Combining IP₃ affinity chromatography and bioinformatics reveals a novel protein-IP₃ binding site on *Plasmodium falciparum* MDR1 transporter

Eduardo Alves^{1,2}; Helder Nakaya^{2,3} Euzébio Guimarães⁴, Célio R. S. Garcia^{2,*}

¹Life Science Department, Imperial College London, London, UK.

²Department of Clinical and Toxicological Analyses of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil.

³Computational Systems Biology Laboratory, INOVA, University of São Paulo, São Paulo, Brazil

⁴Federal University of Rio Grande do Norte, Pharmacy Department, Health Science Center, Natal, Brazil.

*To whom correspondence should be addressed email: cgarcia@usp.br

Abstract

Intracellular Ca^{2+} mobilization induced by second messenger IP_3 controls many cellular events in most of the eukaryotic groups. Despite the increasing evidence of IP_3 -induced Ca^{2+} in apicomplexan parasites like *Plasmodium*, responsible for malaria infection, no protein with potential function as an IP_3 -receptor has been identified. The use of bioinformatic analyses based on previously known sequences of IP_3 -receptor failed to identify potential IP_3 -receptor candidates in any *Apicomplexa*. In this work, we combine the biochemical approach of an IP_3 affinity chromatography column with bioinformatic meta-analyses to identified potential vital membrane proteins that present binding with IP_3 in *Plasmodium falciparum*. Our analyses reveal that PF3D7_0523000, a gene that codes a transport protein associated with multidrug resistance, as a potential target for IP_3 . This work provides a new insight for probing potential candidates for IP_3 -receptor in *Apicomplexa*.

Introduction

The inositol 1,4,5-triphosphate (IP₃) is an important second messenger that regulates cytosolic Ca²⁺ in a variety of Eukaryotic organism^(1, 2). Briefly, the activation of phospholipase C (PLC) mediated by surface receptor breaks phosphatidylinositol 4,5-bisphosphate (PIP₂) into soluble short life second messenger IP₃ that binds into IP₃ receptor (IP₃R) culminating in intracellular Ca²⁺ release^(3,4).

The phylum *Apicomplexa* includes unicellular eukaryotes parasites like *Plasmodium*, the etiology agent of malaria infection, and possesses the metabolic enzymes responsible for generation and degradation of IP₃, see review⁽⁵⁾. IP₃ can mobilize Ca²⁺ from intracellular stores in isolate and permeabilize blood stage *P. chabaudi*⁽⁶⁾ and in intact *P. falciparum* within red blood cells (RBCs)⁽⁷⁾. Within RBCs, parasites manage to maintain the Ca²⁺ stores full even under low Ca²⁺ environment⁽⁸⁾. An increasing number of reports supporting the existence of intracellular Ca²⁺ release induced by IP₃ in malaria parasites^(6, 7, 9-12) suggesting the existence of a Ca²⁺ channel sensitive to IP₃, the IP₃R.

The IP₃R it is a well know described protein in vertebrates that contains around four to six transmembrane domains (TMDs), see review⁽¹³⁾. Prole and Taylor⁽¹⁴⁾ used the sequence of mammal N-terminal IP₃R binding domain and the amino-terminal RIH (Ryanodine and IP₃R homology) domains to perform a BLAST (Basic Local Alignment Search Tool) on the genome of diverse parasites. However, this work failed to find any potential candidate for IP₃R in *Apicomplexa*. So far, no apicomplexan IP₃R candidate has been identified through bioinformatics approaches. Moreover, there is no publication that attempted to use biochemical approach like an IP₃ affinity chromatography column in *Apicomplexa* to identify proteins that might bind to IP₃.

Hirata and collaborators⁽¹⁵⁾ managed to enrich proteins from rat brain sample that has an affinity to IP₃ like IP₅-phosphatase and IP₃ 3-kinase using an analogous IP₃ affinity chromatography column 2-O-[4-(5-aminoethyl-2-hydroxyphenylazo) benzoyl]-1,4,5-tri-O-phosphono-myo-inositol trisodium salt-Sepharose 4B. Using a similar column, Kishigami and collaborators⁽¹⁶⁾ managed to identify the PLC protein from octopus' eyes *Todarodes pacificus* and reported that squid rhodopsin also has an affinity to IP₃. Nevertheless, besides the potential of these columns to enrich proteins that bind to IP₃, no IP₃R has ever been identified using a IP₃-affinity column alone.

By adapting the protocol from Hirata/Kishigami^(15, 16), we created a column containing IP₃ conjugated with biotin linked with a high-performance sepharose-streptavidin and challenged with proteins from asynchronous *P. falciparum* blood-stage. Using a IP₃-free column containing only sepharose-streptavidin as reference, we selected only the candidates exclusive on the IP₃-column to undergo a series of bioinformatic meta-analyses. Our approach targeted for candidates with at least one transmembrane domain, considered essential, conserved among most apicomplexan species, and with unknown or nor-clear function. Finally, the candidate that fit all these criteria were used as targets for *in silico* molecular docking against IP₃.

Using this strategy, we identified the *P. falciparum* multidrug resistance protein 1 (*PfMDR1*), a vital and conserved membrane protein that has potential to bind to IP₃. This protein is located on parasite food vacuole, a Ca²⁺ storage compartment⁽¹⁷⁾. Combined, the affinity column and bioinformatic successfully provided a candidate for a membrane protein associated to an intracellular Ca²⁺ compartment that binds to IP₃ in malaria.

