

Identification of potential inhibitors of casein kinase 2 alpha of *Plasmodium falciparum* with potent *in vitro* activity

Kaira C. P. Tomaz,¹ Tatyana A. Tavella,¹ Joyce V. B. Borba,^{1,2} Luis C. Salazar-Alvarez,¹ João E. Levandoski,³ Melina Mottin,² Bruna K. P. Sousa,² José T. Moreira-Filho,² Vitor M. Almeida,⁴ Leandro C. Clementino,¹ Catarina Bourgard,^{1,5} Katlin B. Massirer,⁴ Rafael M. Couñago,^{4,6} Carolina H. Andrade,^{2,7,8} Per Sunnerhagen,⁵ Elizabeth Bilsland,⁹ Gustavo C. Cassiano,^{1,10} Fabio T. M. Costa¹

AUTHOR AFFILIATIONS See affiliation list on p. 13.

ABSTRACT Drug resistance to commercially available antimalarials is a major obstacle in malaria control and elimination, creating the need to find new antiparasitic compounds with novel mechanisms of action. The success of kinase inhibitors for oncological treatments has paved the way for the exploitation of protein kinases as drug targets in various diseases, including malaria. Casein kinases are ubiquitous serine/threonine kinases involved in a wide range of cellular processes such as mitotic checkpoint signaling, DNA damage response, and circadian rhythm. In *Plasmodium*, it is suggested that these protein kinases are essential for both asexual and sexual blood-stage parasites, reinforcing their potential as targets for multi-stage antimalarials. To identify new putative PfCK2α inhibitors, we utilized an *in silico* chemogenomic strategy involving virtual screening with docking simulations and quantitative structure-activity relationship predictions. Our investigation resulted in the discovery of a new quinazoline molecule (**542**), which exhibited potent activity against asexual blood stages and a high selectivity index (>100). Subsequently, we conducted chemical-genetic interaction analysis on yeasts with mutations in casein kinases. Our chemical-genetic interaction results are consistent with the hypothesis that **542** inhibits yeast Cka1, which has a hinge region with high similarity to PfCK2α. This finding is in agreement with our *in silico* results suggesting that **542** inhibits PfCK2α via hinge region interaction.

KEYWORDS malaria, antimalarials, virtual screening, docking, quinazoline

Malaria is an ancient parasitic disease characterized by high intermittent fevers. The disease results from infection by protozoa of the genus *Plasmodium* and transmitted to humans through the bite of female *Anopheles* mosquitoes. For thousands of years, malaria has been one of the most impactful public health problems in tropical and subtropical areas of the world. According to the latest World Malaria Report from the World Health Organization, the malaria mortality rate decreased by about 50% between the period of 2000 and 2015, falling from 30 to 15 deaths per 100,000 people at risk. In the following years, mortality continued to fall but at a slower rate, reaching 13 deaths per 100,000 populations in 2019 (1). In 2020, however, the mortality rate rose again and fell back to the level observed 5 years earlier (1). The global decline in malaria cases and deaths over the last two decades can be attributed to vector control strategies, rapid diagnosis, and early treatment of patients. However, resistance to commonly used antimalarials and insecticides is a key factor contributing to the slower decline in malaria mortality in recent years (1). Hence, the development of additional antimalarial drugs is critical, especially those with novel biological targets.

Protein kinases have been studied as potential targets for the development of new drugs, as these proteins are important regulators of a variety of cellular process such as metabolism, proliferation, cell motility, apoptosis, and differentiation (2). Following

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Address correspondence to Gustavo C. Cassiano, gcapatti@hotmail.com, or Fabio T. M. Costa, fabiotmc72@gmail.com.

Kaira C. P. Tomaz and Tatyana A. Tavella contributed equally to this article. The order of authors was determined by each individual's contribution to the study.

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genome sequencing and protein characterization of the primary malaria-causing species, at least 85 protein kinases have been identified in the *Plasmodium* kinome (3, 4). As most of these proteins have been shown to be essential to all stages of the *Plasmodium* life cycle, protein kinases have been highlighted as potential candidates for the development of prophylactic, curative, and transmission-blocking drugs. Today, several protein kinase inhibitors are under development as antimalarial drugs, with one molecule (MMV390048) in phase II trials (5).

Casein kinases are ubiquitous serine/threonine kinases that were first named based on their ability to phosphorylate casein *in vitro*. This family of kinases can be subdivided further into two groups: casein kinase 1 (CK1) and casein kinase 2 (CK2). CK1 is a small family within the kinome, with seven isoforms in mammals (α , β , γ_1 , γ_2 , γ_3 , δ , and ϵ) (6), displaying a highly conserved kinase domain. Members of this family are involved in a wide range of cellular processes such as mitotic checkpoint signaling (7), DNA damage response (8), Wnt signaling (9), and circadian rhythm (10). CK2 is a constitutively expressed serine/threonine protein kinase composed of two alpha catalytic (CK2 α , encoded by *CSNK21*, and CK2 α' , encoded by *CSNK22*) and two beta regulatory (CK2 β and CK2 β') subunits and has been implicated in cell cycle regulation (11), apoptosis (12), and DNA repair (13).

Unlike the human tetrameric CK2, *PfCK2* is formed by one single catalytic (α) and two regulatory (β_1 and β_2) subunits. The activation mechanism of *PfCK2* also appears to be different from its human counterpart: *PfCK2* undergoes autophosphorylation at Thr⁶³, a residue located in domain I of the catalytic subdomain, but not at a conserved Tyr¹⁸⁶, located within the activation loop (14). *In vitro* phosphorylation of chromatin proteins by *PfCK2* α , added to the wide distribution of this enzyme in the nucleus of parasite, indicates that *PfCK2* is involved in chromatin assembly and dynamics (15). Failure to disrupt *pfck2a* using a reverse genetic approach suggests that this protein kinase is essential for the asexual blood stage of development (16, 17). Importantly, *PfCK2* has recently been shown to be essential for both asexual and sexual blood-stage parasites, as conditional knockdown of *PfCK2* α prevented the transition of stage IV gametocytes into transmission-competent stage V parasites, reinforcing its potential as a target for multi-stage antimalarials (18).

Despite the importance of *PfCK2* to the biological cycle of malaria parasites, no specific and potent inhibitor against this protein kinase has yet been reported. Although there is high sequence identity between parasite CK2 and their human counterpart, some marked differences in the protein structures could be favorable in the search for specific inhibitors. Recently, the crystal structure of *PfCK2* α complexed with ATP was solved, providing insights into the structural basis for inhibition. Some amino acid substitutions in the vicinity of where the adenine base binds promote changes in the ATP-binding pocket (19).

