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

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ABSTRACT

Intracellular Ca²⁺ mobilization induced by second messenger IP₃ controls many cellular events in most of the eukaryotic groups. Despite the increasing evidence of IP₃-induced Ca²⁺ in apicomplexan parasites like Plasmodium, responsible for malaria infection, no protein with potential function as an IP₃-receptor has been identified.

The use of bioinformatic analyses based on previously known sequences of IP₃-receptor failed to identify potential IP₃-receptor candidates in any Apicomplexa. In this work, we combine the biochemical approach of an IP₃ affinity chromatography column with bioinformatic meta-analyses to identify potential vital membrane proteins that present binding with IP₃ 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 IP₃. This work provides a new insight for probing potential candidates for IP₃-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 organisms (Michell, 2011; Berridge, 2009). 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 (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 IP₃, see review (Garcia et al., 2017). IP₃ can mobilize Ca²⁺ 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 Ca²⁺ stores full even under a low Ca²⁺ environment (Gazzarini et al., 2003). An increasing number of reports supporting the existence of intracellular Ca²⁺ release induced by IP₃ 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 Ca²⁺ channel sensitive to IP₃, the IP₃R.

The IP₃R is a well known 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 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 or suggested through bioinformatics approach. Moreover, there is no publication that attempted to use a biochemical approach like an IP₃ affinity chromatography column in Apicomplexa to identify proteins that might bind to IP₃.

Hirata and collaborators (Hirata et al., 1990) managed to enrich proteins from rat brain sample that has an affinity to IP₃, like IP₃-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-myoinositol 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 IP₃. Nevertheless, besides the potential of these columns to enrich proteins that bind to IP₃, no IP₃R has ever been identified using an
IP$_3$-affinity chromatography column

For the column, it was used a commercial high performance Sepharose substrate bound to streptavidin (GE Heathacare 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$_2$PO$_4$, 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 helix cases and their position from the peptide/protein amino acid sequence.

Phenotype score, conservation, and function predictions

The phenotype score used the determine gene essentiality of each candidate was obtained from the work of Zhang et al. (2018) available on PlasmoDB (https://plasmodb.org/plasmo). To identify ortholog 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 (Schweid 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 diveral cation at the intracellular domain was used to guide the inspect a hypothetical IP$_3$-Mg$^{2+}$-interaction. IP$_3$ was manually positioned inside the ATP cavity to mimic an IP$_3$-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**

**IP₃-affinity chromatography data**

Adapting the protocol based on Hirata/Mishigami (Hirata et al., 1990; Kishigami et al., 2001), 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 (Fig. 1).

The access code of the brute data on mass spectrometry analyses from the eluate samples of the IP₃-sepharose column containing at least one exclusive peptide were detected from the IP₃-sepharose column. In comparison, 494 proteins were detected from the sepharose matrix alone (Fig. 1). All proteins exclusively present on IP₃-sepharose were selected (total 201 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 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 IP₃R in vertebrates, invertebrates and single eukaryotes organism possess a TMDs, so we used this feature as the second step to select potential candidates for IP₃R. Fig. 1.

Table 1 summarizes 26 proteins exclusively found at IP₃-biotin-streptavidin-sepharose column containing 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 sequesters cytosolic IP₃ (Usui-Aoki et al., 2005), did not result in viable parasites (Pecenin et al., 2018) suggesting a vital role of IP₃ signaling in *P. falciparum*. Accordingly, the next step to narrow the number of potential candidates that might act as IP₃R in malaria is to focus on essential genes. To deem whether a gene is essential, we considered only conserved genes among multiples species within 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.

| 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_1135400       | Apical membrane antigen 1 | 1 | No |
| PF3D7_0827960       | Protein diulifide-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 DNA-arguneric or argyimidic site) lysate 1 | 1 | Yes |
| PF3D7_1132600       | Conserved Plasmodium protein, unknown function | 1 | No |
| PF3D7_1038000.1     | Antigen UB05 | 2 | Yes |
| PF3D7_1016900       | Early transcribed membrane protein 10.3 | 2 | No |
| PF3D7_1002100       | EMF1-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 |

Apicomplexan parasites (Garcia et al., 2017), so in our analyses we considered only conserved genes among multiples species within 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
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 shock 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 IP₃.

**IP₃-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 IP₃ binding was estimated by the SwissDock server (Fig. 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 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 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 (Ferris et al., 1996; Bezprozvanny and Ehrlich, 1993) including the inhibition of Ca²⁺ flux regulated by IP₃R 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 PfRACK1 can inhibit host IP₃-mediated Ca²⁺ signaling by direct interaction with IP₃R (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 PfMDR1 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 IP₃R still remains a
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\(_{3}\)-R 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\(_{3}\)-R.

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 to IP\(_{3}\) initially enriching proteins with IP\(_{3}\) attached to a high-performance streptavidin-sepharose substrate to 2001 ). In this work, we used a biotin-inositol 1,4,5-triphosphate to IP\(_{3}\) search for an IP\(_{3}\) R 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\(_{3}\)-R 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\(_{3}\)-R.

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Ca\(^{2+}\) is a second messenger that regulates a variety of vital functions in apicomplexan parasites (Nagamune and Sibley, 2006; Docampo et al., 2014; Busu and Garcia, 2012). Accordingly, the use of 2-aminoethoxydiphenyl borinate (2-APB), a pharmacological drug that inhibits IP\(_{3}\)-R, abolished spontaneous Ca\(^{2+}\)-mobilization and compromise intracellular development of blood stage P. falciparum (Enomoto et al., 2012). Pecennin and collaborator (Pecennin et al., 2018) failed to obtain any viable parasite expressing IP\(_{3}\)-spponge. 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\(_{3}\)-R 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\(_{3}\)-R 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\(_{3}\)-ana- logues drugs like adenohostin 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 IP3 compared to IP3-binding core of mammalian IP3R (ΔG = −10.3 kcal/mol on 23 °C) (Ding et al., 2010) and a lower affinity when compared to IP3-binding with N-terminal region of mammalian IP3R (ΔG = −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 IP3.

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 Ca2+ 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 IP3R-like candidate. Moreover, the in vivo and in vitro treatment with IP3R inhibitor 2-aminoethoxydiphenyl borinate (2-APB) is associated with reversing resistance to antimalarial chloroquine in P. falciparum and P. chabaudi parasites, presumably by disrupting Ca2+ homeostasis (Mossaad et al., 2015). Multiple antimalarial drugs can also disrupt the Ca2+ dynamic on the parasite (Lee et al., 2018; Gazarian et al., 2007), nevertheless, there is no direct evidence that suggests the MDR1 acts as a Ca2+ 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 IP3. The next step is to obtain functional evidence that these four candidates act as a protein sensitive to IP3. One suggestion is expressing them on a triple IP3R knock-out cell lines like DT40 chicken B cell (Winding and Berchtold, 2001) and test its sensitivity to mobilize Ca2+ with IP3.

Considering that agents that disrupt IP3R 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 Ca2+ signaling, but it will also present a potential new target for pharmacological treatment. This work aims to stimulate the use of IP3-affinity column with bio-informatic strategies as a potential tool to identify proteins that might act as IP3R 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 IP3.

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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.

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Supplementary materials
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