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Update and elucidation of *Plasmodium* kinomes: Prioritization of kinases as potential drug targets for malaria

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ABSTRACT

Malaria is a tropical disease caused by *Plasmodium* spp. and transmitted by the bite of infected *Anopheles* mosquitoes. Protein kinases (PKs) play key roles in the life cycle of the etiological agent of malaria, turning these proteins attractive targets for antimalarial drug discovery campaigns. As part of an effort to understand parasite signaling functions, we report the results of a bioinformatics pipeline analysis of PKs of eight *Plasmodium* species. To date, no *P. malariae* and *P. ovale* kinome assemble has been conducted. We classified, curated and annotated predicted kinases to update *P. falciparum, P. vivax, P. yoelii, P. berghei, P. chabaudi, and P. knowlesi* kinomes published to date, as well as report for the first time the kinomes of *P. malariae* and *P. ovale*. Overall, from 76 to 97 PKs were identified among all *Plasmodium* spp. kinomes. Most of the kinases were assigned to seven of nine major kinase groups: AGC, CAMK, CMGC, CK1, STE, TKL, OTHER; and the *Plasmodium-specific* group FIKK. About 30% of kinases have been deeply classified into group, family and subfamily levels and only about 10% remained unclassified. Furthermore, updating and comparing the kinomes of *P. vivax* and *P. falciparum* allowed for the prioritization and selection of kinases as potential drug targets that could be explored for discovering new drugs against malaria. This integrated approach resulted in the selection of 37 protein kinases as potential targets and the identification of investigational compounds with moderate *in vitro* activity against asexual *P. falciparum* (3D7 and Dd2 strains) stages that could serve as starting points for the search of potent antimalarial leads in the future.

1. Introduction

Malaria is a tropical infectious disease and still a massive problem in many parts of the world, caused by *Plasmodium* spp. parasites, through the contaminated *Anopheles* mosquito bite. Five *Plasmodium* species are known to infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* e *P. knowlesi,* causing approximately 228 million clinical cases and 405,000 deaths in 2018 [1]. *P. falciparum* cause the most severe form of malaria whereas *P. vivax* is the other predominant strain causing human disease, which causes a relapsing form of the disease. Moreover, the rodent parasites *P. berghei*, *P. chabaudi*, and *P. yoelii* are model organisms for the study of parasite biology and treatment [2].

Malaria control and elimination is likely to require the application of different strategies, including vector control, drug combination therapies and vaccines. Despite of significant progress in this field, one particular concern is the emerging resistance to current drugs within the parasite [3], and the resistance to artemisinin, a core component of current front-line artemisinin combination therapies (ACTs), in South East Asia [4]. Also, many experimental technical difficulties such as culturing the parasite (specially *P.*

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vivax), *in vivo* models of the disease, and stage-specific culturing hampered knowledge gaps in the understanding of its fundamental biology, molecular and cell responses, which has challenged the selection of effective drugs and targets [5]. Therefore, there is an urgent need for new drug classes with novel mechanisms of action to tackle the problem of resistance and to improve clinical efficacy.

Whole genome sequence technology has aided the search for new drugs based on the knowledge of biological targets of the most diverse *Plasmodium* species [6]. Protein kinases are responsible for catalyzing the transfer of a phosphate group from one donor molecule to another acceptor, and therefore are key controllers of signal transduction pathways that regulate essential cellular processes such as growth, development, and reproduction in eukaryotic cells [7,8]. Dysregulation of phosphorylation plays a role in many diseases such as cancer and Alzheimer' disease, which has led to the identification of protein kinases as attractive targets and prompted the search for kinase inhibitors [9,10]. Factors such as structural and functional divergence between human and parasite kinases, the presence of kinases that are not present in the human kinome (Calcium Dependent protein Kinases – CDPKs and FIKKs, named after the 4-amino acid chain sequence Phe–Ile–Lys–Lys), and the essentiality of these proteins in the parasite life cycle, boosted by the success in developing drugs targeting human kinases, made *Plasmodium* kinases attractive targets for next generation antimalarials [11]. Therefore, the improvement of the understanding of *Plasmodium* kinases is crucial for fueling this class of proteins as drug targets for antimalarial discovery.

Eukaryotic protein kinases (ePK) are characterized by their highly conserved catalytic domain with about 300 amino acids. This domain is constituted of eleven subdomains with conserved motifs with important roles in kinase functions and stability. Proteins with kinase activity that don't have the conserved kinase domain are called atypical protein kinases (aPK) [12,13]. The ensemble of protein kinases of an organism's genome is called kinome [14]. Kinases are classified into nine groups according to functional categories with distinguishing sequence and structural features: (i) the AGC group: protein kinases A, G, and C; (ii) CAMK group: Ca+/CAM-dependent kinases; (iii) CMGC group: Cyclin Dependent Kinase (CDK), Mitogen Activated Protein Kinase (MAPK), Glycogen Synthase 3 Kinase (GSK3), and CDC-like kinase (CLK); (iv) CK1 group: casein kinase 1 (CK1); (v) STE group: homologues of yeast sterile 7, 11, and 20 (STE7, STE11, STE20, respectively); (vi) RGC group: receptor guanylate cyclases; (vii) TK group: tyrosine kinase; (viii) TKL group: tyrosine kinase like; and (ix) "OTHER" group: several kinase families that do not fit within any of the other main kinase groups [12]. These kinase groups and the cell processes in which they participate, are well characterized in humans and model organisms, constituting the base for kinase classification in other organisms [15].

Here we have developed and applied an integrated chemogenomics and bioinformatics pipeline to update *P. falciparum, P. vivax, P. yoelii, P. berghei, P. chabaudi, and P. knowlesi* kinomes published to date, as well as report for the first time the kinomes of *P. malariae* and *P. ovale*. We have applied a different pipeline for kinase classification that allowed for a deeper group/family/subfamily classification not provided by other studies. Furthermore, updating and comparing the kinomes of *P. vivax* and *P. falciparum* allowed for the prioritization and selection of kinases as potential drug targets that could be explored for discovering new drugs against malaria. This integrated approach resulted in the selection of 37 protein kinases as potential targets and the identification of investigational compounds with moderate *in vitro* activity against asexual *P. falciparum* (3D7 and Dd2 strains) stages that could serve as starting points for the search of potent antimalarial leads in the future.