Material and Methods

***P. falciparum* Culture:** *P. falciparum* (D37) parasites were maintained in culture as described⁽¹⁸⁾. Briefly, *P. falciparum* were cultured in RPMI media supplemented with 50 mg/L hypoxanthine; 40 mg/L gentamycin; 435 mg/L NaHCO₃; 2% haematocrit of A⁺ human red blood cells and 10% A⁺ human blood serum in an atmosphere of 5% CO₂; 3% O₂; 92% N₂ at 37°C. Media was changed every 24 h and RBCs replaced every 48 h. Parasitemia and the development stage of cultures were determined by Giemsa-stained smears.

***P. falciparum* protein sample:** Total *P. falciparum* protein extract was obtained from 2.5 L of unsynchronized culture, at 8% parasitemia. Culture was washed three times in PBS (300 g, 5 minutes) and parasites isolated from erythrocytes using 0.03% (w/v) saponin (Sigma) on PBS containing protease inhibitors: antiplaque, pepstatin, chymostatin and leupeptin (Sigma) at concentrations of 20 µg/mL each and 500 µM benzamidine (Sigma). Isolate parasites were centrifuge on 1300 g for 10 minutes at 4°C and washed three times in PBS with protease inhibitors. The isolate parasite samples were resuspended in 50 mM TRIS-HCl buffer pH 7.4 containing 2 mM EDTA, 0.1% Triton X-100, protease inhibitors and 1 mM PMSF. Samples were sonicated on SONIC (Vibracell) 50% potency for 20 seconds for 3 times on ice (10-second interval between each sonication) follow by at 1300 g centrifugation for 10 minutes at 4°C for removal of the insoluble pellet. DNase and RNase (final concentration 200 ng/µL each) were added on soluble pellet and incubate for one hour at 37 °C. The samples were passed through a 0.45 µm filter. The amount of protein was quantitated using Pierce's BCA protein assay kit.

IP₃-affinity chromatography column: To build the column, it was used a commercial high performance Sepharose substrate bound to streptavidin (GE Healthacare Life Science) and biotin-conjugated IP₃ (Echelom Biosciences). The streptavidin-sepharose column was equilibrated by washing once with 10x volume of ice-cold, 0.45 µm filter binding buffer (20 mM NaH₂PO₄, 150 mM NaCl, 20 mM LiCl and 2 mM EDTA, pH 7.5). The columns were mounted in a 15 mL sterile falcon tube. For each column it was used 1.25 ml of equilibrated Sepharose-streptavidin resuspended in binding buffer mixed with 20 µg of IP₃-biotin. The columns were left by constant stirring for 12 hours at 4°C in a dark environment and then centrifuged for 1 minute, 300 g at 4 °C. The supernatant containing excess IP₃-biotin was removed and columns were washed five times with 2 ml of ice-cold binding buffer to remove any free IP₃-biotin. Two distinct columns were assembled: one containing IP₃-biotin-sepharose-streptavidin and other containing sepharose-streptavidin only. In each column was added 2.5 mg of *P. falciparum* protein extract. The column volume was adjusted with ice-cold binding buffer with protease inhibitors until a final volume of 5 ml. The columns were incubated at 4°C under gentle, steady shaking in a light-protected environment for 12 hours. After incubation, the columns were centrifuged for 1 minute at 300 g at 4 °C and the supernatant discarded. Each column was washed seven times with ice-bound binding buffer with protease inhibitors. To elute the proteins, 1mL of an ice-cold elution buffer (8 M Guanidin-HCl, 20 mM LiCl, 2 mM EDTA, pH: 1.5 with protease inhibitors) was added on each column and followed by constant stirring for 1 hour at 4 °C. At the end of this incubation the columns were centrifuged for 1 minute at 300 g and the supernatant collected in sterile low binding protein eppendorfs.

Mass spectrometry: The protein samples were applied on 8% polyacrylamide gel and run at low voltage (60 v) until the bands were discriminated. After the run, the gel was fixed and stained following the recommendations of the "Colloidal Blue Staining Kit" from Invitrogen. The sections of the gel containing visible bands were cut and sent for analysis on a mass spectrometer at Taplin Mass Spectrometry, Harvard Medical School (<https://taplin.med.harvard.edu/>) for protein identification. All identified proteins containing at least one exclusive peptide match was considered for analyses.

Transmembrane domain prediction: To detect the presence of a transmembrane domain, the whole amino acid sequence from the protein identified at mass spectrometry were analysed using the public HMMTOP program version 2.0 (www.enzim.hu/hmmtop/). This program predicts the number of transmembrane helices and their position from peptide/protein amino acid sequence.

Phenotype score, conservation and function predictions: The phenotype score used the determinate gene essentiality of each candidate was obtained from the work of Zhang and collaborators⁽¹⁹⁾ that is available on PlasmoDB (<https://plasmodb.org/plasmo>). To identify the presence of a candidate orthologs among *Apicomplexa* group, we use the OrthoMCL database (<https://orthomcl.org/orthomcl>). For function prediction, we consulted the gene annotation information provided by PlasmoDB.

In silico docking with IP₃: The primary sequence of MDR1 (Gene - PF3D7_0523000, plasmodb.org) was used to build its probable 3D structure by homology modelling. The server SwissModel⁽²⁰⁾ was employed to automatically build the models optimized to bind IP₃ at various locations inside MDR1 homology. Blind molecular docking simulation were carried out to obtain possible interactions for the intermembrane domain as predicted by the TMHMM Server⁽²¹⁾. The SwissDock⁽²²⁾ server enabled the study of IP₃ intermembrane MDR1 domain binding poses. Additionally, the IP₃-Ion-MDR1 binding was further investigated using the multidrug transporter permeability (P)-glycoprotein is an adenosine triphosphate (ATP)-binding cassette (PDB id: 6C0V). The later ability to bind simultaneously ATP and a divalent cation at the intracellular domain was used to guide the inspect a hypothetical IP₃-Ion-MDR1 interaction. IP₃ was manually positioned inside the ATP cavity to mimic an IP₃-Mg²⁺ interaction. The binding conformation was optimized with molecular mechanics by means of the UCSF⁽²³⁾ chimera minimize structure tools.