Here, we report a strategy for discovering new putative inhibitors of *PfCK2* α using an integrated structure- and ligand-based virtual screening with docking simulations through three-dimensional (3D) models of the protein's structure predicted by AlphaFold and quantitative structure-activity relationship (QSAR) models for predicting antiplasmodial activity of the top-ranked virtual hits. Subsequently, 168 compounds were finally selected for experimental validation, and 11 of these compounds exhibited >50% inhibition of the growth of *Plasmodium falciparum* asexual blood-stage at 1 μ M. Among the compounds, a quinazoline (**542**) presented the highest selectivity index (SI) and was chosen for chemical-genetic interaction assays for target validation.

MATERIALS AND METHODS

PfCK2 α structural predictions

The structure of *PfCK2* was available at the AlphaFold protein structure database (20). Quality assessment was performed using MolProbity Server (21).

Protein and ligand preparation

The chemical structures were retrieved from ChemBridge database (DiverSet, with approximately 50,000 compounds). Ligands were prepared using the LigPrep tool (Schrödinger Release 2019-4: LigPrep; Schrödinger, LLC, New York, NY) with OPLS2005 force field. All possible ionization states were generated for each ligand at pH 7.5 $\pm$ 0.5 using Epik. The identification of protein binding sites is necessary to define the grid center for docking studies. Thus, we identified these sites with the KLIFS database (<https://klifs.net/>) (22), which provides information about the residues of the catalytic site of human kinases. By aligning the amino acid sequences of the orthologs with the *Plasmodium* kinases, we identified the active site of our target protein. The PockDrug web server (23) was used for the identification of pockets of the protein that are “druggable” (ability of a molecule to bind to a certain region of the protein) using geometry, hydrophobicity, and aromaticity as parameters (23). The modeled structure of the PfCK2 was imported using Protein Preparation Wizard tool (Schrödinger Release 2019-4: Protein Preparation Wizard; Schrödinger, LLC). First, hydrogen atoms were added, and protonation states were adjusted at pH 7.5. Finally, a system energy minimization was performed using the OPLS-2005 force field. The receptor grid was generated using the Receptor Grid Generation tool and centered at the ATP-binding site.

Virtual screening

Virtual screening was performed in three steps by using Glide software v.11.2. First, the data set was carefully curated via the following: exclusion of salts and metals, pKa normalization, Lipinski's rule of 5 (24), Veber filter (25), and removal of undesirable properties, such as PAINS and aggregators, using the FILTER software (v.2.2.1, Openeye Scientific Software). Duplicates were detected and removed using ISIDA Duplicates (26). We screened all compounds via the high-throughput virtual screening (HTVS). In the next step, the compounds that presented the lowest docking scores from HTVS were evaluated by standard precision (SP) docking process. In the third stage, the top lowest docking score compounds from SP docking are retained and further evaluated using extra-precision docking to obtain the top molecules, which were ranked based on the lowest docking score. The Pymol software (The PyMOL Molecular Graphics System, v.1.2r3pre; Schrödinger, LLC) was used for the visual inspection of 3D docking poses and to help the identification of non-covalent interactions among proteins and ligands.

The top molecules were then submitted to QSAR models based on deep learning previously published by our group (27). Finally, the compounds predicted as actives against *P. falciparum* (3D7 models) were selected for experimental validation.

Drug activity assays against *P. falciparum* asexual blood stage

The *P. falciparum* chloroquine-sensitive (3D7) and multi-drug resistant (Dd2) strains used in this study were cultured at 4% hematocrit in type O + human red blood cells (Hematology Center of University of Campinas) and maintained at 37°C in an atmosphere containing 1% O₂, 5% CO₂, and 94% N₂, in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% pooled A⁺ human serum (Hematology Center of University of Campinas) (28). Drug inhibition assays were performed as previously described (29). Briefly, D-sorbitol synchronized ring cultures (0.5% parasitemia and 2% hematocrit) (30) were plated in 96-well plates in the presence of different concentrations of the compounds (5.0–0.002 μ M), in the drug vehicle dimethyl sulfoxide (DMSO). After 72 hours of incubation, parasitemia was assessed by fluorometry using SybrGreen fluorescent dye. EC₅₀ values were calculated by plotting log dosing vs growth inhibition (expressed as percentage relative to the drug-free control).

Cytotoxicity assays in HepG-2 and MCF-7 cells

Cytotoxicity of compounds was assessed by PrestoBlue and MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium chloride] reduction assay for quantification of cellular

reductase enzyme activity as an indirect measurement of cell viability using human epithelial breast cancer cell line (MCF-7), human hepatocarcinoma cell line (HepG-2), and fibroblast-like cell lines derived from monkey kidney tissue (COS-7 cells) (31). Briefly, cells were cultivated at 5% CO₂ and 37°C using Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum. For this experiment, cells were seeded at a density of 10⁴ cells/well in a 96-well plate prior to incubation with a serial dilution of compounds of interest and controls for 24 hours (MCF-7 and HepG-2 cells) or 72 hours (HepG-2 and COS-7 cells). After drug treatment, MCF-7 or HepG-2 cells were next incubated with resazurin (PrestoBlue) for 1 hour followed by formation of resorufin by viable cell reductase enzymes (32). Fluorescence reading was measured by POLARstar Omega microplate-reader at 544-nm excitation and 590-nm emission. For HepG-2 and COS-7 cells after 72-hour drug treatment, MTT was added to the wells and absorbance reading was performed on a CLARIOstar plate reader (BGMtech) at a wavelength of 570 nm (OD570). Cellular viability was expressed as a percentage relative to vehicle treated control. The CC₅₀ was defined as the concentration that reduced the fluorescence or absorbance of treated cells to 50% when compared to non-treated controls.

Yeast chemical-genetic interactions

Chemical-genetic interactions were investigated using haploid deletion strains from the commercial yeast deletion library (Yeast Knockout Collection Haploid Mat-a/*Trans*OMIC technologies). Strains selected for these assays were *cka1Δ* (deletion of the gene encoding the alpha catalytic subunit of casein kinase 2); *cka2Δ* (deletion of the gene encoding the alpha' catalytic subunit of casein kinase 2); *eap1Δ* (deletion of the gene encoding the eIF4E-associated protein, which has a positive genetic interaction with *cka1Δ* and a negative genetic interaction with *cka2Δ*); and *his3Δ* (wild-type control with the same antibiotic resistance markers as the test strains). Data for genetic interactions were taken from the Global Yeast Genetic Interaction Network (<https://thecellmap.org/>) (33). After preparing the YPD medium (1% Bacto yeast extract, 2% Bacto peptone, and 2% glucose), we grew individual strains at 30°C in 2.5 mL of YPD medium in 15-mL conical tubes until saturation. The yeast cultures were diluted to OD₆₀₀ of 0.2 for chemical-genetic assays (2× final cell density). Compound **542** stored in DMSO at a concentration of 10,000 μM was diluted in the culture medium (YPD) with 2% glucose to a concentration of 100 μM. The same volume as DMSO was diluted in a culture medium used as the negative control. The OD of each strain was adjusted to 0.2. The experiment was conducted in a 384-well plate with a total volume of 70 μL in each well. The plate assembly was done to preserve the humidity of the environment, so the columns and external lines were filled with only culture medium, forming a humid environment and avoiding losses by evaporation. In the center, each strain was placed in triplicate for the control and compound **542**. Each well was placed with 35 μL of culture with OD of 0.2 and 35 μL of the solution containing the compound **542** or DMSO as the negative control, forming a suspension with OD of 0.1- and 50.0-μM inhibitor concentration.