2. Methods

The general workflow of this study is presented in Fig. 1.

2.1. Definition and prediction of Plasmodium species kinomes

We have updated the kinomes of the species *P. falciparum, P. vivax, P. ovale, P. yoelii, P. berghei, P. chabaudi, P. knowlesi* and *P. malariae* using a modified and refined bioinformatics pipeline previously described [16]. The proteomes of *Plasmodium* species were used as input sequences to the program Kinannote v.1.0 [17], which was executed using the default protocol. Kinnanote is a software that identifies and classifies protein kinases of eukaryotic protein kinase (ePK) families using hidden Markov models (HMM) and BLAST search against a locally curated version of KinBase database (https://www.kinbase.com). The classification process done by a typical Kinannote run follows a pipeline composed by three phases. In phase 1, the input sequences are analyzed to identify the subset of kinases among the non-kinases; a relaxed cutoff is applied to reduce the searching space while keeping the divergent kinases. The kinase candidates are searched using position-specific score matrices (PSSM) and BLAST against the KinBase database. Phase 1 ends with the BLAST results being parsed and prepared for the phase 2. In phase 2, the BLAST results are analyzed to identify the conserved kinases with low HMM scores. The identified kinases are then classified as twilight hits and high-confidence protein kinases, using their PSSM scores combined with an optimized HMM cutoff to produce a high-quality set of kinases. These parameters are better described by [17]. Finally, this high-quality set of kinases is then parsed to phase 3 and fully classified into groups, families, and subfamilies. Kinnanote avoids the elimination of proteins with the kinase subdomain, but below the cutoffs that would list them as ePKs. To this end, the software flags such proteins as "unclassified" and "partially classified", allowing their further curation. The annotations of *P. falciparum, P. yoelii* and *Toxoplasma gondii* deposited on PlasmoDB (https://plasmodb.org/plasmo) were used as a reference to further classify the "unclassified" and "partially classified" kinases, to improve the classification, and find proteins that were not detected by Kinannote. InterproScan v.5.18 (https://www.ebi.ac.uk/interpro/search/sequence-search) was used to elucidate and locate kinase domains of the classified proteins. Then, the updated kinomes of *Plasmodium* species were compared with each other and with previously published kinomes of different *Plasmodium* species based on the number of kinases and depth of classification.

2.2. Phylogenetic tree construction

One phylogenetic tree for each kinase group containing kinases from all eight *Plasmodium* species was built to study the relationships within the *Plasmodium* spp. kinases. For each group, only the catalytic domains were kept for automatic multiple sequence alignment (MSA) using MAFFT v. 7.215 [18] in most accurate mode (L-INS-i; parameters *--localpair --maxiterate 1000*). The option "L-INS-i" is a strategy available in this version of the software that is recommended to be used when working with input lists containing less than 200 sequences. The command used to run this option requires two other arguments: (*i*) "localpair", which indicates that the alignment will be done locally, with only one domain to be aligned; (*ii*) "maxiterate 1000", which relates to the maximum number of cycles to obtain the most accurate alignment for the sequences in the input list. MAFFT v.7 parameters and procedures are well documented at https://mafft.cbrc.jp/alignment/software/algorithms/algorithms.html. Next, MUSCLE v. 3.8.31 [19] was used to refine the obtained alignments using the parameter *--refine*. This