Protein-protein interaction network: Using *Plasmodium* interactome data⁽²⁴⁾, we looked for the proteins that interact with the MDR1. The protein annotation and functions were also retrieved from the original publication. Network was generated using Cytoscape⁽²⁵⁾.

Results

IP₃-affinity chromatography data

Adapting the protocol based on Hirata/Mishigami^(15, 16), we use an IP₃ affinity chromatography column with protein homogenate from unsynchronized asexual blood stages of isolated *P. falciparum* as the first step to identified potential proteins that have a similar function to IP₃R receptor in a mammal.

The access code of the brute data on mass spectrometry analyses from the eluate samples of IP₃-affinity chromatography column can be found in Supplemental Material Table 1. At least 700 proteins from *P. falciparum* containing at least one exclusive peptide were detected from the IP₃-sepharose column. In comparison, 494 proteins were detected from the sepharose matrix alone (Figure 1). With both columns' information, all proteins exclusively present on IP₃-sepharose were selected (total 206 proteins) for the bioinformatic meta-analyses (Sup. Table 2).

Once the proteins exclusive for IP₃-column were identified (Sup. Table 2), the first bioinformatic approach aimed to select proteins that contains a transmembrane domain (TMD). The IP₃R is a protein attached at the membrane, a TMD is an important structure to anchor proteins through biological membranes by its physical properties like the length and hydrophilicity of the transmembrane span⁽²⁶⁾, every IP₃R in vertebrates, invertebrates and single eukaryotes organism possess a TMDs, so we use this feature as the second step to select potential candidates for IP₃R. Figure 1.

The table 1 summarizes the list of 26 proteins exclusively found at IP₃-biotin-streptavidin-sepharose column that contains at least one TMDs. Transfection of *P. falciparum* to constitutively express IP₃-sponge, a protein containing a modified IP₃ binding domain based on mouse IP₃R that sequester cytosolic IP₃⁽²⁷⁾, does not result in viable parasites⁽²⁸⁾ suggesting a vital role of IP₃ signalling in *P. falciparum*. With this information, the next step to narrow the number of potential candidates that might act as IP₃R in malaria is focus on essential genes. To deem whether a gene is essential, we considered only the genes that score lower than 0.5 on its mutagenic index of phenotype graphic (data provide by PlasmoDB). That decrease the number of candidates to 11 (Fig. 1, table I).

For the next step on the bioinformatic meta-analyses, we considered only the genes that is conserve among multiples species within *Apicomplexa* phylum. Only four essential candidates with TMDs domains met this criterium: multidrug resistance protein 1 (MDR1); a heat shock protein 40, type II (HSP40); aspartate carbamoyltransferase (ATCase) and antigen UB05. PlasmoDB access code: PF3D7_0523000, PF3D7_0501100, PF3D7_1344800 and PF3D7_1038000 respectively. Among these 4 candidates, only MDR1 and antigen UB05 has unknow or unclear function. The HSP40 is a cochaperone protein with conserved J-domain that regulates other heat socks protein 70 (HSP70)⁽²⁹⁾ and the ATCase is an enzyme important for the pyrimidine biosynthetic pathway⁽³⁰⁾.

IP₃-MRD1 binding modelling and protein interactions network

The MDR1 model provide by the SwissModel server proved to be quite similar to human P-glycoprotein ABCB1 receptor, protein data bank id: 7A69⁽³¹⁾. The sequence alignment proved that a homology model could be built with fair quality with an identity of 29.7% and similarity of 48.2% (Pairwise Sequence Alignment EMBOSS Water server,

<https://www.ebi.ac.uk/Tools/emboss/>). Two binding position at the transmembrane domain of MRD1 and IP₃ binding was estimated by the SwissDock server (Figure 2).

The pocket 1 (binding energy -15.7 kcal/mol) proved to be the best IP₃ docking position. The site is a lysin rich domain able to form various hydrogen bonds with IP₃. The second-best bind pocket proved to be less favored as derived from the lower interaction energy (-11.4 kcal/mol). Another binding possibility investigated was the interaction with the same pocket ATP binding. The interaction involves the presence of a divalent cation (green spheres) like Mg²⁺ intercalating with IP₃. The MDR1 is an ATP-binding cassette (ABC) transporter family member that is associated to multidrug drug resistance due the ability to translocating amphiphilic compounds⁽³²⁾. The translocation of a substrate across the membrane by proteins like *P. falciparum* MDR1 requires an ATP binding on Q-loop site that cause a rearrangement of TM⁽³³⁾. The binding on IP₃-divalent cation on the MDR1 Q-loop site suggests a potential competition between ATP and IP₃. Interestingly, ATP is known to allosterically modulate the functional of mammal IP₃R^(34, 35) including the inhibition of Ca²⁺ flux regulated by IP₃R under high concentration of ATP⁽³⁵⁾.

To help uncover the cellular function of MDR1 protein, we searched for proteins that interact with MDR1 in the *Plasmodium* interactome data⁽²⁴⁾ (Figure 3). It is interesting to note that MDR1 interacts with receptor for activated C kinase (RACK1, PF3D7_1148000). The *Pf*RACK1 can inhibit host IP₃-mediated Ca²⁺ signaling by direct interaction with IP₃R⁽³⁶⁾. This data suggests that *Pf*MDR1 has the potential to associate or have similar functions to other receptors that coordinate signaling events regulated by protein kinase C.

