Current Research in Microbial Sciences 4 (2023) 100179

Contents lists available at ScienceDirect

Current Research in Microbial Sciences

journal homepage: www.sciencedirect.com/journal/current-research-in-microbial-sciences

Combining IP_3 affinity chromatography and bioinformatics reveals a novel protein-IP_3 binding site on *Plasmodium falciparum* MDR1 transporter

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ARTICLE INFO

Keywords:
Plasmodium falciparum
IP_3 receptor
Malaria
Signaling
MDR transporter

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 identify 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 (IP_3) is an important second messenger that regulates cytosolic Ca^{2+} in a variety of Eukaryotic organisms (Michell, 2011; Berridge, 2009). Briefly, the activation of phospholipase C (PLC) mediated by surface receptor breaks phosphatidylinositol 4,5-bisphosphate (PIP_2) into soluble short life second messenger IP_3 that binds into IP_3 receptor (IP_3R), culminating in intracellular Ca^{2+} release (Streb et al., 1983; Berridge and Irvine, 1984).

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 IP_3, see review (Garcia et al., 2017). IP_3 can mobilize $Ca^{2+}$from intracellular stores in isolate and permeabilize blood-stage *P. chabaudi* (Passos and Garcia, 1998) and in intact *P. falciparum* within red blood cells (RBCs) (Alves et al., 2011). Within RBCs, parasites manage to maintain the Ca^{2+} stores full even under a low Ca^{2+}environment (Gazarini et al., 2003). An increasing number of reports supporting the existence of intracellular Ca^{2+}release induced by IP_3 in malaria parasites (Passos and Garcia, 1998; Alves et al., 2011; Beraldo et al., 2007; Martin et al., 1994; Enomoto et al., 2012; Raabe et al., 2011) suggest the existence of a Ca^{2+} channel sensitive to IP_3, the IP_3R.

The IP_3R is a well know described protein in vertebrates and contains around four to six transmembrane domains (TMDs), see review (Mikoshiba, 2007). Prole and Taylor (2011) used the sequence of mammal N-terminal IP_3R binding domain and the amino-terminal RIH (Ryanodine and IP_3R 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 IP_3R in *Apicomplexa*. So far, no apicomplexan IP_3R candidate has been identified or suggested through bioinformatics approach. Moreover, there is no publication that attempted to use a biochemical approach like an IP_3 affinity chromatography column in *Apicomplexa* to identify proteins that might bind to IP_3.

Hirata and collaborators (Hirata et al., 1990) managed to enrich proteins from rat brain sample that has an affinity to IP_3, like IP_5-phosphatase and IP_3 3-kinase using an analogous IP_3 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 (Kishigami et al., 2001) managed to identify the PLC protein from octopus' eyes *Todarodes pacificus* and reported that squid rhodopsin also has an affinity to IP_3. Nevertheless, besides the potential of these columns to enrich proteins that bind to IP_3, no IP_3R has ever been identified using an

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https://doi.org/10.1016/j.crmicr.2022.100179

Available online 18 December 2022

IP_3-affinity column alone.

By adapting the protocol from Hirata/Kishigami (Hirata et al., 1990; Kishigami et al., 2001), we created a column containing IP_3 conjugated with biotin linked with a high-performance sepharose-streptavidin and challenged with proteins from asynchronous *P. falciparum* blood-stage. Using an IP_3-free column containing only sepharose-streptavidin as a reference, we selected only the candidates exclusive on the IP_3-column to undergo a series of bioinformatic meta-analyses. Our approach targeted 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 IP_3.

Using this strategy, we identified the *P. falciparum* multidrug resistance protein 1 (*Pf*MDR1) as a vital and conserved membrane protein that has the potential to bind to IP_3. This protein is located on the parasite food vacuole, a Ca^{2+} storage compartment (Biagini et al., 2003). Combined, our IP_3 affinity column and bioinformatic approach successfully narrow to provide the first small list of malaria proteins candidates with quintessential features expected from an IP_3R.