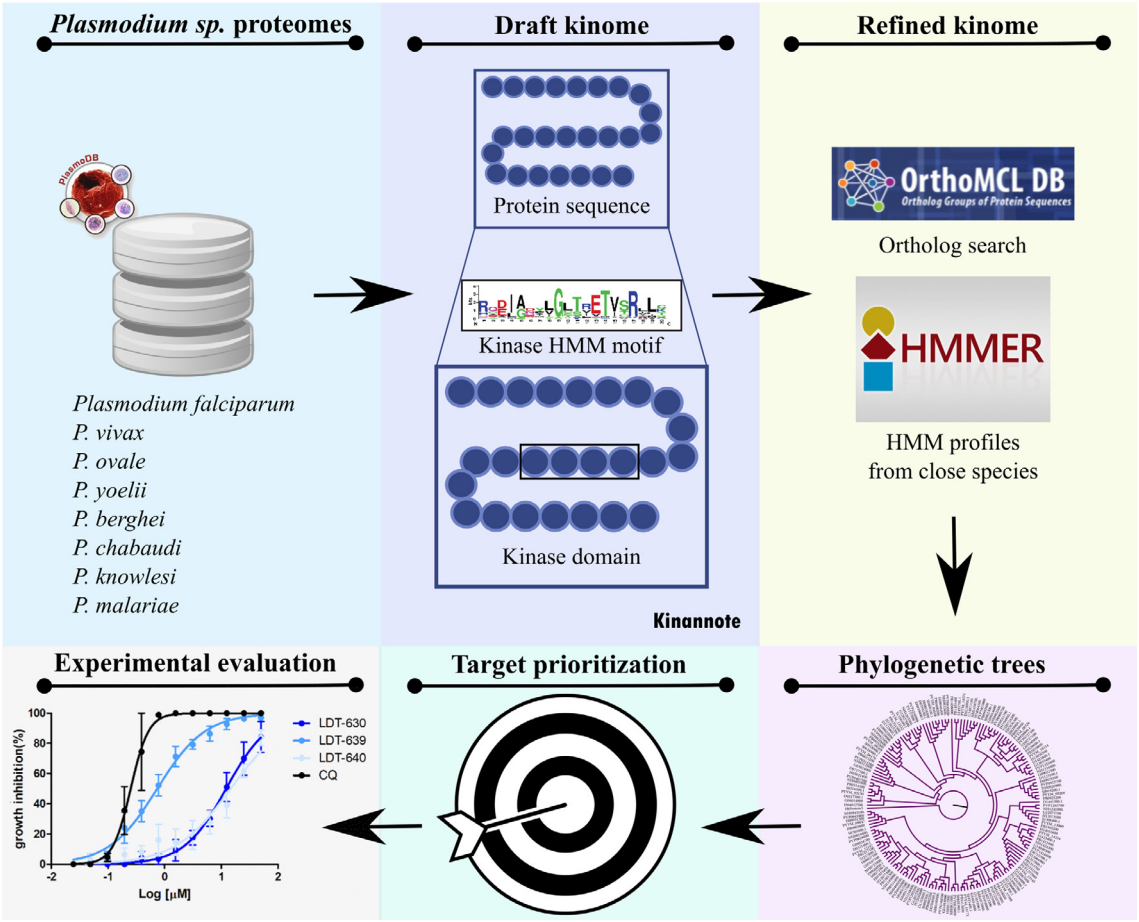


Fig. 1. Chemogenomics and bioinformatics pipeline used to update *Plasmodium falciparum, P. vivax, P. yoelii, P. berghei, P. chabaudi, and P. knowlesi* kinomes, elucidate the kinomes of *P. malariae and P. ovale,* to prioritize kinases as drug targets, and select drugs for testing. First, we searched for kinase motifs in proteomes of each *Plasmodium* species using Kinannote software. Then, the draft kinome was refined comparing the kinase classifications with ortholog kinases of close related organisms using both OrthoMCL and HMMer. Then, the refined kinomes were used to build phyolgenetic trees of each kinase group, comparing those kinases among species. We further conducted target prioritization approaches to select and experimentally test some approved drugs and investigational compounds related to those targets.

parameter makes MUSCLE analyze an existing MSA and re-align it, aiming to re-score and fine tune the alignments in the input file. The detailed description of MUSCLE parameters and procedures are available at http://www.drive5.com/muscle/muscle.html. Biopython scripts [20] were used to convert between the MSA formats generated by the distinct tools. ProtTest3 v. 3.4.2 was used to select the best-fit model of amino acid replacement according to the Akaike information criterion measure [21]. PhyML v. 20131022 [22] was used to infer maximum likelihood trees with 1000 bootstrap replicates using the amino acid substitution model chosen in the previous step. FigTree v. 1.4.3 (available at https://tree.bio.ed.ac.uk/software/figtree/) was used to perform tree visualization and export.

2.3. Drug target prioritization

A target prioritization approach was performed by constructing a protein network interaction (PNI) of *P. vivax* kinase proteins via STRING [23] v. 10.0 (http://string-db.org/) web server. The obtained PNI was used as input to Cytoscape, where we used a plugin named CytoNCA v. 3.3.0 [24], which allows the calculation of different centrality measures for PNIs. Even though the plugin can calculate several centrality measures, we used only degree centrality and subgraph centrality in the first step of our target prioritization. These centrality measures are well detailed elsewhere [24]. Both degree and subgraph centrality can highlight the local importance of a node within a network. After calculating and visually inspecting the values obtained for degree and subgraph centralities, we created a subset with the top-ranked nodes of the obtained PNI for *P. vivax* kinases. Afterwards, a second target prioritization approach was conducted based on an essentiality search where *P. vivax* kinases were selected when they were homologous to *P. falciparum* kinases with experimental lethal phenotypes after mutations, found at PlasmoDB (plasmodb.org).

2.4. Compound selection for experimental evaluation

The FASTA sequences of each prioritized target were used to interrogate two different publicly available databases that provide detailed information on drugs and their targets: DrugBank and Stitch. The search strategy was based on the principle of homology, where each query (*P. vivax* targets) was compared for matches to known drug targets contained in each database. We set a threshold of E-value $\leq 10^{-30}$ to consider the target as acceptable and we considered approved drugs, compounds in clinical trials and experimental compounds for this search. Then, a list of drugs/compounds and their possible targets was compiled. To investigate which of the predicted drugs have already been tested against *Plasmodium* species, we undertook a literature search using PubMed and SciFinder engines. The details of the search were: ("drug name" [MeSH Terms] OR "drug name" [All Fields]) AND ("Plasmodium" [MeSH Terms] OR "Plasmodium" [All Fields]). For

the remaining compounds, that have not been tested against Plasmodium, we predicted their antiplasmodial activity using a previously developed Quantitative Structure-Activity Relationships (QSAR) classification model for *P. falciparum* 3D7 and W2 strains [25]. The compounds predicted to be active by the QSAR models were purchased for *in vitro* experimental evaluation.

2.5. Experimental materials

All compounds experimentally evaluated were purchased from MolPort® and further diluted in DMSO to make a stock solution of 10 mM. Chloroquine was purchased from Sigma-Aldrich®.

2.6. Plasmodium falciparum in vitro culture

P. falciparum laboratory strains 3D7 and Dd2 were continuously cultivated using a modified candle jar method, as previously described [26]. Briefly, parasites were maintained in O^+ erythrocytes at 37 °C in a low oxygen atmosphere (5% CO_2, 5% O_2 and 90% N_2). The erythrocytes were suspended in RPMI-1640 medium supplemented with 0.05 mg/mL gentamycin and 10% A+ human serum in a final hematocrit of 5%. Parasitemia was monitored daily by Giemsa-stained smears. Synchronic ring-stage parasite cultures were obtained by two consecutive rounds of treatments with a 5% solution of D-sorbitol every 48 h. Blood bag supply was provided by the University of Campinas Blood Center and all experiments and procedures were approved by the Ethical Committee Research from UNICAMP (protocol number CAAE 15413019.5.0000.5404).