Discussion and conclusion

Phylogenetic analyses and comparative genomic managed to reveal both unique and conserved proteins related to calcium signalling pathways on apicomplexan parasites^(14, 37, 38), nevertheless the IP₃R still remain a major missing piece of this Ca²⁺ signalling toolkit. The conflict with the pharmacological and functional studies using exogenous IP₃ that supports the existence of protein sensitive to IP₃ that is capable to mobilize Ca²⁺ with the bioinformatic data that constantly failed to point an IP₃R candidate suggests this receptor in *Apicomplexa* has a different and unique structure compared to the IP₃-binding core domain from other eukaryotes. The search for an IP₃R in *Apicomplexa* requires a different strategy that do not rely exclusively on bioinformatic tools as BLAST (Basic Local Alignment Search Tool) based on previously known IP₃R.

The use of IP₃ affinity chromatography column has being successfully reported to concentrate proteins that interact with high affinity to IP₃ analogues⁽¹⁵⁾ as well as retained key components from IP₃-Ca²⁺ signalling from proteins extract from tissues⁽¹⁶⁾. In this work, we used a biotin-inositol 1,4,5-triphosphate attached to a high-performance streptavidin-sepharose substrate to initially enrich proteins with IP₃ affinity from unsynchronized isolate *P. falciparum* blood culture. One of the major limitations of using chromatography affinity column based on a short life IP₃ molecule is the number of proteins that are naturally present at sample homogenate that degrades this second messenger. *P. falciparum* contains proteins that can dephosphorylate or phosphorylate IP₃ like inositolpolyphosphate 5-phosphatase and inositol 1,4,5-trisphosphate 3-kinase⁽³⁹⁾. In this protocol, we tried to overcome this limitation by keeping all the binding and elution steps under low temperature while adding LiCl in every buffer. LiCl has being previously used to inhibit the dephosphorylation of IP₃⁽⁴⁰⁻⁴²⁾. Another risk of using an IP₃ affinity column is assume that protein(s) that might act as IP₃R in *Plasmodium* do not bind/interact with strong affinity with the sepharose-streptavidin substrate alone. In this protocol, we excluded all 494 proteins that bind with the sepharose-free IP₃ column as a potential IP₃R candidate (Fig.1).

From the 206 proteins identified exclusively from IP₃-sepharose column, 180 did not contained any TMDs suggesting the protocol used to extract the proteins from parasite lysate benefit mostly soluble proteins that do not strongly interact with lipid bilayers. For future trials, this protocol can be strongly optimized by using a protein extraction that target membrane proteins (MPs). The IP₃R in mammals is a MP protein containing 6 TMDs⁽⁴³⁾. The presence of a TMDs is an important aspect of any MPs to physically interact with biological membranes⁽²⁶⁾. It is fair to predict that any protein with potential function of IP₃R should have TMDs to able to interact with membranes. The table I list all candidates with TMDs exclusively from IP₃-column.

Ca²⁺ is a second messenger that regulate a variety of vital functions in apicomplexan parasites^(37, 44, 45). Accordingly, the use of 2-aminoethoxydiphenyl borinate (2-APB), a pharmacological drug that inhibit IP₃R, abolished spontaneous Ca²⁺ mobilization and compromise intracellular development of blood stage *P. falciparum*⁽¹¹⁾. Pecenin and collaborator⁽²⁸⁾ failed to obtain any viable parasite expressing IP₃-sponge. These data suggest that IP₃-Ca²⁺ signalling pathway has a vital role during intraerythrocytic development of *P. falciparum* and support our hypotheses that a potential candidate for IP₃R in *Plasmodium* not only has to present a TMDs, but also has to be essential. A prediction of gene essentiality in *P. falciparum*, based on the work of Zhang and collaborators⁽¹⁹⁾ is available for consultation at PlasmoDB website.

The pharmacological evidence that supports IP₃-Ca²⁺ signalling pathway in *Apicomplexa* group is not exclusive to malaria parasites, but also present in *Toxoplasma gondii*⁽⁴⁶⁻⁴⁸⁾ and *Babesia bovis*⁽⁴⁹⁾. The strategy to pinpoint the potential candidate for IP₃R in apicomplexan should not rely on gene only exclusive to *Plasmodium* species. Adding this extra meta-analysis step, the list of potential candidates presented exclusively on IP₃-sepharose column is finally reduced to four proteins: a MDR1; a HSP40; an ATCase and antigen UB05. Among those four, only MDR1 and antigen UB05 has an unclear function.

The small number of candidates makes the use of more computational demanding bioinformatic analyses more feasible. A molecular docking allows to target the structural protein complexes from our candidate list against potential ligand as IP₃ or other potent IP₃-analogues drugs like adenophostin A⁽⁵⁰⁾.

Molecular docking on IP₃ on *P. falciparum* MDR1 protein revealed two potential binding sites on TMD: pocket site 1 (binding energy -15.7 kcal/mol) and pocket site 2 (-11.4 kcal/mol), see Figure 2. This data suggests that MDR1 pocket 1 has a higher affinity to IP₃ compared to IP₃-binding core of mammal IP₃R ($\Delta G = -10.3$ kcal/mol on 23°C)⁽⁵¹⁾ and a lower affinity when compare to IP₃-binding with N-terminal region of mammal IP₃R ($\Delta G = -79.5$ kcal/mol)⁽⁵²⁾. Nevertheless, the binding of ATP on Q-Loop site on nucleotide-binding domain (NBD) likely cause profound changes on the TMD region⁽³³⁾ making hard to predict the real affinity of MDR1 protein with IP₃.