Material and methods

P. falciparum culture

P. falciparum (D37) parasites were maintained in culture as described (Trager and Jensen, 1976). Briefly, *P. falciparum* were cultured in RPMI media supplemented with 50 mg/L hypoxanthine; 40 mg/L gentamycin; 435 mg/L $NaHCO_3$; 2% hematocrit of A^+ human red blood cells and 10% A^+human blood serum in an atmosphere of 5% CO_2; 3% O_2; 92% N_2 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. The culture was washed three times in PBS (300 g, 5 min) 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 min 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 s for 3 times on ice (10 s interval between each sonication) follow by a 1300 g centrifugation for 10 min at 4 °C for removal of the insoluble pellet. DNAse and RNAse (final concentration 200 ng/µL each) were added on soluble pellet and incubated 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.

IP_3-affinity chromatography column

For the column, it was used a commercial high performance Sepharose substrate bound to streptavidin (GE Healthacare Life Science) and biotin-conjugated IP_3 (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 NaH_2PO_4, 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 IP_3-biotin. The columns were left by constant stirring for 12 h at 4 °C in a dark environment and then centrifuged for 1 min, 300 g at 4 °C. The supernatant containing excess IP_3-biotin was removed and columns were washed five times with 2 ml of ice-cold binding buffer to remove any free IP_3-biotin. Two distinct columns were assembled: one containing IP_3-biotin-sepharose-streptavidin and other containing only sepharose-streptavidin. In each column was loaded with 2.5 mg of *P. falciparum* protein extract and the 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 h and finally centrifuged for 1 min at 300 g at 4 °C to discard the supernatant. Each column was washed seven times with ice-bound binding buffer with protease inhibitors. To elute the proteins, 1 mL 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 followed by constant stirring for 1 h at 4 °C. At the end of incubation, the columns were centrifuged for 1 min at 300 g, and the supernatant was collected in sterile low binding protein Eppendorf.

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 gel sections 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 were considered for analyses.

Transmembrane domain prediction

To detect a transmembrane domain's presence, the whole amino acid sequence from the protein identified at mass spectrometry was analysed using the public HMMTOP program version 2.0 (www enzim.hu/hmmtop/). This program predicts the number of transmembrane helicases and their position from the 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 et al. (2018) available on PlasmoDB (https://plasmodb.org/plasmo). To identify orthologs candidates among the *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 IP_3

The primary sequence of MDR1 (Gene - PF3D7_0523000, plasmodb.org) was used to build its probable 3D structure by homology modeling. The server SwissModel (Schwede et al., 2003) was employed to automatically create the models optimized to bind IP_3 at various locations inside MDR1 homology. Blind molecular docking simulations were carried out to obtain possible interactions for the intermembrane domain as predicted by the TMHMM Server (Krogh et al., 2001). The SwissDock (Grosdidier et al., 2011) server enabled the study of IP_3 intermembrane MDR1 domain binding poses. Additionally, the IP_3-Ion-MDR1 binding was further investigated using the multidrug transporter permeability (P)-glycoprotein is 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 IP_3-Ion-MDR1 interaction. IP_3 was manually positioned inside the ATP cavity to mimic an $IP3\text{-}Mg^{2+}$ interaction. The binding conformation was optimized with molecular mechanics employing the UCSF (Pettersen et al., 2004) chimera minimize structure tools.

Protein-protein interaction network

Using *Plasmodium* interactome data (Hillier et al., 2019), we looked for the proteins that interact with the MDR1. The protein annotation and functions were also retrieved from the original publication. The network was generated using Cytoscape (Shannon et al., 2003).

Results

IP_3-affinity chromatography data

Adapting the protocol based on Hirata/Mishigami (Hirata et al., 1990; Kishigami et al., 2001), we use an IP_3 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 IP_3R receptor in a mammal (Fig. 1).

The access code of the brute data on mass spectrometry analyses from the eluate samples of the IP_3-affinity chromatography column can be found in Supplemental Material Table 1. At least 695 proteins from *P. falciparum* containing at least one exclusive peptide were detected from the IP_3-sepharose column. In comparison, 494 proteins were detected from the sepharose matrix alone (Fig. 1). All proteins exclusively present on IP_3-sepharose were selected (total 201 proteins) for the bioinformatic meta-analyses (Sup. Table 2).