2.7. In vitro assays for P. falciparum growth inhibition

Parasite growth inhibition was determined in 96-well plates using ring stage synchronized parasites at 0.5% parasitemia and 2% hematocrit. Compounds were tested in duplicate using a 12-point serial dilution starting at 50 μM. Chloroquine was used as an antimalarial positive control. After 72 h of incubation, susceptibility of parasites to drugs was evaluated using the SYBR Green method [27]. Then, 100 μL of homogenized parasite culture was transferred to a new black 96-well plate together with 100 μL of lysis buffer (20 mM Tris, 5 mM EDTA, 0.008% wt/vol saponin, 0.08% vol/vol Triton X-100 and 0.4 μL/mL of SYBR Green) and incubated in the dark for 1 h. Afterwards, fluorescence reading was measured with aid of a microplate reader (CLARIOstar, Labtech BMG) at 490 nm excitation and 540 nm emission. EC_{50}s were calculated by plotting Log doses *versus* parasite growth inhibition (related to drug-free controls) in GraphPad Prism v.5 (GraphPad Software, La Jolla, California, USA). Results are expressed as mean of three independent assays.

2.8. Cytotoxicity assays

Cytotoxicity was evaluated in two mammalian cell lines: human hepatoma cells (HepG2) and fibroblast-like cells derived from monkey kidney tissue (COS-7) using the MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium chloride) reduction method [28]. The cells were cultivated in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 0.05 mg/mL gentamycin in a 5% CO_2 atmosphere at 37 °C. After harvest, cells were seeded in 96-well plates at 10^4 cells/well and incubated for 72 h in the presence of test compounds in serial dilutions starting at 100 μM. After incubation, MTT was added, and the optical density was measured at 570 nm (CLARIOstar, Labtech BMG). Cell viability was expressed as percentage of untreated control and CC_{50} values were obtained by plotting Log doses *versus* cell viability in GraphPad Prism v.5 (GraphPad Software, La Jolla, California, USA). Results represent at least three independent assays.

3. Results

3.1. Definition and prediction of Plasmodium species kinomes

Using Kinannote v.1.0 software, the *Plasmodium spp*. proteomes were screened for searching the whole set of kinases encoded by their respective sequences. Initially, the software provides an output containing the so called “draft” classification, which is the pre-curated kinome. By default, Kinannote algorithm classified sequences into groups, families, and subfamilies. Those proteins partially classified and those without a formal classification or undefined/unpredicted domains were labeled as “unclassified” kinases and further curated and classified through annotations gathered from PlasmoDB and InterproScan v.5.18 runs.

The number of proteins classified only at the group level reduced for all species, as they were further classified at family or subfamily levels. However, the number of proteins classified at group level increased if one compares the draft and the final classification, since unclassified proteins were classified after further curation and, consequently, were assigned to their respective kinase group. Table 1 shows an overview of the classification made by Kinannote (draft) and the final classification comparing our final classified kinome with the previously published *Plasmodium* spp. kinomes [29].

In addition, our approach resulted in a better classification of kinases into subfamily levels – [before classification/after classification] – e.g., *P. falciparum* [8/27], *P. berghei* [6/25], *P. knowlesi* [7/30], *P. ovale* [7/26], *P. malariae* [5/24], *P. chabaudi* [8/20], *P. yoelii* [7/23], *P. vivax* [8/26]. Our approach also reduced the initial number of unclassified proteins – *P. falciparum* [25/12], *P. berghei* [23/8], *P. knowlesi* [23/5], *P. ovale* [26/7], *P. malariae* [22/7], *P. chabaudi* [20/8], *P. yoelii* [22/5], *P. vivax* [25/12]. The work by [29] was the only one that systematically classified kinases into group, family, and subfamily levels. Most of the previous studies classified the kinases mainly into groups and/or subfamilies level [30–32], which was a bottleneck to the comparison between our work and others. Detailed information of each classified kinase can be found at Supplementary Materials.

3.2. Comparison of Plasmodium species kinomes

Our pipeline enabled the identification and classification of the kinome of eight *Plasmodium* species and, in general, all of them presented members of seven major kinases groups: AGC, CAMK, CK1, CMGC, STE, TKL or OTHER. FIKK kinases (Fig. 2), a species-specific kinase group of the Apicomplexan phylum, were found in all species, and was well represented in *P. falciparum*, with 21 proteins classified into this group. The largest group represented in all species was CAMK followed by CMGC and OTHER. No STE proteins were found in *P. berghei*, *P. chabaudi* and *P. yoelii*. Fig. 2 shows the number of proteins classified into major kinase groups for each *Plasmodium* species investigated in this work.

3.3. Phylogenetic tree construction

We have constructed a phylogenetic tree for each major kinase group of the whole set of *Plasmodium* species. From phylogenetic trees it is possible to infer those kinases sharing similarities in their catalytic sites and their common ancestor, allowing more inferences about the possible interaction between them and some inhibitors. Kinases classified into the same group were frequently at the same evolutionary branches in the phylogenetic tree, showing consistency with our classification pipeline. Figures showing each group’s phylogenetic tree are available on the supplementary material.

Table 1
Plasmodium spp. **kinome classification and comparison with previously published kinomes.** The classification into families and subfamilies was enriched and some kinases that were not classified in Kinannote were added.

Organism	Prediction approach	Group	Family	Subfamily	Unclassified	Total of Kinases
P. vivax	Kinannote	14	39	8	25	86
	Consensus	10	33	26	12	81
	Published*	8	35	10	12	65
P. falciparum	Kinannote	12	41	8	39	100
	Consensus	34	25	27	11	97
	Published*	30	35	9	19	93
P. berghei	Kinannote	11	38	6	23	78
	Consensus	12	31	25	8	76
	Published*	7	33	11	18	69
P. knowlesi	Kinannote	13	38	7	23	81
	Consensus	14	28	30	5	77
	Published*	7	36	9	13	65
P. malariae	Kinannote	10	44	5	22	81
	Consensus	13	34	24	7	78
P. ovale	Kinannote	12	39	7	26	84
	Consensus	14	33	26	7	78
P. chabaudi	Kinannote	13	39	6	20	78
	Consensus	16	31	22	8	77
	Published*	12	30	11	17	70
P.yoelii	Kinannote	10	38	7	22	77
	Consensus	12	36	23	5	76
	Published*	9	30	10	13	62

*[29].