In *P. falciparum* the MDR1 gene encodes for a 162.2 kilo Daltons P-glycoprotein located on the digestive vacuole (DV)⁽⁵³⁾ with unclear function, but the polymorphisms within this protein are associated to increase *in vitro* resistance against multiple antimalarial drugs like quinine⁽⁵⁴⁻⁶¹⁾. The MDR1 display a role as a transporter protein bringing solutes into DV and it is consisted of two distinct homologous regions: one cytosolic nucleotide-binding domain (NBD) and a substrate-binding consisting of 11 TMDs^(62, 63). Interestingly, in malaria parasites the DV is an acid compartment known to be a dynamic intracellular Ca²⁺ store^(17, 64-66), making the subcellular location of MDR1 protein suitable for a IP₃R-like candidate. Moreover, the *in vivo* and *in vitro* treatment with IP₃R inhibitor 2-aminoethoxydiphenyl borinate (2-APB) is associated to reverse resistance to antimalarial chloroquine in *P. falciparum* and *P. chabaudi* parasites, presumably by disrupting Ca²⁺ homeostasis⁽⁶⁷⁾.

Multiple antimalarial drugs can also disrupt Ca^{2+} dynamic on parasite^(68, 69), nevertheless there is not direct evident that suggest the MDR1 acts as a Ca^{2+} gate. Considering that agents that disrupt IP_3R channels such as 2-APB block malaria *in vitro* growth^(9, 11, 28), identified this receptor in *Plasmodium* will not only add crucial missing information on malaria Ca^{2+} signalling but it will also present a potential new target for pharmacological treatment. This work aims to stimulate the use of IP_3 -affinity column with bioinformatic strategies as a potential tool to identify proteins that might act as IP_3R in *Apicomplexa*. The MDR1 seems to be a promising candidate to be validated, however, it is initial step from a long rewarding task of finding the *Apicomplexa* channel sensitive to IP_3 .

Conflict of Interest

The authors declare no conflict of financial or commercial interests.

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References:

1. Michell RH. Inositol and its derivatives: their evolution and functions. *Adv Enzyme Regul.* 2011;51(1):84-90.
2. Berridge MJ. Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta.* 2009;1793(6):933-40.
3. Streb H, Irvine RF, Berridge MJ, Schulz I. Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature.* 1983;306(5938):67-9.
4. Berridge MJ, Irvine RF. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature.* 1984;312(5992):315-21.
5. Garcia CRS, Alves E, Pereira PHS, Bartlett PJ, Thomas AP, Mikoshiba K, et al. IP_3 Signaling in Apicomplexan Parasites. *Curr Top Med Chem.* 2017;17(19):2158-65.
6. Passos AP, Garcia CR. Inositol 1,4,5-trisphosphate induced Ca^{2+} release from chloroquine-sensitive and -insensitive intracellular stores in the intraerythrocytic stage of the malaria parasite *P. chabaudi*. *Biochem Biophys Res Commun.* 1998;245(1):155-60.
7. Alves E, Bartlett PJ, Garcia CR, Thomas AP. Melatonin and IP_3 -induced Ca^{2+} release from intracellular stores in the malaria parasite *Plasmodium falciparum* within infected red blood cells. *J Biol Chem.* 2011;286(7):5905-12.
8. Gazarini ML, Thomas AP, Pozzan T, Garcia CR. Calcium signaling in a low calcium environment: how the intracellular malaria parasite solves the problem. *J Cell Biol.* 2003;161(1):103-10.

9. Beraldo FH, Mikoshiba K, Garcia CR. Human malarial parasite, *Plasmodium falciparum*, displays capacitative calcium entry: 2-aminoethyl diphenylborinate blocks the signal transduction pathway of melatonin action on the *P. falciparum* cell cycle. *J Pineal Res.* 2007;43(4):360-4.
10. Martin SK, Jett M, Schneider I. Correlation of phosphoinositide hydrolysis with exflagellation in the malaria microgametocyte. *J Parasitol.* 1994;80(3):371-8.
11. Enomoto M, Kawazu S, Kawai S, Furuyama W, Ikegami T, Watanabe J, et al. Blockage of spontaneous Ca^{2+} oscillation causes cell death in intraerythrocytic *Plasmodium falciparum*. *PLoS One.* 2012;7(7):e39499.
12. Raabe AC, Wengelnik K, Billker O, Vial HJ. Multiple roles for *Plasmodium berghei* phosphoinositide-specific phospholipase C in regulating gametocyte activation and differentiation. *Cell Microbiol.* 2011;13(7):955-66.
13. Mikoshiba K. IP_3 receptor/ Ca^{2+} channel: from discovery to new signaling concepts. *J Neurochem.* 2007;102(5):1426-46.
14. Prole DL, Taylor CW. Identification of intracellular and plasma membrane calcium channel homologues in pathogenic parasites. *PLoS One.* 2011;6(10):e26218.
15. Hirata M, Yanaga F, Koga T, Ogasawara T, Watanabe Y, Ozaki S. Stereospecific recognition of inositol 1,4,5-trisphosphate analogs by the phosphatase, kinase, and binding proteins. *J Biol Chem.* 1990;265(15):8404-7.
16. Kishigami A, Ogasawara T, Watanabe Y, Hirata M, Maeda T, Hayashi F, et al. Inositol-1,4,5-trisphosphate-binding proteins controlling the phototransduction cascade of invertebrate visual cells. *J Exp Biol.* 2001;204(Pt 3):487-93.
17. Biagini GA, Bray PG, Spiller DG, White MR, Ward SA. The digestive food vacuole of the malaria parasite is a dynamic intracellular Ca^{2+} store. *J Biol Chem.* 2003;278(30):27910-5.
18. Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science.* 1976;193(4254):673-5.
19. Zhang M, Wang C, Otto TD, Oberstaller J, Liao X, Adapa SR, et al. Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. *Science.* 2018;360(6388).
20. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Res.* 2003;31(13):3381-5.
21. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol.* 2001;305(3):567-80.
22. Grosdidier A, Zoete V, Michielin O. SwissDock, a protein-small molecule docking web service based on EADock DSS. *Nucleic Acids Res.* 2011;39(Web Server issue):W270-7.
23. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem.* 2004;25(13):1605-12.
24. Hillier C, Pardo M, Yu L, Bushell E, Sanderson T, Metcalf T, et al. Landscape of the *Plasmodium* Interactome Reveals Both Conserved and Species-Specific Functionality. *Cell Rep.* 2019;28(6):1635-47 e5.
25. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 2003;13(11):2498-504.