The findings of this study, which pertain to chemical-genetic interactions in Cka1 yeast, are backed by data that are openly accessible on the Mendeley Data public database at <https://data.mendeley.com/datasets/zc8kxkr5vh/1>.

Human protein kinase selectivity panel

The human kinase selectivity panel used here is based on the displacement of a fluorescent tracer by competing test compounds. When the tracer binds to the kinase ATP-binding pocket, a high TR-FRET signal is obtained (excitation at 340 nm and emission at 665/615 nm). When test compounds compete with the fluorescent tracer for the kinase ATP-binding pocket, a lower TR-FRET signal is detected. Fluorescent tracers used were T236, T178, T199, T222, and T1710, all purchased from Thermo Fischer Scientific. Proteins in the selectivity panel were expressed in-house using plasmid pNIC-Bio3 for expression in *Escherichia coli* and pFB-Bio5 for expression in insect cells.

These vectors introduced a C-terminal Avi-tag sequence for the lysine biotinylation (GLNDIFEAQKIEWHE) of the recombinant protein. For the expression in *E. coli*, each target kinase was co-expressed with BirA biotin ligase previously cloned into the plasmid pCDF-BirA, which confers resistance to spectinomycin. For Sf9 cells, the biotinylation was performed by a secreted BirA, which was co-expressed along with the target kinase. In both expression systems, 0.14-mM biotin was added to the cell culture medium. Purified, biotinylated kinases were incubated for an hour at room temperature with Eu-Streptavidin (Thermo Fisher, #PV6025), the appropriate fluorescent tracer containing Alexa Fluor-647, and the test compounds. Final concentrations used in the assay were protein, 15 nM; tested compounds, 1 μ M; control inhibitors (staurosporine for T222 and T236, dasatinib for T178, and SB-202190 for T199), 10 μ M. Data were collected using a ClarioStar plate reader (BMG LabTech) and normalized to 0–100% tracer bound based on negative (0% bound, 10- μ M reference inhibitor) and positive (100% bound, DMSO only) controls.

RESULTS AND DISCUSSION

Sequence analysis and alignment

The primary sequence of CK2 α proteins was extracted from the UniProt database. Human CK2 α and CK2 α' are highly conserved (88.5% identity), and the amino acid sequence alignments of *Plasmodium* casein kinases with their human closest orthologue showed high identity: 68.8% (*Pf*CK2 α to *h*CK2 α) and 68.2% (*Pv*CK2 α to *h*CK2 α). The sequence identity between *Pf*CK2 α and *Pv*CK2 α is 97%. Highly conserved regions among protein kinase subdomains are present in *Pf*CK2 α (Fig. 1). We can highlight the glycine-rich loop, characterized by the motif GXGXXG in subdomain I (the third glycine is substituted by a serine) and the invariant lysine in subdomain II, which interacts with the α -phosphate and β -phosphate of ATP. The glutamate in subdomain III, which forms a salt bridge with the invariant lysine of subdomain II, the catalytic loop HRDXXXXN in subdomain VIb, which contains the essential aspartate that is the catalytic residue acting as a base acceptor, are also overlapped in both sequences. Unlike other protein kinases, both human and parasite CK2 α feature a DWG motif in the subdomain VII rather than a DFG motif: the glutamate and the arginine, localized in subdomains VIII and XI, respectively, which form a salt bound that provides structural stability of the C-terminal lobe, and the aspartate in subdomain IX, which forms hydrogen bonds with the backbone to stabilize the structural stability of the catalytic loop of subdomain VI (Fig. 1).

*Pf*CK2 α three-dimensional modeling

Three-dimensional structures are needed to conduct docking calculations. By the time our studies took place, there was only one crystal structure resolved for *Pf*CK2 α (PDB ID: 5XVU) (19), corresponding to a D179S inactive mutant. Due to this mutation at the DWG motif of catalytic binding site, we used the *Pf*CK2 α 3D structure modeled with Asp179 instead of Ser179 that was deposited in the AlphaFold (20) protein structure database (<https://alphafold.ebi.ac.uk/>, ID = Q8IIR9). The per-residue confidence score (based on the predicted local-distance difference test) for most residues was very high (>90) and was only low (<60) in the N- and C-terminal regions. The statistical quality of the models was evaluated using MolProbity (Table 1).

Virtual screening

To identify potential inhibitors of *Pf*CK2 α , we performed independent and parallel structure-based virtual screening (SBVS) against the 3D model of *Pf*CK2 α using Glide software (34). The Chembridge DIVERSet Library was used for the virtual screening. After filtering to prioritize drug-like compounds, we selected a subset of 35,782 compounds. The docking was performed in three-tiered Glide protocol using hierarchical filtering