Once the proteins exclusive for IP_3-column were identified (Sup. Table 2), the first bioinformatic approach aimed to select proteins that contain at least one transmembrane domain (TMD). The TMD is an important structure to anchor proteins through biological membranes by its physical properties like the length and hydrophilicity of the transmembrane span (Cosson et al., 2013), every IP_3R in vertebrates, invertebrates and single eukaryotes organism possess a TMDs, so we used this feature as the second step to select potential candidates for IP_3R. Fig. 1.

Table 1 summarizes 26 proteins exclusively found at IP_3-biotin-streptavidin-sepharose column containing at least one TMDs. Transfection of *P. falciparum* to constitutively express IP_3-sponge, a protein containing a modified IP_3 binding domain based on mouse IP_3R that sequestrates cytosolic IP_3 (Usui-Aoki et al., 2005), did not result in viable parasites (Pecenin et al., 2018)' suggesting a vital role of IP_3 signaling in *P. falciparum*. Accordingly, the next step to narrow the number of potential candidates that might act as IP_3R in malaria is to focus on essential genes. To deem whether a gene is essential, we considered only the candidates that scored lower than 0.5 on its mutagenic index of phenotype graphic (data provided by PlasmoDB). That decreases the number of candidates to 11 (Fig. 1, Table 1).

There is pharmacological evidence of the IP_3R in multiple apicomplexan parasites (Garcia et al., 2017), so in our analyses we considered only conserved genes among multiples species withing the *Apicomplexa* phylum as the fourth step for candidate screening. 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

Table 1
The table I: The list of 26 proteins exclusively found at IP_3-biotin-streptavidin-sepharose column that contains at least one TMDs.

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

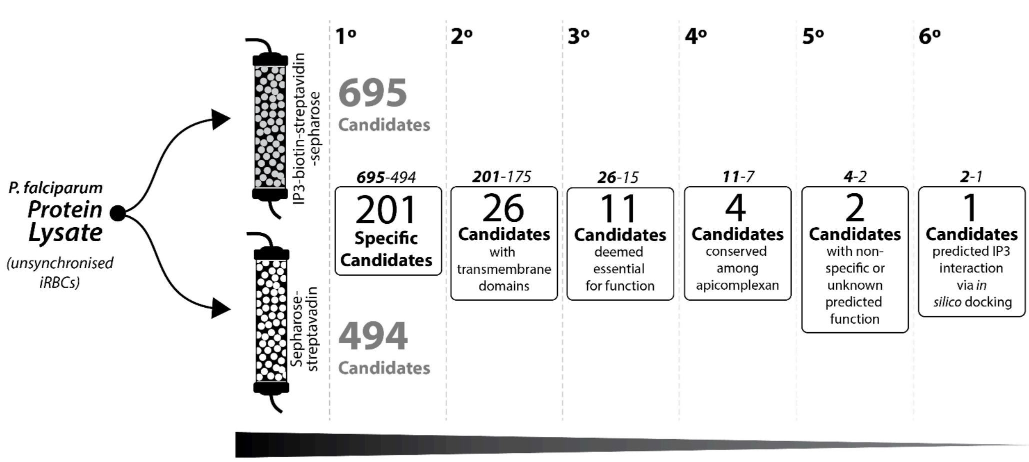

Fig. 1. Schematic approached to pinpoint potential candidates for IP_3R from isolates blood stage *P. falciparum*. 1° step: selection of proteins that are exclusively found on IP_3-Biotin-streptavidin-sepharose column. 2° step: selection of proteins that contains at least one transmembrane domain. 3° 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 A*picomplexa* group. 5° step: selection of protein with unknow or non-specific metabolic function. 6° step: candidates with positive in-silico docking against IP_3.

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) (Walsh et al., 2004), and the ATCase is an enzyme important for the pyrimidine biosynthetic pathway (Simmer et al., 1990). The MDR1 was the only candidate with information available to build a 3D structure by homology modeling to perform an in-silico binding with IP_3.

IP_3-MRD1 binding modeling and protein interactions network

The MDR1 model provides by the SwissModel server proved to be quite similar to the human P-glycoprotein ABCB1 receptor, protein data bank id: 7A69 (Nosol et al., 2020). 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 IP_3 binding was estimated by the SwissDock server (Fig. 2).