26. Cosson P, Perrin J, Bonifacino JS. Anchors aweigh: protein localization and transport mediated by transmembrane domains. *Trends Cell Biol.* 2013;23(10):511-7.
27. Usui-Aoki K, Matsumoto K, Koganezawa M, Kohatsu S, Isono K, Matsubayashi H, et al. Targeted expression of Ip3 sponge and Ip3 dsRNA impairs sugar taste sensation in *Drosophila*. *J Neurogenet.* 2005;19(3-4):123-41.
28. Pecenin MF, Borges-Pereira L, Levano-Garcia J, Budu A, Alves E, Mikoshiba K, et al. Blocking IP3 signal transduction pathways inhibits melatonin-induced Ca(2+) signals and impairs *P. falciparum* development and proliferation in erythrocytes. *Cell Calcium.* 2018;72:81-90.
29. Walsh P, Bursac D, Law YC, Cyr D, Lithgow T. The J-protein family: modulating protein assembly, disassembly and translocation. *EMBO Rep.* 2004;5(6):567-71.
30. Simmer JP, Kelly RE, Rinker AG, Jr., Zimmermann BH, Scully JL, Kim H, et al. Mammalian dihydroorotase: nucleotide sequence, peptide sequences, and evolution of the dihydroorotase domain of the multifunctional protein CAD. *Proc Natl Acad Sci U S A.* 1990;87(1):174-8.
31. Nosol K, Romane K, Irobalieva RN, Alam A, Kowal J, Fujita N, et al. Cryo-EM structures reveal distinct mechanisms of inhibition of the human multidrug transporter ABCB1. *Proc Natl Acad Sci U S A.* 2020;117(42):26245-53.
32. Koenderink JB, Kavishe RA, Rijpma SR, Russel FG. The ABCs of multidrug resistance in malaria. *Trends Parasitol.* 2010;26(9):440-6.
33. Jones PM, O'Mara ML, George AM. ABC transporters: a riddle wrapped in a mystery inside an enigma. *Trends Biochem Sci.* 2009;34(10):520-31.
34. Ferris CD, Haganir RL, Snyder SH. Calcium flux mediated by purified inositol 1,4,5-trisphosphate receptor in reconstituted lipid vesicles is allosterically regulated by adenine nucleotides. *Proc Natl Acad Sci U S A.* 1990;87(6):2147-51.
35. Bezprozvanny I, Ehrlich BE. ATP modulates the function of inositol 1,4,5-trisphosphate-gated channels at two sites. *Neuron.* 1993;10(6):1175-84.
36. Sartorello R, Amaya MJ, Nathanson MH, Garcia CR. The plasmodium receptor for activated C kinase protein inhibits Ca(2+) signaling in mammalian cells. *Biochem Biophys Res Commun.* 2009;389(4):586-92.
37. Nagamune K, Sibley LD. Comparative genomic and phylogenetic analyses of calcium ATPases and calcium-regulated proteins in the apicomplexa. *Mol Biol Evol.* 2006;23(8):1613-27.
38. Ladenburger EM, Sehring IM, Korn I, Plattner H. Novel types of Ca2+ release channels participate in the secretory cycle of *Paramecium* cells. *Mol Cell Biol.* 2009;29(13):3605-22.
39. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature.* 2002;419(6906):498-511.
40. Elabbadi N, Ancelin ML, Vial HJ. Characterization of phosphatidylinositol synthase and evidence of a polyphosphoinositide cycle in *Plasmodium*-infected erythrocytes. *Mol Biochem Parasitol.* 1994;63(2):179-92.
41. Irvine RF, Anggard EE, Letcher AJ, Downes CP. Metabolism of inositol 1,4,5-trisphosphate and inositol 1,3,4-trisphosphate in rat parotid glands. *Biochem J.* 1985;229(2):505-11.

532 42. Thomas AP, Alexander J, Williamson JR. Relationship between inositol
533 polyphosphate production and the increase of cytosolic free Ca^{2+} induced by vasopressin in
534 isolated hepatocytes. *J Biol Chem*. 1984;259(9):5574-84.

535 43. Joseph SK. The inositol triphosphate receptor family. *Cell Signal*. 1996;8(1):1-7.

536 44. Docampo R, Moreno SN, Plattner H. Intracellular calcium channels in protozoa. *Eur J*
537 *Pharmacol*. 2014;739:4-18.

538 45. Budu A, Garcia CR. Generation of second messengers in *Plasmodium*. *Microbes*
539 *Infect*. 2012;14(10):787-95.

540 46. Lourido S, Moreno SN. The calcium signaling toolkit of the Apicomplexan parasites
541 *Toxoplasma gondii* and *Plasmodium* spp. *Cell Calcium*. 2015;57(3):186-93.

542 47. Chini EN, Nagamune K, Wetzel DM, Sibley LD. Evidence that the cADPR signalling
543 pathway controls calcium-mediated microneme secretion in *Toxoplasma gondii*. *Biochem J*.
544 2005;389(Pt 2):269-77.