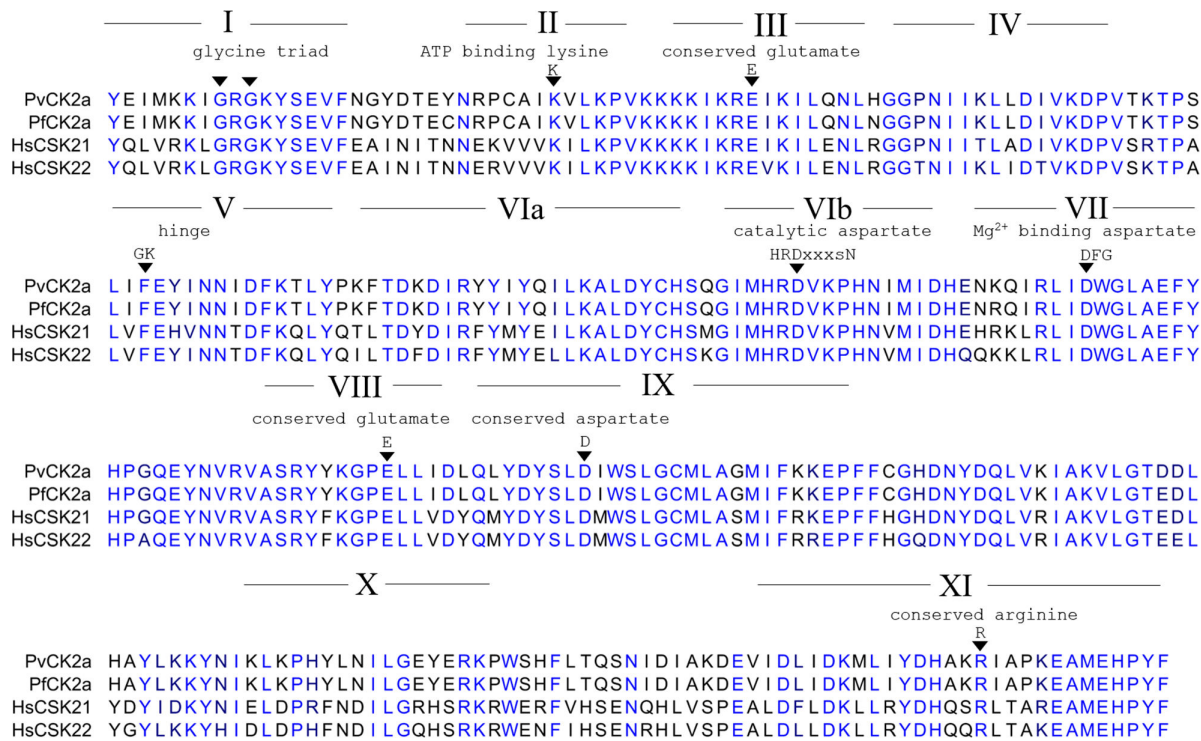


FIG 1 Amino acid sequence alignment of protein kinase domains of CK2α sequences of *P. falciparum* and *Plasmodium vivax* with the homolog sequence in humans. Protein kinase domains were identified using Pfam database. Conserved residues are shown in blue. The 11 subdomains of the protein kinase domain are indicated with roman numerals. The arrows indicate amino acids highly conserved among protein kinase subdomains. The alignment was performed with CLC Sequence Viewer v. 8 (gap open cost 10, gap extension cost 1, end gap cost as any other). GK, gatekeeper.

(HTVS, SP, and XP precision modes) (35). Once docking was completed, a docking score of -7.0 kcal/mol was chosen as the cutoff for the next step for *PfCK2*. The top ranked compounds (2,268) were then screened using a previously developed QSAR phenotypic model (27) to identify novel compounds that may have antimalarial activity. Finally, 168 potential inhibitors of *PfCK2α* were selected, obtained, and subjected to biological testing (Fig. 2A).

Biological evaluation

Compounds were evaluated in a fluorescence-based assay against the asexual blood stage of *P. falciparum* (3D7 strain). For an initial activity assessment, a single concentration of $5 \mu\text{M}$ was used. Nineteen out of 168 compounds that showed more than 60% of

TABLE 1 MolProbity statistical quality of the final model of *PfCK2α* after refinement

	<i>PfCK2α</i>
Clash score	0.71 (99th percentile) ^a
Poor rotamers	0 ^b
Favored rotamers	305 (98.7%) ^c
Ramachandran outliers (%)	0.3 ^d
Ramachandran favored (%)	95.2 ^e
MolProbity score	1.06 (100th percentile) ^{a,f}

^a100th percentile is the best among structure of comparable resolution; 0th percentile is the worst.

^bGoal: <0.3%.

^cGoal: >98%.

^dGoal: <0.05%.

^eGoal: >98%.

^fMolProbity score combines the clash score, poor rotamers, and Ramachandran favored evaluations into a single score, normalized to be on the same scale as X-ray resolution.

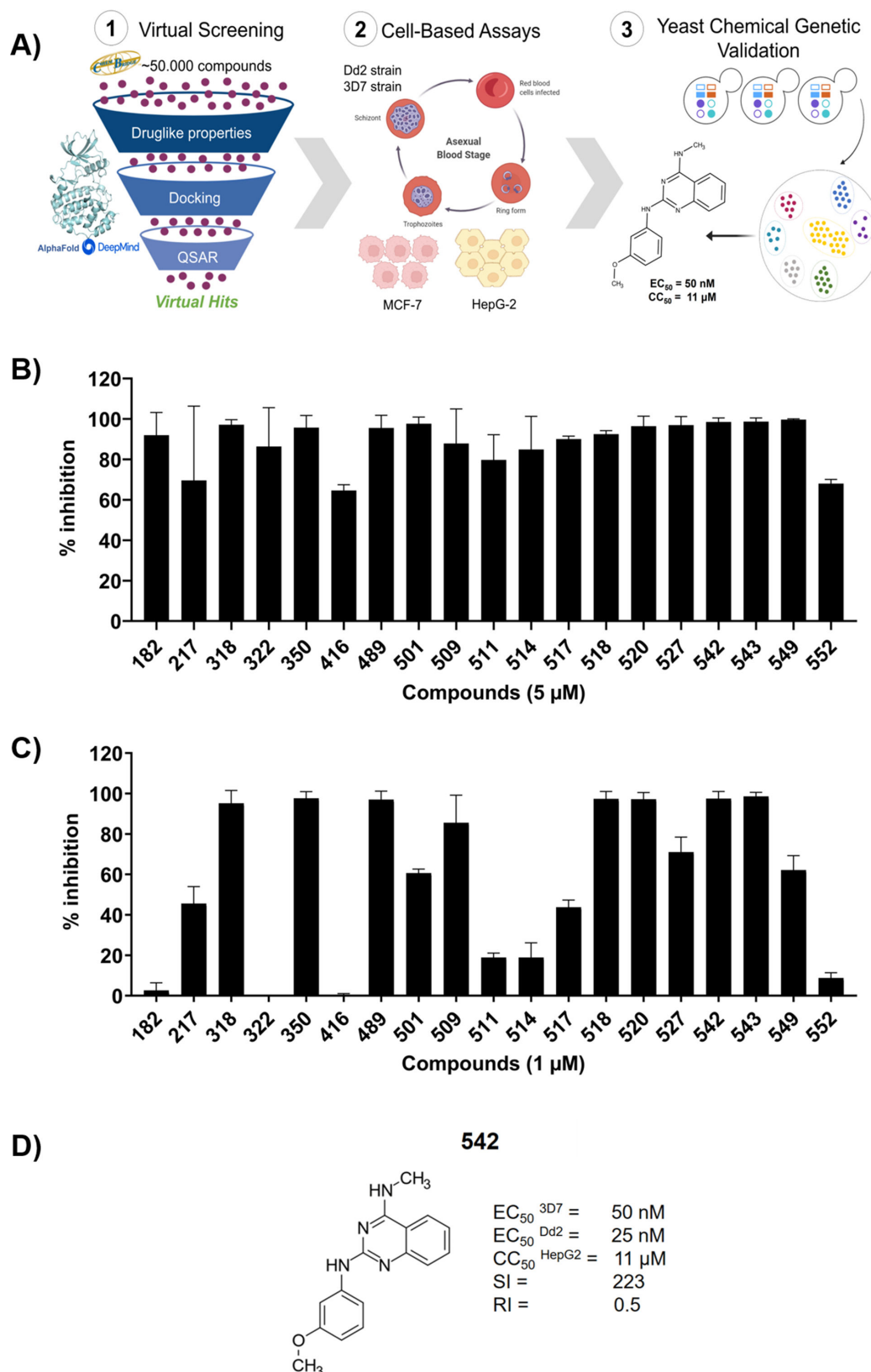


FIG 2 (A) Workflow of the virtual screening strategy. First, the Chembridge Library with ~50 K compounds was passed through the drug-likeness filter, then through the three molecular docking filters available in ascending order of precision to select the molecules according to an established threshold (−7 kcal/mol). Finally, we used the QSAR model built by Neves (Continued on next page)