The pocket 1 (binding energy −15.7 kcal/mol) proved to be the best IP_3 docking position. The site is a lysin rich domain able to form various hydrogen bonds with IP_3. 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 Mg^{2+} intercalating with IP_3. The MDR1 is an ATP-binding cassette (ABC) transporter family member associated with multidrug drug resistance due to translocating amphiphilic compounds (Koenderink et al., 2010). The translocation of a substrate across the membrane by proteins like *P. falciparum* MDR1 requires an ATP binding on Q-loop site that causes a rearrangement of TM (Jones et al., 2009). The binding on IP_3-divalent cation on the MDR1 Q-loop site suggests a potential competition between ATP and IP_3. Interestingly, ATP is known to allosterically modulate the functional of mammal IP_3R (Ferris et al., 1990; Bezprozvanny and Ehrlich, 1993)' including the inhibition of Ca^{2+}flux regulated by IP_3R under a high concentration of ATP (Bezprozvanny and Ehrlich, 1993).

To help uncover the cellular function of MDR1 protein, we searched for proteins that interact with MDR1 in the *Plasmodium* interactome data (Hillier et al., 2019) (Fig. 3). The data suggests that MDR1 interacts with activated C kinase receptors (RACK1, PF3D7_1148000). The *Pf*RACK1 can inhibit host IP_3-mediated Ca^{2+}signaling by direct interaction with IP_3R (Sartorello et al., 2009). The interaction with eukaryotic translation initiation factor 2 (EIF2, PF3D7_1410600), EIF2β (PF3D7_1010600), EIF2γ (PF3D7_1410600) and serine/threonine protein kinase (PF3D7_1148000) suggests that *Pf*MDR1 can associate or have similar functions to other receptors and nuclear factors that coordinate signaling events regulated by protein kinase.

Discussion and conclusion

Phylogenetic analyses and comparative genomic data revealed both unique and conserved proteins related to calcium signaling pathways on apicomplexan parasites (Prole and Taylor, 2011; Nagamune and Sibley, 2006; Ladenburger et al., 2009), nevertheless, the IP_3R still remains a