545 48. Lovett JL, Marchesini N, Moreno SN, Sibley LD. *Toxoplasma gondii* microneme
546 secretion involves intracellular Ca^{2+} release from inositol 1,4,5-triphosphate
547 (IP₃)/ryanodine-sensitive stores. *J Biol Chem*. 2002;277(29):25870-6.

548 49. Florin-Christensen J, Suarez CE, Florin-Christensen M, Hines SA, McElwain TF, Palmer
549 GH. Phosphatidylcholine formation is the predominant lipid biosynthetic event in the
550 hemoparasite *Babesia bovis*. *Mol Biochem Parasitol*. 2000;106(1):147-56.

551 50. Mak DO, McBride S, Foscett JK. ATP-dependent adenophostin activation of inositol
552 1,4,5-trisphosphate receptor channel gating: kinetic implications for the durations of
553 calcium puffs in cells. *J Gen Physiol*. 2001;117(4):299-314.

554 51. Ding Z, Rossi AM, Riley AM, Rahman T, Potter BV, Taylor CW. Binding of inositol
555 1,4,5-trisphosphate (IP₃) and adenophostin A to the N-terminal region of the IP₃ receptor:
556 thermodynamic analysis using fluorescence polarization with a novel IP₃ receptor ligand.
557 *Mol Pharmacol*. 2010;77(6):995-1004.

558 52. Chandran A, Chee X, Prole DL, Rahman T. Exploration of inositol 1,4,5-trisphosphate
559 (IP₃) regulated dynamics of N-terminal domain of IP₃ receptor reveals early phase
560 molecular events during receptor activation. *Sci Rep*. 2019;9(1):2454.

561 53. Cowman AF, Karcz S, Galatis D, Culvenor JG. A P-glycoprotein homologue of
562 *Plasmodium falciparum* is localized on the digestive vacuole. *J Cell Biol*. 1991;113(5):1033-42.

563 54. Sidhu AB, Verdier-Pinard D, Fidock DA. Chloroquine resistance in *Plasmodium*
564 *falciparum* malaria parasites conferred by pfcrt mutations. *Science*. 2002;298(5591):210-3.

565 55. Sidhu AB, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA.
566 Decreasing pfmdr1 copy number in *Plasmodium falciparum* malaria heightens susceptibility
567 to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. *J Infect Dis*.
568 2006;194(4):528-35.

569 56. Sanchez CP, Stein WD, Lanzer M. Dissecting the components of quinine
570 accumulation in *Plasmodium falciparum*. *Mol Microbiol*. 2008;67(5):1081-93.

571 57. Basco LK, Le Bras J, Rhoades Z, Wilson CM. Analysis of pfmdr1 and drug susceptibility
572 in fresh isolates of *Plasmodium falciparum* from sub-Saharan Africa. *Mol Biochem Parasitol*.
573 1995;74(2):157-66.

574 58. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. Pgh1 modulates sensitivity and
575 resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature*. 2000;403(6772):906-
576 9.

59. Cowman AF, Galatis D, Thompson JK. Selection for mefloquine resistance in *Plasmodium falciparum* is linked to amplification of the *pfmdr1* gene and cross-resistance to halofantrine and quinine. *Proc Natl Acad Sci U S A*. 1994;91(3):1143-7.
60. Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC. The tyrosine-86 allele of the *pfmdr1* gene of *Plasmodium falciparum* is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. *Mol Biochem Parasitol*. 2000;108(1):13-23.
61. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, et al. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. *Lancet*. 2004;364(9432):438-47.
62. Friedrich O, Reiling SJ, Wunderlich J, Rohrbach P. Assessment of *Plasmodium falciparum* PfMDR1 transport rates using Fluo-4. *J Cell Mol Med*. 2014;18(9):1851-62.
63. Rohrbach P, Sanchez CP, Hayton K, Friedrich O, Patel J, Sidhu AB, et al. Genetic linkage of *pfmdr1* with food vacuolar solute import in *Plasmodium falciparum*. *EMBO J*. 2006;25(13):3000-11.
64. Garcia CR, Ann SE, Tavares ES, Dluzewski AR, Mason WT, Paiva FB. Acidic calcium pools in intraerythrocytic malaria parasites. *Eur J Cell Biol*. 1998;76(2):133-8.
65. Borges-Pereira L, Thomas SJ, Dos Anjos ESAL, Bartlett PJ, Thomas AP, Garcia CRS. The genetic Ca(2+) sensor GCaMP3 reveals multiple Ca(2+) stores differentially coupled to Ca(2+) entry in the human malaria parasite *Plasmodium falciparum*. *J Biol Chem*. 2020;295(44):14998-5012.
66. Varotti FP, Beraldo FH, Gazarini ML, Garcia CR. *Plasmodium falciparum* malaria parasites display a THG-sensitive Ca2+ pool. *Cell Calcium*. 2003;33(2):137-44.
67. Mossaad E, Furuyama W, Enomoto M, Kawai S, Mikoshiba K, Kawazu S. Simultaneous administration of 2-aminoethyl diphenylborinate and chloroquine reverses chloroquine resistance in malaria parasites. *Antimicrob Agents Chemother*. 2015;59(5):2890-2.
68. Lee AH, Dhingra SK, Lewis IA, Singh MK, Siriwardana A, Dalal S, et al. Evidence for Regulation of Hemoglobin Metabolism and Intracellular Ionic Flux by the *Plasmodium falciparum* Chloroquine Resistance Transporter. *Sci Rep*. 2018;8(1):13578.
69. Gazarini ML, Sigolo CA, Markus RP, Thomas AP, Garcia CR. Antimalarial drugs disrupt ion homeostasis in malarial parasites. *Mem Inst Oswaldo Cruz*. 2007;102(3):329-34.