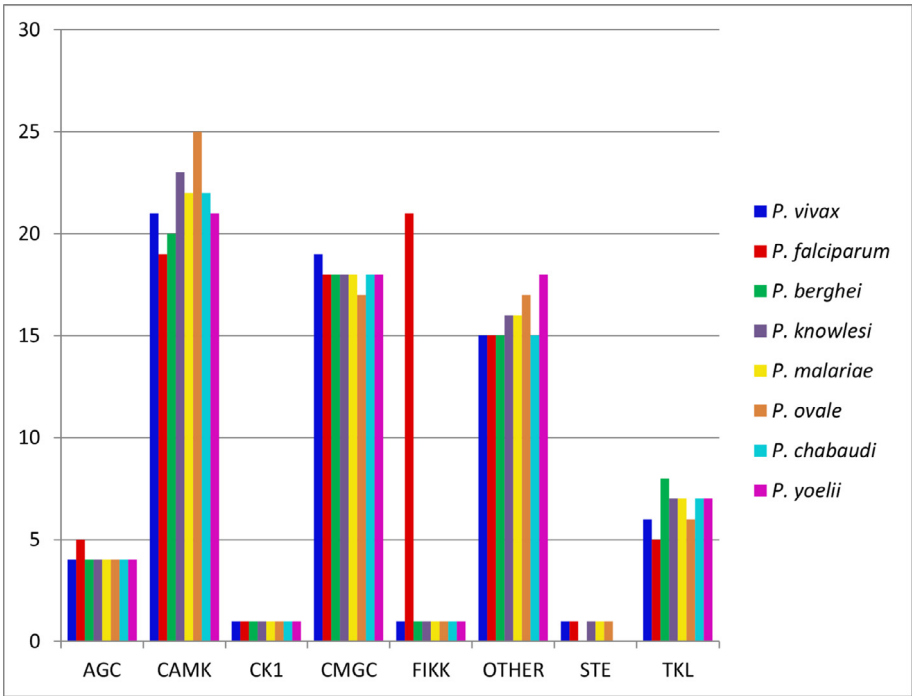


Fig. 2. Distribution of protein kinases of each *Plasmodium* specie into eight kinase groups.

3.4. Drug target prioritization

We conducted a target prioritization using *P. vivax*, the most distributed species of the genus and the most endemic species in Brazil. Also, combined drug therapies used for *P. falciparum* have failed in controlling *P. vivax* due to its ability to form hypnozoites, the latent stage of malaria. Since we can find homologous proteins in most of the species, we believe that the approach can be generalized to other species. The set of classified kinases of *P. vivax* was submitted to STRING v.10.0 (http://string-db.org/) web server. Next, the protein–protein interaction network obtained was the input to CytoNCA plugin implemented on Cytoscape, and the following centrality metrics were calculated: betweenness, closeness, and degree centralities. Based on the calculated values, a subset of 15 proteins was selected and prioritized as potential drug targets for *P. vivax*. Fig. 3 displays the network of prioritized targets.

A second strategy for target prioritization was done searching for experimentally validated essential *P. falciparum* kinases that were homologous to our set of *P. vivax* protein kinases. With this strategy, 27 kinases were prioritized and four among those were also prioritized in the string interaction network. Table 2 shows all prioritized kinases from both approaches. Among the 37 prior-

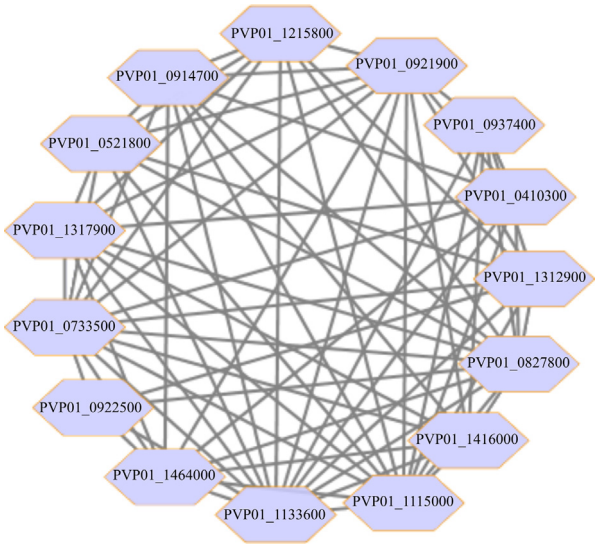


Fig. 3. Target prioritization approach. A protein–protein interaction network of *P. vivax* kinome proteins was constructed using the web server STRING and a sub-network was extracted using CytONCA, a Cytoscape plugin that calculates graph centrality measures. According to this criterion, the most important nodes were output into that graph, leading to the target selection.

itized targets using both approaches (Table 2), 13 belong to CMGC group, eight to CAMK group, six to OTHER group, four to AGC group, two to atypic protein kinase group (RIO, PI4K), one to TKL group, one to CK1 group and one orphan kinase (Protein kinase 7 - PK7). One kinase classified as essential was not classified into any group by our kinome classification approach (unclassified).

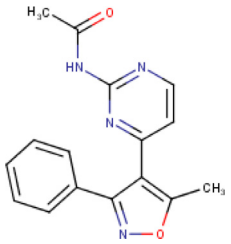
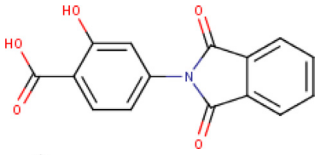
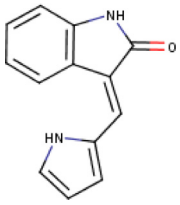
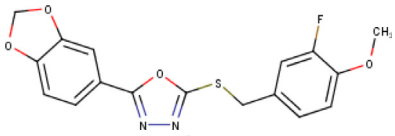
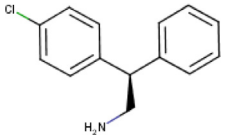
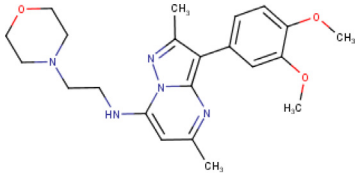
3.5. Compound selection for experimental evaluation