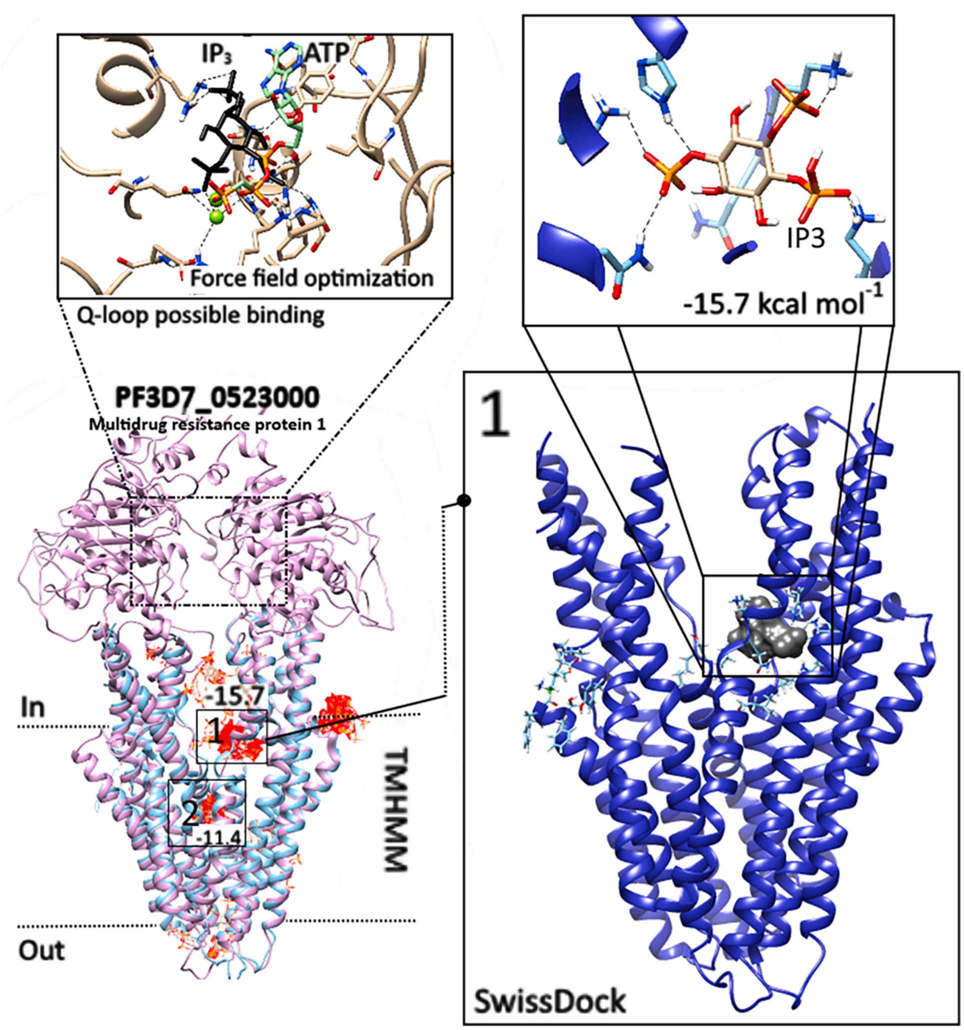

Fig. 2. Schematic representation of the SwissDock most energetically favored binding poses of IP_3−MDR1. The cytosolic nucleotide-binding domain (upper part) display an IP_3 associated with divalent cation (green spheres) interacting on the same ATP binding pocket. The transmembrane domains (lower part) display two possible pocket sites on IP_3 interaction and their respective interaction energy values (kcal/mol). On the right side, details of the IP_3−MDR1 pocket 1 interaction, a region rich on lysine..

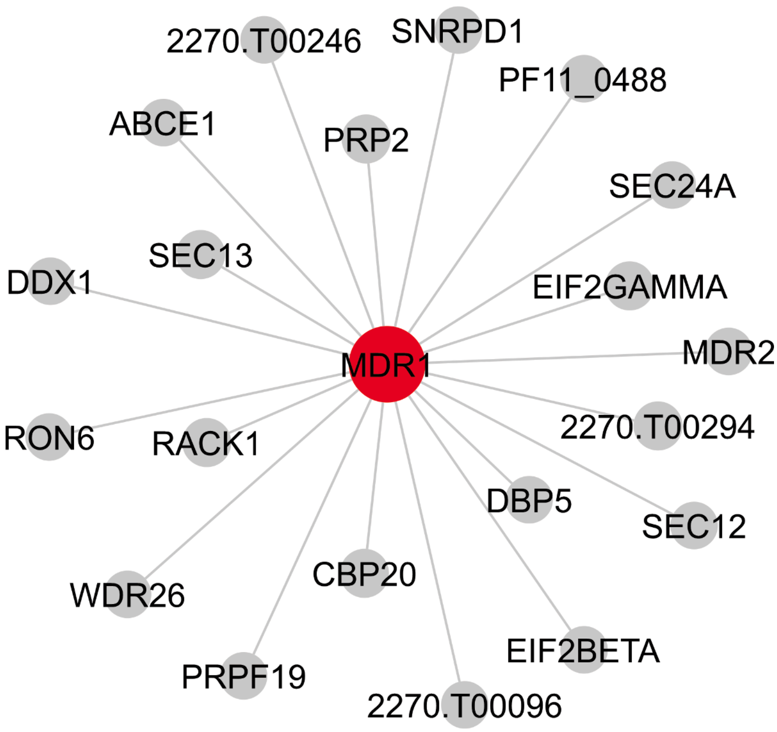

Fig. 3. Proteins that interact with MDR1. The network shows the proteins (gray nodes) that interact with MDR1 (red node) according to the *Plasmodium* interactome data (Hillier et al., 2019). Gene codes: 2270.T00246, translation initiation factor (PF3D7_0607000). SNRPD1, small nuclear ribonucleoprotein Sm D1(PF3D7_1125500). ABCE1, ABC transporter E family member 1 (PF3D7_1,368,200). 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).

major missing piece of this Ca^{2+}signaling toolkit. Studies using exogenous IP_3 on malaria parasites support the existence of protein sensitive to IP_3 that is capable to trigger a Ca^{2+} response (Passos and Garcia, 1998; Alves et al., 2011). The constant failed to identify this protein in *Apicomplexa* suggests this group has a distinct and unique structure compared to the IP_3-binding core domain from other eukaryotes. The search for an IP_3R in *Apicomplexa* requires a different strategy that does not rely exclusively on bioinformatics tools as BLAST (Basic Local Alignment Search Tool) based on previously known IP_3R.