Figure legends:

Figure 1: Schematic approached to pinpoint potential candidates for IP₃R from isolates blood stage *P. falciparum*. 1° step: selection of proteins that are exclusively found on IP₃-Biotin-streptavidin-sepharose column. 2° step: selection of proteins that contains at least one transmembrane domain. 3° step: selection of protein that are considered essential for malaria parasite during red blood stage development. 4° step: Selection of proteins that are conserve among most species within *Apicomplexa* group. 5° step: selection of protein with unknow or non-specific metabolic function. 6° step: candidates with positive *in silico* docking against IP₃.

Figure 2: Schematic representation of the SwissDock most energetically favored binding poses of IP₃-MDR1. The cytosolic nucleotide-binding domain (upper part) display an IP₃ associated with divalent cation (green spheres) interacting on the same ATP binding pocket. The transmembrane domains (lower part) display two possible pocket sites on IP₃ interaction and their respective interaction energy values (kcal/mol). On the right side, details of the IP₃-MDR1 pocket 1 interaction, a region rich on lysine.

Figure 3: Proteins that interact with MDR1. The network shows the proteins (grey nodes) that interact with MDR1 (red node) according to the *Plasmodium* interactome data⁽²⁴⁾. Gene codes: 2270.T00246, translation initiation factor (PF3D7_0607000). SNRPD1, small nuclear ribonucleoprotein Sm D1 (PF3D7_1125500). ABCE1, ABC transporter E family member 1 (PF3D7_1368200). PRP2, pre-mRNA-splicing factor ATP-dependent RNA helicase PRP2 (PF3D7_1231600). PF11_0488, serine/threonine protein kinase (PF3D7_1148000). DDX1, ATP-dependent RNA helicase DDX1 (PF3D7_0521700). SEC13, protein transport protein SEC13 (PF3D7_1230700). SEC24A, transport protein Sec24A (PF3D7_1361100). EIF2GAMMA, eukaryotic translation initiation factor 2 subunit gamma (PF3D7_1410600). RON6, rhoptry neck protein 6 (PF3D7_0214900). RACK1, receptor for activated C kinase (PF3D7_0826700). MDR2, multidrug resistance protein 2 (PF3D7_11447900). 2270.T00294, ATP-dependent RNA helicase MTR4, (PF3D7_0602100). DBP5, ATP-dependent RNA helicase DBP5 (PF3D7_1459000). SEC12, guanine nucleotide-exchange factor (PF3D7_1116400). WDR26, WD repeat-containing protein 26 (PF3D7_0518600). CBP20, nuclear cap-binding protein subunit 2 (PF3D7_0415500). PRPF19, pre-mRNA-processing factor 19 (PF3D7_0308600). EIF2BETA, eukaryotic translation initiation factor 2 subunit beta (PF3D7_1010600). 2270.T00096, cleavage stimulation factor subunit 1 (PF3D7_0620500).

Tables:

Table I:

Gene code (PlasmoDB)	Predicted function/ annotation	Number TMDs	Essential gene
PF3D7_1001500	early transcribed membrane protein 10.1	2	Yes
PF3D7_0501300	skeleton-binding protein 1	1	No
PF3D7_1133400	Apical membrane antigen 1	1	No
PF3D7_0827900	Protein disulfide-isomerase	1	Yes
PF3D7_0918000	glideosome-associated protein 50	2	No
PF3D7_1364100	6-cysteine protein P92	1	No
PF3D7_0523000	Multidrug resistance protein 1	11	Yes
PF3D7_0202500	Early transcribed membrane protein 2	1	No
PF3D7_0817500	histidine triad nucleotide-binding protein 1	1	No
PF3D7_0402100	Plasmodium exported protein (PHISTb), unknown function	1	No
PF3D7_0501200	parasite-infected erythrocyte surface protein	3	No
PF3D7_0501100	heat shock protein 40, type II	1	yes
PF3D7_1252100	rhoptry neck protein 3	3	Yes
PF3D7_1237700	conserved protein, unknown function	5	Yes
PF3D7_0801800	mannose-6-phosphate isomerase, putative	1	No
PF3D7_0731300	Plasmodium exported protein (PHISTb), unknown function	1	Yes
PF3D7_0702500	Plasmodium exported protein, unknown function	2	No
PF3D7_1344800	aspartate carbamoyltransferase	1	Yes
PF3D7_1332600	DNA-(apurinic or apyrimidinic site) lyase 1	1	No
PF3D7_1105300	Conserved Plasmodium protein, unknow function	1	Yes

PF3D7_1038000.1	Antigen UB05	2	Yes
PF3D7_1016900	early transcribed membrane protein 10.3	2	No
PF3D7_1002100	EMP1-trafficking protein	1	No
PF3D7_1476600	Plasmodium exported protein, unknown function	1	No
PF3D7_1458100	protein PET117, putative	1	No
PF3D7_0508000	6-cysteine protein	1	Yes

649 The table I: The list of 26 proteins exclusively found at IP₃-biotin-streptavidin-sepharose
650 column that contains at least one TMDs.

P. falciparum
**Protein
Lysate**
(unsynchronised
*i*RBCs)

1°
700
Candidates

700-494

206
Specific
Candidates

494
Candidates

2°
206-180
26
Candidates
with transmembrane
domains

3°
26-15
11
Candidates
deemed essential
for function

4°
11-7
4
Candidates
conserved among
apicomplexan

5°
4-2
2
Candidates
with non-specific or
unknown
predicted function

6°
2-1
1
Candidates
predicted IP3
interaction
via *in silico* docking