The FASTA sequences of the 37 prioritized targets were used to search for known drug targets in the publicly available databases DrugBank and TDR targets using an E-value threshold of 10^{-30}. This analysis resulted in the prediction of 15 targets associated with 1,884 drugs. The 1,884 drugs were then submitted to a virtual screening using previously developed QSAR models [25] for the prediction of their inhibitory activity against *P. falciparum* 3D7 and W2 strains. A literature search was conducted for the 71 compounds predicted as active (inhibitors) by both ML models, to verify if they had already been tested against *Plasmodium* spp. After the literature search, six compounds that were not tested as antimalarials were selected for experimental evaluation. The selected compounds were related to five out of the 37 prioritized targets: two compounds for *P.vivax*' NimA related kinase 1 (*Pv*NEK1) (homolog protein on DrugBank = NimA related kinase 1 - NEK2), one compound for *P.vivax*' 1-phosphatidylinositol 4 kinase (*Pv*PIK4) (homolog protein on DrugBank = phosphatidylinositol 4 kinase beta - PI4KB), one compound for *Pv*PKA (homolog protein on DrugBank = Cyclic adenosine 3′,5′-monophosphate (cAMP) - dependent protein kinase catalytic subunit alpha), one compound for *Pv*CK1 (homolog protein on DrugBank = CK1), and one compound for *Pv*GSK3 (homolog protein on DrugBank = GSK3). The overall results of DrugBank and TDR targets queries are available in Table 3.

Table 2
Prioritized targets for *P. vivax* from essentiality and network centrality approaches.

ID P. vivax PlasmoDB	Name	Kinase classification (group/family/subfamily)	Target prioritization approach
PVP01_1312900	Protein Kinase G (PKG)	AGC/PKG	STRING
PVP01_0827800	Glycogen Synthase Kinase 3 (GSK3)	CMGC/GSK/GSK3	STRING
PVP01_0733500	Protein Kinase A (PKA)	AGC/PKA	STRING/ Essentiality
PVP01_0914700	Mitogen Activated Protein Kinase 2 (MAPK2)	CMGC/MAPK/MAPK2	STRING
PVP01_1317900	Mitogen Activated Protein Kinase 1 (MAPK1)	CMGC/MAPK/MAPK1	STRING
PVP01_0921900	Tyrosine Kinase Like 2 (TLK2)	TKL/TKL2	STRING
PVP01_1115000	Protein Kinase 5 (PK5)	CMGC/CDK/CDC2	STRING
PVP01_1133600	Cysteine-rich RLK (receptor-like protein kinase) 5 (CRK5)	CMGC/CDK	STRING
PVP01_1215800	–	CMGC/CDK	STRING
PVP01_0521800	Cysteine-rich RLK (receptor-like protein kinase)4 (CRK4)	CMGC/CDK	STRING/ Essentiality
PVP01_0410300	–	CAMK	STRING/ Essentiality
PVP01_0937400	Caseine Kinase 1 (CK1)	CK1/CK1/CK1-D	STRING/ Essentiality
PVP01_1416000	Protein Kinase 9 (PK9)	CAMK/CAMKL	STRING
PVP01_0922500	–	AGC/PDK1	STRING
PVP01_1464000	Protein Kinase B (PKB)	AGC/AKT	STRING
PVP01_0313900	–	Unclassified	Essentiality
PVP01_0313300	Cyclin Dependent Protein Kinase 4 (CDPK4)	CAMK/CDPK/CDPK4	Essentiality
PVP01_0407500	Cyclin Dependent Protein Kinase 1 (CDPK1)	CAMK/CDPK/CDPK1	Essentiality
PVP01_0411500	Protein Kinase 7 (PK7)	PK7	Essentiality
PVP01_1223600	–	CMGC/CDK	Essentiality
PVP01_1216900	Cyclin Dependent Protein Kinase 5 (CDPK5)	CAMK/CDPK/CDPK5	Essentiality
PVP01_0108600	BUD32	OTHER/BUD32	Essentiality
PVP01_0511300	Vacuolar Protein Sorting 15 (VPS15)	OTHER/VPS15	Essentiality
PVP01_0529500	RIO2	Atypical/RIO/RIO2	Essentiality
PVP01_0909200	Casein Kinase 2 (CK2)	CMGC/CK2	Essentiality
PVP01_0915400	CDC-like kinase 3 (CLK3)	CMGC/DYRK/PRP4	Essentiality
PVP01_0923700	Cyclin Dependent Protein Kinase7 (CDPK7)	CAMK/CDPK/CDPK7	Essentiality
PVP01_0945900	–	CAMK/CAMKL	Essentiality
PVP01_0948600	–	OTHER/TLK	Essentiality
PVP01_0814500	MLK(mixed lineage kinase) – related kinase (MRK)	CMGC/CDK	Essentiality
PVP01_0822900	Cysteine-rich RLK (receptor-like protein kinase)4 (CRK4)	CMGC/CDK	Essentiality
PVP01_1024200	phosphatidylinositol 4 kinase (PI4K)	Atypical/PI4K	Essentiality
PVP01_0724600	–	OTHER	Essentiality
PVP01_1261600	CDC-like kinase 1 (CLK)	CMGC/CLK	Essentiality
PVP01_0831000	Aurora Kinase 2 (ARK2)	OTHER/AUR	Essentiality
PVP01_0830100	Cyclin Dependent Protein Kinase 3 (CDPK3)	CAMK/CDPK/CDPK3	Essentiality
PVP01_1446500	NimA related kinase 1 (NEK1)	OTHER/NEK	Essentiality

Table 3
Compounds prioritized and selected for experimental evaluation.