The use of IP_3 affinity chromatography column has been successfully reported to concentrate proteins that interact with high affinity to IP_3 analogues (Hirata et al., 1990) and retained key components from IP_3—Ca^{2+} signaling from proteins extract from tissues (Kishigami et al., 2001). In this work, we used a biotin-inositol 1,4,5-triphosphate attached to a high-performance streptavidin-sepharose substrate to initially enriching proteins with IP_3 affinity from unsynchronized isolate *P. falciparum* blood culture. One of the significant limitations of using chromatography affinity column based on a short life IP_3 molecule is the number of naturally present proteins at sample homogenate that degrade this second messenger. *P. falciparum* contains proteins that can dephosphorylate or phosphorylate IP_3 like inositol polyphosphate 5-phosphatase and inositol 1,4,5-trisphosphate 3-kinase (Gardner et al., 2002). 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 been previously used to inhibit the dephosphorylation of IP_3 (Elabbadi et al., 1994; Irvine et al., 1985; Thomas et al., 1984). Another risk of using an IP_3 affinity column assumes that protein(s) that might interact with IP_3 in *Plasmodium* do not bind/interact with strong affinity with the sepharose-streptavidin substrate alone. We excluded all 494 proteins that bind with the sepharose-free IP_3 column as a potential IP_3R candidate (Fig. 1).

From the 201 proteins identified exclusively from the IP_3-sepharose column, 175 did not contain any TMDs suggesting the protocol used to extract the proteins from parasite lysate benefited mostly soluble proteins that do not strongly interact with lipid bilayers. This protocol can be optimized for future trials by using a protein extraction that targets membrane proteins (MPs). The IP_3R in mammals is an MP protein containing 6 TMDs (Joseph, 1996). The presence of TMDs is an essential aspect of any MPs to physically interact with biological membranes (Cosson et al., 2013). It is fair to predict that any protein with the potential function of IP_3R should have TMDs to interact with membranes. Table 1 list all candidates with TMDs exclusively from IP_3-column.

$Ca^{2+}$is a second messenger that regulates a variety of vital functions in apicomplexan parasites (Nagamune and Sibley, 2006; Docampo et al., 2014; Budu and Garcia, 2012). Accordingly, the use of 2-aminoethoxydiphenyl borinate (2-APB), a pharmacological drug that inhibits IP_3R, abolished spontaneous Ca^{2+}mobilization and compromise intracellular development of blood stage *P. falciparum* (Enomoto et al., 2012). Pecenin and collaborator (Pecenin et al., 2018) failed to obtain any viable parasite expressing IP_3-sponge. These data suggest that the IP_3—Ca^{2+} signaling pathway has a vital role during intraerythrocytic development of *P. falciparum* and support our hypotheses that a potential candidate for IP_3R 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 (Zhang et al., 2018) is available for consultation at the PlasmoDB website.

The pharmacological evidence that supports the IP_3—Ca^{2+} signaling pathway in the *Apicomplexa* group is not exclusive to malaria parasites but also present in *Toxoplasma gondii* (Lourido and Moreno, 2015; Chini et al., 2005; Lovett et al., 2002) and *Babesia bovis* (Florin-Christensen et al., 2000). The strategy to pinpoint the potential candidate for IP_3R 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 the IP_3-sepharose column is finally reduced to four proteins: a MDR1; HSP40; an ATCase, and antigen UB05. Among those four, only MDR1 and antigen UB05 currently have an undefined function.

The small number of candidates makes the use of more computational demanding bioinformatic analyses more feasible. A molecular docking allows us to target the structural protein complexes from our candidate list against potential ligand as IP_3 or other potent IP_3-analogues drugs like adenophostin A (Mak et al., 2001).