FIG 2 (Continued)

et al. (27) to increase our screening accuracy rate. Candidate compounds were then tested against *P. falciparum* asexual stages and mammalian cell lines. The chemical-genetic interaction profile of the compound with the highest selectivity index was evaluated in *Saccharomyces cerevisiae* deletion mutants (B and C) *Plasmodium* growth inhibition of the most active compounds. *P. falciparum* 3D7 blood-stage parasites were cultured for 72 hours in the presence of compounds at 5 μ M (B) or 1 μ M (C). Values are normalized as percentage of non-treated parasites; error bars represent the standard error. Inhibition is displayed as a mean of two independent experiments. (D) Structure representation of the most promising molecule (**542**) and activities against *P. falciparum* and mammalian cell lines. RI, resistance index (ratio of EC₅₀ for Dd2 to EC₅₀ for 3D7); SI, selectivity index (ratio of EC₅₀ for 3D7 to CC₅₀ for HepG-2).

parasite inhibition at 5 μ M were rescreened at 1 μ M (Fig. 2B and C). Of these, 11 showed greater than 50% inhibition at 1 μ M.

To better understand the structure-activity relationship (SAR) of the active compounds, we performed experimental assays to determine the EC₅₀ values of these 11 compounds (Table S1). Compound **318** was excluded from this analysis because it is the only compound in the series that lacks the quinazoline core. Figure 3 shows a summary of the SAR study. From this analysis, we can see that changes in R1 position have more significant impact on the compound's antiplasmodial activity compared to changes in R2 or R3. In addition, we found that the presence of electron-donating groups, which render the benzene ring more electron-rich and activate it toward electrophilic aromatic substitution reactions, leads to increased *in vitro* potency of the series. This positive correlation between the resonance effect of the substituent groups in R1 (anisole > phenol > dimethylaniline > isopropylbenzene > benzamide) and compound activity is depicted in Fig. 3. Compound **509** yields benzamide with a para-substituted amide functionality. Notably, amides exhibit greater electronegativity compared to isopropyl alcohols and dimethylamines. However, the absence of a meta-substituted amide within our compound set restricts a more comprehensive analysis of this distinct functional group. Additionally, our observations indicate a preference for hydrogen atoms over more voluminous substituents in R3. This assertion finds support in the case of

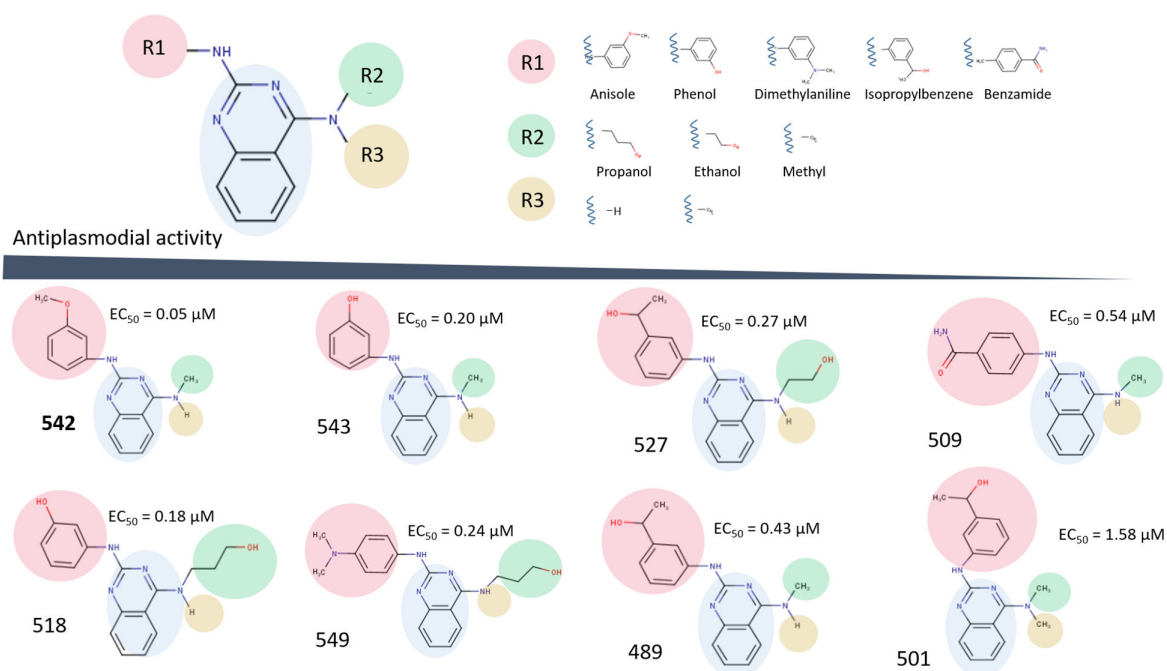


FIG 3 Structure-activity relationship of the quinazoline series. Potential PfCK2 α inhibitors are arranged based on their antimalarial activity, with the lowest Pf3D7 EC₅₀ values (most potent) on the left and the highest Pf3D7 EC₅₀ values (less potent) on the right. The common quinazoline core (blue) is shared by all the compounds selected. The compounds can be distinguished by the substitution groups present in R1 (pink), R2 (green), and R3 (yellow).

compound **501**, where the presence of a methyl group instead of a hydrogen atom coincides with the lowest EC_{50} value in the series. Furthermore, the docking studies conducted on compound **542** revealed that the nitrogen atom forms a hydrogen bond with the CK1 binding site. This interaction would be unattainable if a methyl group was present in the R3 position. Exploring R2 substituents yielded limited insights, primarily stemming from the scarcity of diverse examples solely differing in this group. However, it is noteworthy that longer chains featuring polar moieties exhibited enhancements in compound **518** relative to **543**. Similarly, in the case of compound **527** compared to **489**, longer chains with polar groups demonstrated improvements. The extension of these chains likely facilitates the establishment of interactions with hydrophobic residues within the binding site.