#	IUPAC name	Structure	Prioritized Target (classification)	Related target (organism)
(1)	*N*-[4-(5-methyl-3-phenyl-1,2-oxazol-4-yl)pyrimidin-2-yl] acetamide		PVX_092440 (CK1/CK1/CK1-D)	CK1-delta (*Rattus norvegicus*)
(2)	4-(1,3-dioxoisoindol-2-yl)-2-hydroxybenzoic acid		PVX_124045 (OTHER/NEK/NEK1)	NEK2 (*H. sapiens*)
(3)	3-(1*H*-pyrrol-2-ylmethylidene)-1*H*-indol-2-one		PVX_124045 (OTHER/NEK/NEK1)	NEK2 (*H. sapiens*)
(4)	2-(1,3-benzodioxol-5-yl)-5-[(3-fluoro-4-methoxyphenyl) methylsulfanyl]-1,3,4-oxadiazole		PVX_119725 (CMGC/GSK/GSK3)	GSK3 (*H. sapiens*)
(5)	(2*R*)-2-(4-chlorophenyl)-2-phenylethanamine		PVX_086975 (AGC/PKA)	PRKACA (*H. sapiens*)
(6)	3-(3,4-dimethoxyphenyl)-2,5-dimethyl-*N*-(2-morpholin-4-ylethyl)pyrazolo[1,5-a]pyrimidin-7-amine		PVX_098050 (Atypical/PI4K)	PI4KB (*H. sapiens*)

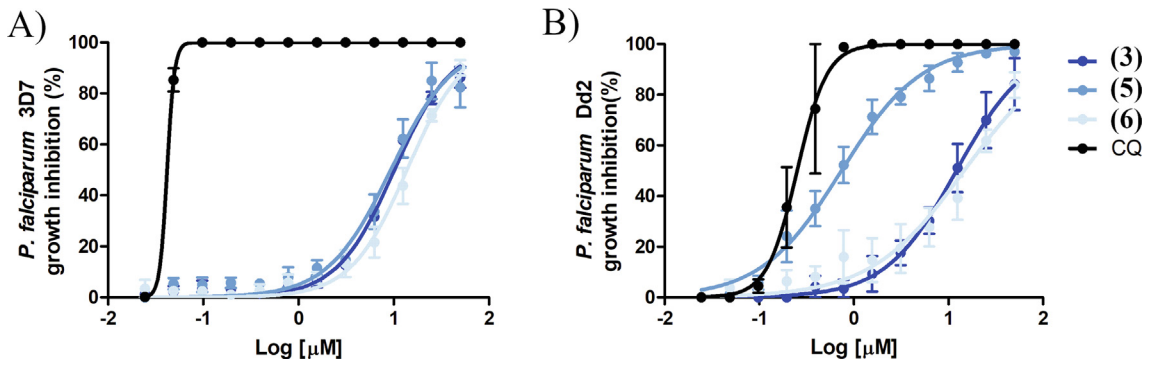


Fig. 4. *In vitro* antimalarial activity of selected compounds against *P. falciparum*. (A) Inhibition curves *in vitro* against chloroquine-sensitive (3D7) (B) and multidrug-resistant (Dd2) *P. falciparum* strains. Data are derived from three independent experiments. CQ = Chloroquine.

3.6. Experimental evaluation

The compounds selected for experimental evaluation were tested against *P. falciparum* 3D7 (chloroquine-sensitive) and Dd2 (multidrug-resistant) strains. Initially, a phenotypic screening at drug concentrations of 5 μM was carried out with the chloroquine-sensitive strain. Three compounds (**(3)**, **(5)**, and **(6)**) inhibited above 30% of parasite growth at this concentration. The

Table 4
Experimental results for the selected compounds tested against chloroquine-sensitive (3D7) and multidrug-resistant (Dd2) *P. falciparum* strains (EC_{50}), and cytotoxicity (CC_{50}) on mammalian cell lines (COS-7 and HepG2).

	EC_{50} (μM)[a]		CC_{50} (μM)[b]	
	*Pf*3D7	*Pf*Dd2	COS-7	HepG2
(3)	10.3 ± 1.3	9.8 ± 1.1	32.8 ± 10.8	13.5 ± 7.6
(5)	9.9 ± 2.5	0.8 ± 0.4	42.8 ± 7.3	44.3 ± 5.2
(6)	13.8 ± 3.8	17.9 ± 5.3	>100	>100
Chloroquine	0.009 ± 0.0	0.057 ± 0.0	ND	ND

[a] EC_{50}: half of the maximum inhibitory concentration in 3D7 and Dd2 strains and their respective standard deviations.
[b] CC_{50}: half the maximum cytotoxic concentration in mammalian cells; ND: not determined. Data derive from at least three independent experiments.

other compounds tested showed poor antiplasmodial activity. Therefore, compounds **(3)**, **(5)**, and **(6)** were further tested to evaluate their EC_{50} values in both parasite strains. While these compounds showed EC_{50} around 10 μM in the 3D7 strain (Fig. 4A), compound **(5)** was able to inhibit the growth of drug-resistant strain (Dd2) in a micromolar scale (0.8 μM; Fig. 4B, Table 4). Moreover, these compounds were tested for their cytotoxicity, and they were not toxic to HepG2 and COS-7 cells, with good CC_{50} values (Table 4).

4. Discussion

Our approach has enabled the update of the kinomes of six *Plasmodium* species and the elucidation for the first time of the kinomes of *P. ovale* and *P. malariae,* two species that also infect humans. Previous studies reported the kinomes of *P. falciparum, P. yoelli, P. berghei, P. chabaudi, P. vivax,* and *P. knowlesi* [29–33] and a comparative analysis of these and ours is available at supplemental material (Supp. Table S1). Overall, our approach was able to identify a higher number of kinases with deeper level of classification for a larger number of *Plasmodium* species among all previous similar works. Moreover, we were able to classify many of the unclassified proteins and in many cases, the kinases were classified until the deeper level of subfamily. Here, we used a more robust approach, that might be useful for further classification, annotation and more importantly as a starting point for understanding *Plasmodium* kinases as drug targets.

We constructed phylogenetic trees for each kinase group to see if our approach correctly classified kinases from different species into the same branches, showing that they are orthologous kinases and probably have the same roles in the parasite cells. As an example, all nine Protein Kinase G (PKG), one from each species, are from the same common ancestor at the AGC group, belonging to the same branch in the phylogenetic tree (Figure S1, Supplemental Material).