Molecular docking on IP_3 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 Fig. 2. This data suggests that MDR1 pocket **1** has a higher affinity to IP_3 compared to IP_3-binding core of mammal IP_3R ($\Delta G = -10.3$ kcal/mol on 23 °C) (Ding et al., 2010) and a lower affinity when compare to IP_3-binding with N-terminal region of mammal IP_3R ($\Delta G = -79.5$ kcal/mol) (Chandran et al., 2019). Nevertheless, the binding of ATP on Q-Loop site on the nucleotide-binding domain (NBD) likely causes profound changes in the TMD region (Jones et al., 2009)' making it hard to predict the actual affinity of the MDR1 protein with IP_3.

In *P. falciparum,* the MDR1 gene encodes for a 162.2 kg Daltons P-glycoprotein located on the digestive vacuole (DV) (Cowman et al., 1991) with unclear function. Still, the polymorphisms within this protein are associated with increases *in vitro* resistance against multiple antimalarial drugs like quinine (Sidhu et al., 2002; Sidhu et al., 2006; Sanchez et al., 2008; Basco et al., 1995; Reed et al., 2000; Cowman et al., 1994; Duraisingh et al., 2000; Price et al., 2004). The MDR1 displays a role as a transporter protein that brings solutes into DV. It consists of two distinct homologous regions: one cytosolic nucleotide-binding domain (NBD) and a substrate-binding consisting of 11 TMDs (Friedrich et al., 2014; Rohrbach et al., 2006). Interestingly, in malaria parasites, the DV is an acid compartment known to be a dynamic intracellular Ca^{2+}store (Biagini et al., 2003; Garcia et al., 1998; Borges-Pereira et al., 2020; Varotti et al., 2003), making the subcellular location of MDR1 protein suitable for an IP_3R-like candidate. Moreover, the *in vivo* and *in vitro* treatment with IP_3R inhibitor 2-aminoethoxydiphenyl borinate (2-APB) is associated with reversing resistance to antimalarial chloroquine in *P. falciparum* and *P. chabaudi* parasites, presumably by disrupting Ca^{2+}homeostasis (Mossaad et al., 2015). Multiple antimalarial drugs can also disrupt the Ca^{2+} dynamic on the parasite (Lee et al., 2018; Gazarini et al., 2007), nevertheless, there is no direct evidence that suggests the MDR1 acts as a Ca^{2+} gate.

The lack of information to build a quality 3D model for in-silico analyses on UB05, HSP40 and ATCase candidates does not exclude them as a potential role in sensing IP_3. The next natural step is to obtain functional evidence that these four candidates act as a protein sensitive to IP_3. One suggestion is expressing them on a triple IP_3R knock-out cell lines like DT40 chicken B cell (Winding and Berchtold, 2001) and test its sensitivity to mobilize Ca^{2+} with IP_3.

Considering that agents that disrupt IP_3R channels such as 2-APB block malaria *in vitro* growth (Beraldo et al., 2007; Enomoto et al., 2012; Pecenin et al., 2018), identify this receptor in *Plasmodium* will not only add crucial missing information on malaria Ca^{2+} signaling, 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 waiting to be validated. Nevertheless, this is just an initial but an important first step from a long rewarding task of finding the *Apicomplexa* channel sensitive to IP_3.

Funding

Celia R. S. Garcia is funded by FAPESP (2017/08684–7; 2018/07177–7). Helder Nakaya is funded by FAPESP2018/14933–2.

Author contribution

All authors have contributed to discuss experimental design, discussing the data and manuscript writing. EA; EG and HN performed experiments.

Declaration of Competing Interest

The authors declare no conflict of financial or commercial interests.

Data availability

Data will be made available on request.

Acknowledgements

We thank Prof. Dr Akio Kishigami for the helpful suggestion on the IP_3-Affinity Column. Ross Tomaino from Taplin Mass Spectrometry Facility for helpful support on mass spectrometry data. Colsan (Associação Beneficente de Coleta de Sangue) for providing the human blood and plasma used on parasite culture.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2022.100179.

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