We further evaluated the cytotoxicity of these 11 most active compounds using two lineages of mammalian cells (MCF-7 and HepG-2). The results are summarized in Table S1. No compound presented greater activity against human cells than against parasites, indicating a high likelihood of selectivity for the parasite. We also tested the most active compound **542** in HepG-2 cells after the same time of incubation (72 hr) used for parasite treatment, to ensure that the high selectivity for **542** was not biased by effects on incubation period ($SI_{HepG-2\ 24h} = 223 \times SI_{HepG-2\ 72h} = 205$). Furthermore, we also tested the compound **542** in a non-cancer cell line (COS-7) ($SI_{COS-7\ 72h} = 294$). In both cases, the same degree of selectivity was observed (Fig. S1).

Compound **542** was selected for the determination of its EC_{50} value against a multi-resistant (Dd2) strain of *P. falciparum* due to its high antiparasitic activity, low cytotoxicity, and chemical structure suitable for optimization (Fig. 2D). The compound exhibited potent activity against both chloroquine-sensitive and multidrug-resistant parasite strains. Chloroquine, on the other hand was shown to be nearly eight times less active against Dd2 resistant strain. In summary, the data suggest that this compound showed no sign of cross-resistance with antimalarials, such as chloroquine, pyrimethamine, and mefloquine. Moreover, compound **542** was threefold more active against Dd2 strain when compared to chloroquine ($EC_{50} = 25$ and 90 nM, respectively) (Fig. 2D).

To assess the selectivity of **542**, we tested the compound at a single concentration ($1\ \mu M$) in a competitive binding assay using fluorescent tracers against a panel of 29 human kinases. Although the human kinome has more than 500 proteins, kinases in the panel covered almost all protein kinase families (Fig. 4). In this panel, binding of the fluorescent tracer to the target kinase by **542** was inhibited $>30\%$ for only two human kinases: GPRK5 (39%) and MAP3K14 (39%). For comparison, we used two known human kinase inhibitors (CRT0066101 and CP673451), which showed a broader kinase binding profile (Fig. 4). Taken together, the high activity against malaria parasites of **542**, in combination with its selectivity profile, offers an attractive starting point for new antimalarial therapies.

Structure-based modeling with predicted *Pf*CK2 α inhibitors

To simulate the ligand-receptor binding process, we docked the most active predicted *Pf*CK2 α inhibitor (**542**) into the ATP-binding pocket, and as expected, this compound was well accommodated inside the active site. The predicted molecular interactions of compound **542** in the ATP-binding site of *Pf*CK2 α are shown in Fig. 5. This compound has a quinazoline core that is a recognized chemical moiety that has been previously shown to inhibit *P. falciparum* growth (36), but for which the mechanism of action (MoA) remains to be determined. The quinazoline core of compound forms hydrophobic interactions with Phe117 (gatekeeper), and Ile120 (hinge) of the ATP-binding site. Additionally, compound **542** forms polar interactions with DWG domain (Asp179). One of the key interactions of ATP and ATP-competitive inhibitors is the H-bonding with the backbone amides in the hinge region, but as the hinge is a conserved region, selectivity can be difficult to achieve. Plasmodial CK2 α s have substitutions in the hinge residues (Glu118, Tyr119, and Ile120) compared to one of its human orthologue CK2 α s (Glu114, His115, and Val116) but are identical with the hinge sequence of CK2 α' (Fig. 1B).

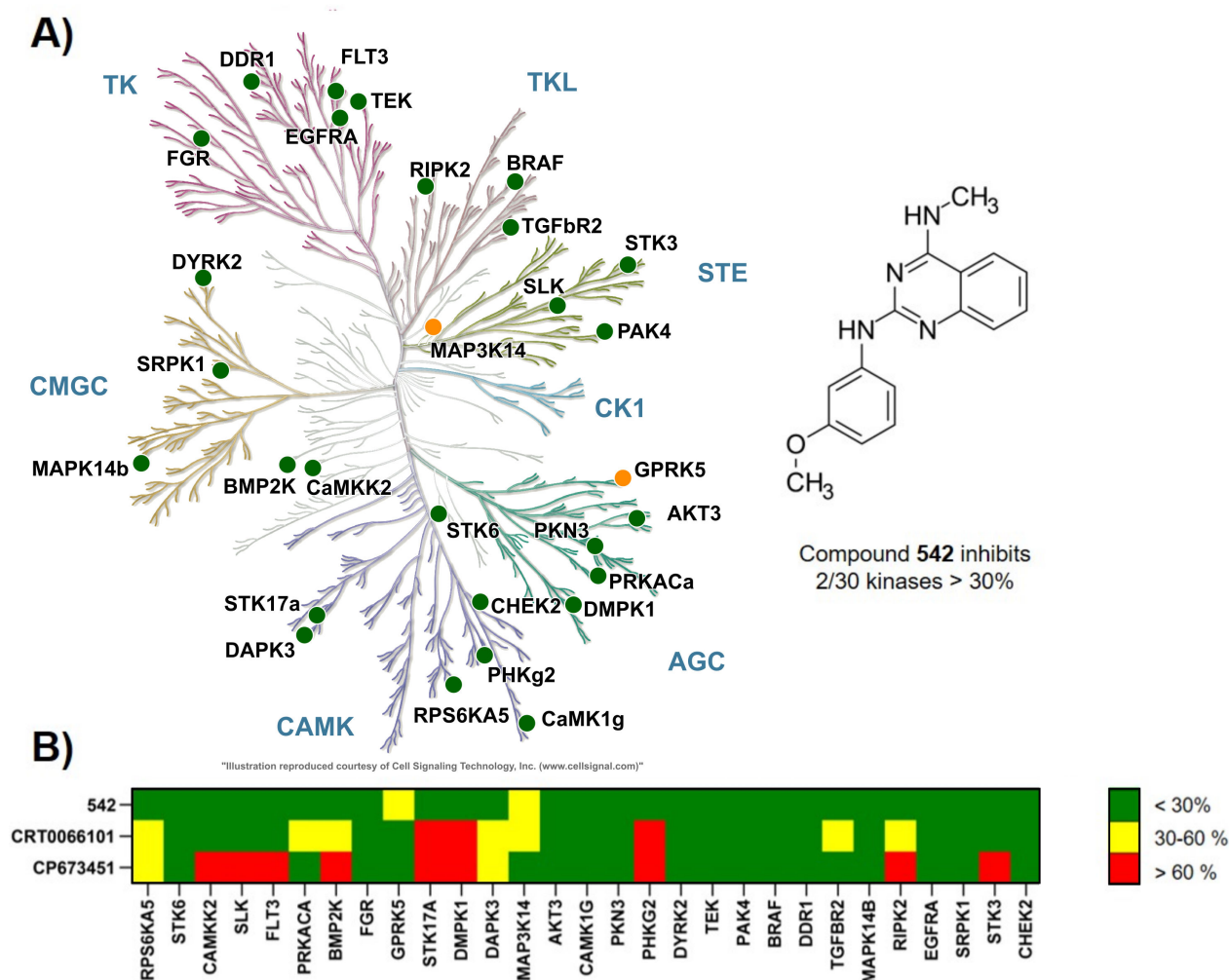


FIG 4 (A) Human kinome dendrogram created using KinMap. Human kinases present in the panel are indicated by colored circles. Orange circles indicate kinases that showed between 30 and 60% binding to compound 542. (B) Representation of tracer displacement caused by compound 542 in different human kinases. Proteins are divided in low (<30%, green), mild (30–60%, yellow), and high (>60%, red) displacement. Compound 542 did not cause high displacement in any of the tested proteins. For comparison, results are also shown for two promiscuous human kinase inhibitors, CRT0066101 and CP673451.