When comparing the kinomes between the *Plasmodium* species, we saw that they have a similar number of kinases in total and that they are also similarly distributed among the kinase groups. The bigger kinome among *Plasmodium* spp. species was that from *P. falciparum*, with 97 kinases at the final classification, while the smaller ones were that from *P.berghei* and *P. yoelii*, with 76 kinases at the final classification. The most representative groups among the species were CAMK, CMGC, OTHER and TKL, while the least representative groups were CK1, FIKK, STE and AGC. An interesting kinase group was FIKK, which is a unique Apicomplexa kinase group. All the species presented only one FIKK protein, while *P. falciparum* presented 21 kinases belonging to the FIKK group. The proteins from this group are known to be secreted into the erythrocytes and to remodel their surface mediating virulence-associated changes in *P falciparum* [34]. Another interesting kinase in *Plasmodium* spp. is PK7. This protein does not follow the HMM signature of any kinase group. In fact, it is a composite, having regions of similarity with many different kinase families, like Mitogen Activated Protein Kinase Kinase (MAPKK, C-terminous similarity) and fungal PKA (*N*-terminus similarity) [35]. For this reason, we did not assign this protein to any group, turning it into an orphan kinase.

Finally, our target prioritization approach led to the identification of 37 potential drug targets in *P. vivax* kinome. A recent perspective on *Plasmodium* kinases as drug targets [36] has discussed some features that could contribute to the selection of good kinase drug targets. This includes (1) the essentiality and vulnerability across different stages of the parasite life cycle; (2) druggability and mode of inhibition; (3) conservation across different *Plasmodium* species and potential for pan-species activity; (4) homology with human kinases and the potential for selectivity; (5) stage specificity, speed of parasite killing, and potential to fulfill target product profile (TCP) requirements; (6) a propensity for resistance; (7) the availability of target-based assays; (8) the availability of structural information. Comparing these features to our prioritized targets, in one of our strategies, kinases where selected if they had experimental evidence of essentiality, fulfilling the first criteria for good kinase targets. Further, many of the selected targets were also pointed as essential to *P. falciparum* erythrocytic schizogony [37]. All the 37 *P. vivax* selected targets have their orthologues in *P. falciparum* kinases belonging to the same OrthoMCL group, fulfilling the third criteria. The fifth criteria was also addressed since many of our selected targets are highly expressed in different *Plasmodium* life stages, including four genes with increased expression (>1.5 fold change) in *P. vivax* hypnozoite stage: PK7, Casein Kinase 2 (CK2, BUD32, and RIO2 [38]. Also, the kinases PKG, CLK3 and PI4K have inhibitors that showed activity in assays testing for asexual blood-stage, transmission-blocking, and prophylactic liver stage antiplasmodium activity [36]. Five of our selected targets have their structures experimentally resolved: PKG (PDB IDs = 4RZ7, 5DYL, 5DZC, 5EZR, 5F0A, 5FET, 5DYK, 5E16, 4OFF, 4OFG), MAPK2 (PDB ID = 3NIE), PK5 (PDB IDS = 11V0B, 1V0O, 1OB3, 1V0P), CDPK4 (PDB IDS = 4RGJ, 4QOX), and CK2 (PDB ID = 5CVU), fulfilling the eighth criteria. *P. falciparum* phosphatidylinositol 4-kinase (*Pf*PI4K) is ubiquitously present in all life stages of the parasite, catalyzing the conversion of phosphatidylinositol to phosphatidylinositol-4-phosphate (PI4P) [39,40]. PI4K is involved in a plethora of cellular processes such as cytokinesis, intracellular signaling, transmembrane transport, and is known as crucial for merozoite development [41]. The strong evidence (genetic, phenotypic and clinical validation, and *in vivo* efficacy) [11] that supports PI4K as an important and validated biological target within the kinome of *P. falciparum* also guided the development of a new set of inhibitors, one of them, MMV390048 [41], is currently undergoing Phase IIa of clinical trials in humans. Despite its success, MMV390048 has reportedly poor solubility and selectivity issues [42,43], which still drives efforts towards both the optimization of known inhibitors and the identification of new potent and selective inhibitors of PI4K of *Plasmodium* [44–51].

Furthermore, our analysis allowed the identification of 1,884 (drugs and investigational compounds from DrugBank and Stitch

databases) associated with those prioritized targets, that could be worth of experimentally validation. For filtering this list down, we predicted the activity against 3D7 and W2 strains of *P. falciparum* using previously developed QSAR models, and this allowed for the identification of 71 compounds, that could be tested against *Plasmodium*. Then, after literature search, six compounds that were not tested as antiplasmodial were selected for experimental evaluation.

The literature search further validated our chemogenomics and bioinformatics approach since some of the selected compounds have been already tested and presented good potency. One example is indirubin-3′-monoxime, a compound predicted to inhibit the *Plasmodium* kinase GSK3, that has been already tested against *Pf*GSK3, with EC_{50} of 22 nM [52]. Nilotinib was tested against *Pf*CDPK1 and inhibited the enzyme with K_D = 0.79 μM [53]. The compound SB203580, a human p38 inhibitor was able to inhibit *P. falciparum* W2 strain with EC_{50} of 12 nM [54]. Therefore, we were confident that our chemogenomics strategy for identifying new antimalarial drugs is valid.

5. Conclusion

This study reported an utmost contribution for future antiplasmodial drug discovery as it reports the update of the kinomes of six *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. berghei*, *P. yoelii*, and *P. chabaudi*, *P. knowlesi*) as well as the elucidation for the first time the kinomes of other two *Plasmodium* species that can cause malaria in humans (*P. ovale* and *P. malariae*), corroborating the data sets published up to now and improving the classification and filling this important gap in the literature. The integrated chemogenomics and bioinformatics pipeline developed here guided the deeper classification of kinases to the subfamily level, serving also as input to target prioritization and subsequent selection of candidates for testing. Finally, we predicted 37 protein kinases that have the potential to be good drug targets for *P. vivax*. This information can be useful for the discovery of new antimalarial compounds. A subset of protein kinases that are essential for the parasite's survival or were central in a protein interaction network was selected as well as some compounds that might inhibit those proteins. Three compounds showed moderate antiplasmodial activity that could be the start-point for further optimization in future studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.07.003.

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