30, 50, 100, and 130 kDa). Staining demonstrated immunolocalization of *Cp*CDPK1, and *Cp*CDPK9 in the whole sporozoite, and only the apical region, respectively. Polyclonal antibodies against both CDPKs showed similar diffuse reactivity in merozoites at 24 h of cultures. Besides, both CDPKs had similar neutralization efficiency on *C. parvum* viability, growth, and survival. Molecular docking studies on *Cp*CDPK1 showed that among 50 compounds investigated, only one compound exhibited potent inhibitory activity, while 10 had significant *in vitro* inhibitory activities. For *Cp*CDPK9 molecular docking, only five compounds showed significant *in vitro* inhibitory effects. Due to different functional domains and expression patterns of *Cp*CDPK1 and *Cp*CDPK9, the investigators concluded that both CDPKs exhibited different functions in *C. parvum* viability, growth, and survival. While *Cp*CDPK1 contributed to *Cryptosporidium* viability, growth, and survival, *Cp*CDPK9 had an essential role in invasion and egress cascade. However, more biological, and immuno-histological studies were recommended to examine *Cryptosporidium* genetic modifications using CRISPR–Cas9 technology. Compiled from **"Comparative characterization of CpCDPK1 and CpCDPK9, two potential drug targets against cryptosporidiosis. Microorganisms 2022 Feb 1; 10(2):333."**

Giardiasis

Previous studies demonstrated the effects of pomegranate peel of *Punica granatum* in protecting intestinal cells against *G. lamblia*, through reducing cyst shedding. However, the mechanism of action was not so far characterized. The objective of the present compilation conducted in Mexico **(Lissethe Palomo-Ligas *et al*.)** was to identify the molecular drug target in *G. lamblia*, and to discover the mechanism of action of polyphenols obtained from *P. granatum*. Two facts encouraged the investigators. First, polyphenols are secondary metabolites that have several biological activities, e.g., antioxidants, antibacterial, anti-parasitic, antiviral and anti-inflammatory effects. Second, in the *Giardia* cytoskeleton, specialized cylindrical microtubules of α and β tubulin, are involved in several functions such as motility, adhesion, cell division, and encystation.

Polyphenolic extract prepared from pomegranate peel was phytochemically identified using microwave-ultrasound technique, and high-performance liquid chromatography (HPLC), respectively. Efficacy of polyphenolic extract was evaluated against *G. lamblia*

trophozoites using parameters for growth, adhesion capacity, morphological damage, and changes on α-tubulin expression.

Using HPLC, several compounds were demonstrated in pomegranate peel polyphenolic extract namely punicalin, punicalagin, luteolin, and ellagic acid. The obtained results showed that the latter caused potent inhibitory effects on trophozoites growth and adhesion capacity. The maximum inhibitory effects were recorded after 48 h for growth (74.36%) and adherence capacity (46.8%) at a dose of 200 μg/ml. Morphological changes were related to *G. lamblia* cytoskeleton including membrane irregularities, and bifurcated flagella with elongation of the normal *G. lamblia* trophozoites shape. Alterations and distribution of α-tubulin expression were also demonstrated in a concentration-independent manner, especially in the ventral disk.

The investigators discussed different mechanisms of actions of polyphenols extracted from pomegranate peel. In addition to their effects on α tubulin, they suggested interactions of polyphenols with membrane lipids that caused membrane irregularities, i.e., microvesicles (MVs). The investigators claimed that these MVs were commonly reported, as plasma membrane buddings, associated with response to stress stimuli. From the present compilation, a list of new molecules was presented for further studies aimed to develop novel drugs against giardiasis. Compiled from **"Polyphenolic extract from *Punica granatum* peel causes cytoskeleton-related damage on *Giardia lamblia* trophozoites *in vitro*. Peer J 2022 Apr 27; 10:e13350."**

Hydatid cyst

Surgery is the ideal treatment for hepatic alveolar hydatid cysts (HAHCs). However, the majority of patients are commonly diagnosed at the advanced stage due to infiltrations to surrounding close (gallbladder, and biliary system) and distant (lung, brain, kidney, and bone) tissues. Medical treatment with Albendazole showed more efficient results than Mebendazole since the former inhibits glucose uptake with marked decrease of the germinal layer glycogen, altered morphological features of endoplasmic reticulum bodies and mitochondria, and formation of lysosomes leading to death. Unfortunately, four issues were reported: 1) Albendazole has a static rather than a killing effect; 2) advanced HAHCs require long-term chemotherapy with adverse side effects including abnormal liver function, leukopenia, and hair loss; 3) high recurrence rates; and 4) high probability of drug resistance due to long term administration. In the last decade, drug repurposing strategy presents new advantages over *de novo* drug discovery since it saves time, cost, efforts, and reduces failure risk. Besides, it is adopted by several pharmaceutical companies as long as the repurposed drug completes the following criteria: assessment of its pharmacological characteristics, formulation considerations, as well as evaluation of its efficacy in *in vitro* and *in vivo* studies, and clinical trials. In the present compilation, **Xiaolei Xu** and his Chinese colleagues reviewed several studies that proposed FDA-approved drugs and the use of nanotechnology, as drug delivery system, with potential therapeutic efficacy against hepatic alveolar hydatid cysts. Interestingly, almost all FDA-approved drugs and nanotechnology delivery systems lacked the clinical trials.

Among the FDA-approved drugs, the reviewers discussed the results obtained from Mefloquine, Nitazoxanide, Amphotericin B, and Bortezomib that were efficiently used as an antimalarial, broad-spectrum antibiotic, anti-fungal, and protease inhibitor, respectively. The reviewers also discussed protein kinases that contribute to signal transduction essentially required for hydatid cyst survival and growth, as well as differentiation and reproductive activity. Sorafenib (mitogen activated protein kinase inhibitor), and Imatinib (tyrosine kinase inhibitor) were also repurposed drugs for HAHCs treatment.

On the other hand, for persistence in the host for a long time, hydatid cyst antigens escape host immune response through immunosuppression and immunoevasion. Several *in vivo* studies demonstrated immunosuppression to specific antigens with activated release of anti-inflammatory cytokines, e.g., interleukin-10 (IL-10), and transforming growth factor β (TGF- β). Prolonged exposure to both cytokines resulted in T cell dysfunction, and T cell apoptosis, respectively. Therefore, the reviewers recommended adjuvant therapy with drugs that improve the hepatic immunity such as TIGIT inhibitors. The latter is a T cell immune receptor with two domains present on natural killer cells. Its main function is regulation of T-cell mediated immunity through binding with CD155 on dendritic cells, and macrophages. Furthermore, the reviewers discussed the role of herbal medicine in HAHCs treatment such as Artemisinins, and the traditional Chinese herbal medicine "Xiaobao Decoction".

Finally, the reviewers discussed the usefulness of combined drug repurposing with nanotechnology. Designing new Albendazole formulations in combination with nanotechnology would certainly increase its bioavailability and water solubility. Several approaches were investigated such as nanoparticles, nanocrystals, and nanocrystalline. Similarly, other drug formulations, e.g., liposomal Amphotericin B showed satisfactory results although, however it needs further validation. Compiled from **"Advances in the pharmacological treatment of hepatic alveolar echinococcosis: From laboratory to clinic. Front Microbiol 2022 Aug 8; 13:953846."**