Yeast chemical-genetic interactions

Although advances in computer-aided drug design and *in silico* docking approaches increase the chances of finding specific inhibitors, these techniques are not yet sufficient to identify drug targets *in vivo*. Due to its high antiparasitic activity and selectivity index, we decided to further explore the potential mechanism of action of compound 542 using chemical-genetic interactions in a yeast model. Genetic interactions between two genes (e.g., gene A and gene B) occur when an unexpected, combined single-deletion mutant phenotype is observed through positive (growth $A\Delta \cdot B\Delta$ – growth $A\Delta$ * growth of $B\Delta$ > 0, fitness enhancement) or negative (growth $A\Delta \cdot B\Delta$ – growth $A\Delta$ * growth of $B\Delta$ < 0, fitness defect) genetic interactions. As chemical perturbations mimic genetic perturbations, we can employ data from genetic interactions to suggest functional interactions between small molecules and gene products. Drug sensitivity can arise when a compound affects an element of a pathway that is parallel to a pathway with a mutant component. Increased growth, or resistance, can arise when the drug target, or a protein from the same pathway as the target, is absent (37, 38).

The *Saccharomyces cerevisiae* genome encodes two CK2as: Cka1 and Cka2. While *CKA1* and *CKA2* are non-essential genes, their double deletion (*cka1Δ cka2Δ*) results in

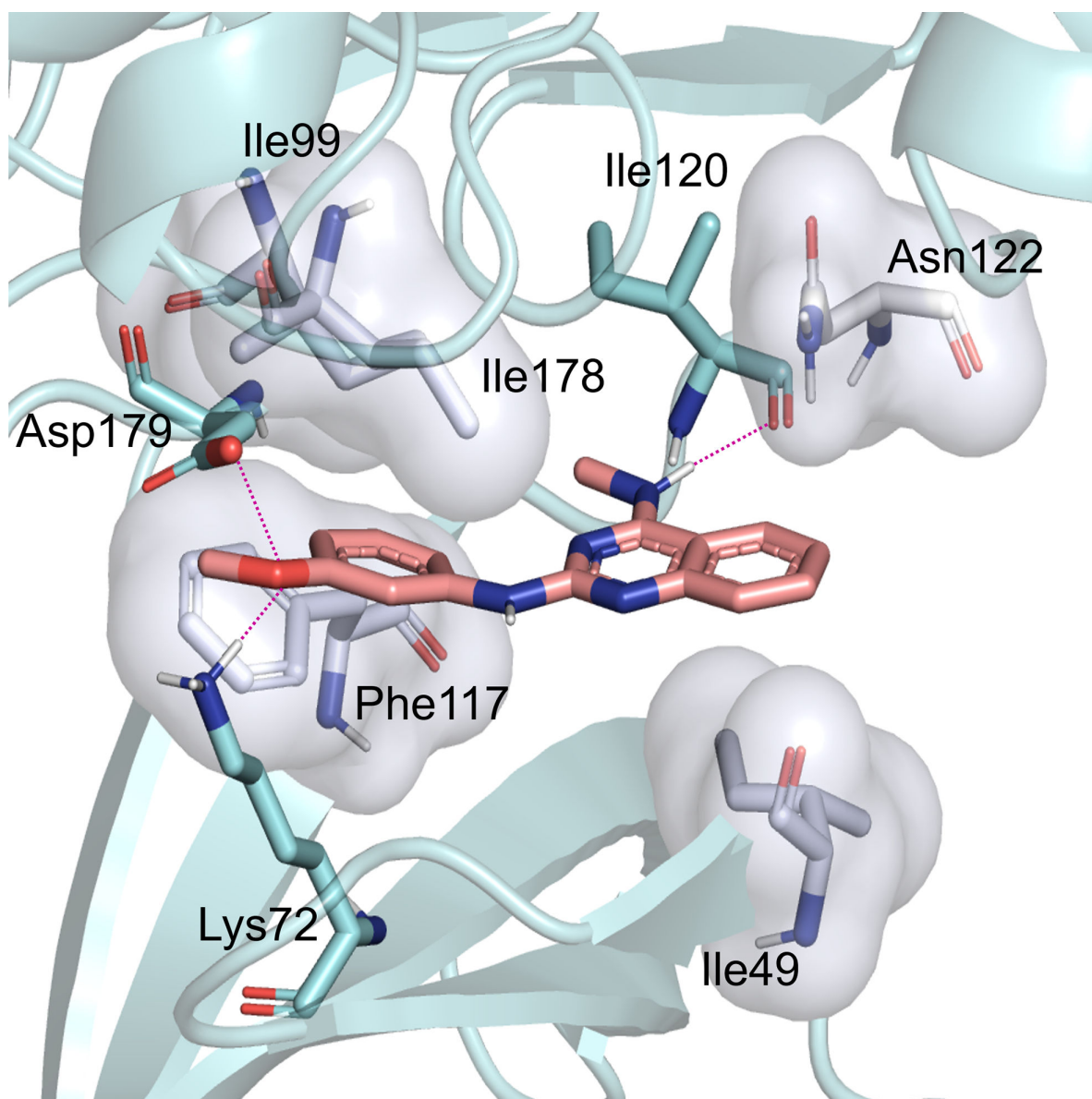


FIG 5 Representation of 3D intermolecular interactions of compound **542** predicted by docking with *PfCK2α*. 3D interactions showing hydrogen bonds in magenta dashed lines and hydrophobic interactions are represented by gray surfaces. Carbon atoms of amino acid residues are colored in pale cyan.

growth defects due to a negative genetic interaction. We hypothesized that adding an inhibitor of Cka2 in a yeast mutant lacking Cka1 (*cka1Δ*) would reproduce the same growth defect phenotype and that a Cka1 inhibitor added to a yeast mutant lacking Cka2 (*cka2Δ*) would yield similar results. Furthermore, treatment of *eap1Δ* mutant (positive genetic interaction with *cka1Δ* and negative genetic interaction with *cka2Δ*) with **542** would be able to suggest if and which of the yeast's CK2as could be inhibited by the test compound.

We then performed multiple sequence alignments of *Plasmodium*, human and yeast CK2as, noting that the *Plasmodium* hinge residues (Glu118, Tyr119, and Ile120) were more similar to the equivalent residues in the yeast Cka1 (Glu153, Tyr154, and Val155) than in Cka2 (Glu125, Glu126, and Ile127); hence, a *Plasmodium* inhibitor targeting the hinge region should inhibit Cka1, but not Cka2, in yeast (Fig. S2).

Thus, to better understand the mode of action of **542**, we chose a wild-type control with the antibiotic resistant marker inserted in a neutral locus (*his3Δ*) and three

single-deletion mutant yeast strains with known genetic interaction profiles to evaluate changes in fitness under treatment with the **542** inhibitor (Fig. 6A and B). Wild-type and *cka1Δ* strains presented no growth defect in the presence of the test compound (Fig. 6C). However, the mutant line *cka2Δ* (synthetic sick with *cka1Δ*) presented fitness defect when combined with **542**, while *eap1Δ* (positive genetic interaction with *cka1Δ* and negative genetic interaction with *cka2Δ*) showed fitness enhancement in the presence of the compound **542**. Hence, the chemical-genetic profile of yeast mutants treated with compound **542** is consistent with the inhibition of yeast Cka1 but not inhibition of Cka2.

Conclusions

Herein, we performed a hierarchical virtual screening by using structure- and ligand-based approaches, leading to the identification of eleven compounds with antiplasmodial activity in the nanomolar range, one of which (**542**) had good selectivity index. The best hit compound, **542**, contains a quinazoline moiety, a known scaffold with reported antiplasmodial activity, but with an unknown MoA. To gain some insight into **542**'s MoA, we performed chemical-genetic interaction analysis using yeast as a model. In agreement with our *in silico* results hypothesizing that **542** inhibits PfCK2α via hinge region interaction, our chemical-genetic interactions suggest that **542** seems to inhibit yeast Cka1, which has a hinge region with high similarity to PfCK2α. The chemical-genetic interaction profile also suggests that **542** does not inhibit Cka2.

Thus, employing *in silico* and *in vitro* experiments, we identified a quinazoline molecule with high antiplasmodial activity against asexual stages and low toxicity against host cells. Our data reinforce the initial hypothesis that suggested compound **542** as a PfCK2α inhibitor. While we have made progress in comprehending the mechanism of action of **542** by employing chemical-genetic interactions in yeast as a

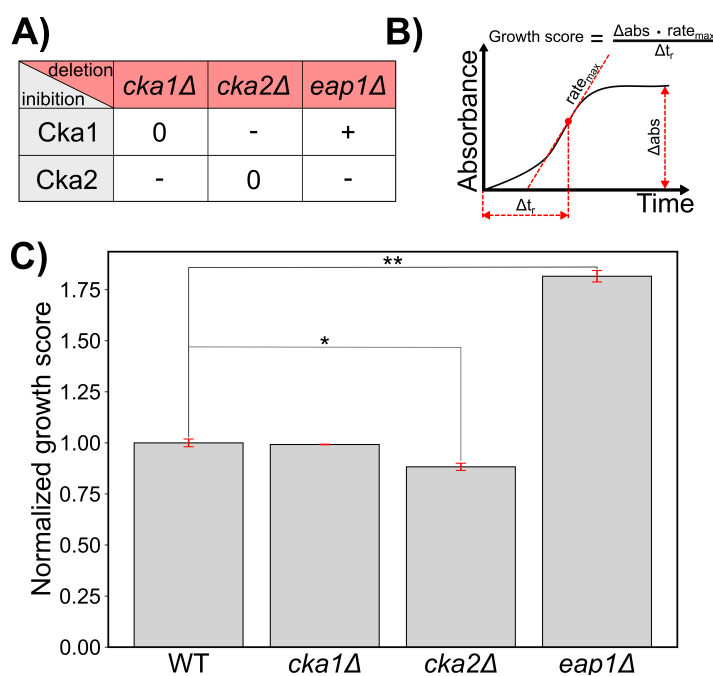


FIG 6 (A) Possible chemical-genetic interaction profiles consistent with Cka1 or Cka2 inhibition based on known genetic interactions (negative genetic interactions: *cka1Δ* with *cka2Δ* and *eap1Δ* with *cka2Δ*; positive genetic interaction *cka1Δ* with *eap1Δ*). (B) Parameters extracted from growth curves of mutant strains grown in the presence of compound **542** or solvent control to determine their respective growth scores. (C) Normalized growth scores of wild type (*his3Δ*) and mutant strains grown in the presence of **542**. Bar graphs represent growth of three biological replicates. Error bars represent standard error based on three biological replicates. Significance was assessed using a two-tailed *t*-test, with statistical significance denoted as follows: * $P < 0.1$, ** $P < 0.01$.

model, additional assays are necessary to validate the target, in addition to the scope of this publication. These assays include *in vitro* kinase inhibition assays, biophysical assays, genome-scale thermal proteome profiling in *P. falciparum*, and genomic analysis of *Plasmodium* 542-resistant lines. Overall, our strategy has identified a promising non-toxic molecule that can undergo further hit-to-lead optimization.

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AUTHOR AFFILIATIONS

¹Laboratory of Tropical Diseases (LDT), Institute of Biology, University of Campinas, Campinas, Brazil

²Laboratory of Molecular Modeling and Drug Design (LabMol), Faculty of Pharmacy, Universidade Federal de Goiás (UFG), Goiânia, Brazil

³Department of Materials and Bioprocesses Engineering, School of Chemical Engineering, University of Campinas, Campinas, Brazil

⁴Centro de Química Medicinal (CQMED), Centro de Biologia Molecular e Engenharia Genética (CBMEG), Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil

⁵Department of Chemistry and Molecular Biology, University of Gothenburg, Gothenburg, Sweden

⁶Structural Genomics Consortium, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

⁷Center for Research and Advancement of Fragments and Molecular Targets (CRAFT), University of São Paulo, São Paulo, Brazil

⁸Center for Excellence in Artificial Intelligence (CEIA), Institute of Informatics, Universidade Federal de Goiás, Goiânia, Brazil

⁹Department of Structural and Functional Biology, Synthetic Biology Laboratory, Institute of Biology, University of Campinas, Campinas, Brazil

¹⁰Global Health and Tropical Medicine (GHTM), Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisbon, Portugal

AUTHOR ORCIDS

Rafael M. Couñago  <http://orcid.org/0000-0003-1847-5090>

Per Sunnerhagen  <http://orcid.org/0000-0002-0967-8729>

Gustavo C. Cassiano  <http://orcid.org/0000-0002-6971-5974>

Fabio T. M. Costa  <http://orcid.org/0000-0001-9969-7300>

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ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Table S1 and Figures S1 and S2 (AAC00589-23-s0001.pdf). Antimalarial and cytotoxicity of tested compounds and alignment of CK2.

Table S2 (AAC00589-23-s0002.xlsx). Sheet shows all compounds tested in this study.

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