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Immunophilin–protein interactions in *Plasmodium falciparum*

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SUMMARY

Immunophilins comprise two protein families, cyclophilins (CYPs) and FK506-binding proteins (FKBPs), and are the major receptors for the immunosuppressive drugs cyclosporin A (CsA) and FK506 (tacrolimus) respectively. Most eukaryotic species have at least one immunophilin and some of them have been associated with pathogenesis of infectious or parasitic diseases or the action of antiparasitic drugs. The human malarial parasite *Plasmodium falciparum* has thirteen immunophilin or immunophilin-like genes but the functions of their products are unknown. We set out to identify the parasite proteins that interact with the major cyclophilins, PfCYP19A and PfCYP19B, and the FKBP, PfFKBP35, using a combination of co-immunoprecipitation and yeast two-hybrid screening. We identified a cohort of putative interacting partners and further investigation of some of these revealed potentially novel roles in parasite biology. We demonstrated that (i) *P. falciparum* cyclophilins interacted with the heat shock protein Hsp70, (ii) treatment of parasites with cyclophilin ligands disrupted transport of the rhoptry-associated protein RAP1, and (iii) PfFKBP35 interacted with parasite histones in a way that might modulate gene expression. These findings begin to elucidate the functions of immunophilins in malaria. Furthermore, the known antimalarial effects of CsA, FK506, and non-immunosuppressive derivatives of these immunophilin ligands could be mediated through these partner proteins.

Key words: malaria, immunophilin, cyclosporin A, FK506, cyclophilin, FK506-binding protein, histone, rhoptry, Hsp70, protein–protein interaction.

KEY FINDINGS

- Immunophilins PfCYP19A, PfCYP19B and PfFKBP35 probably interact with diverse *P. falciparum* proteins.
- PfCYP19B interacts specifically with PfHsp70.
- Cyclophilin ligands disrupt transport of RAP1 in cultured parasites.
- PfFKBP35 interacts with *P. falciparum* histones, possibly influencing epigenetic modifications.

INTRODUCTION

Correct protein folding depends on the *cis-trans* isomerisation of X-Pro bonds, where X is any other amino acid (Brandts *et al.*, 1975). Uniquely among naturally occurring amino acids, peptidyl-prolyl bonds have a relatively low difference in free energy between the *cis*- and *trans*-conformations. X-Pro bonds spontaneously adopt their intended conformations only extremely slowly, and this effectively limits the rate of folding of some proteins (Fischer & Schmid, 1990). Catalysis of *cis-trans* isomerisation can be mediated by four classes of peptidyl-prolyl *cis-trans* isomerase (PPIase) (Galat, 2003): cyclophilins (CYPs), FK506-binding proteins (FKBPs), Pin1/parvulins and trigger factors. Almost all organisms characterised possess at least one protein from one of these families of PPIases (Galat, 2003). There is no significant sequence homology between the four groups, but they do exhibit some overlap in sequence specificity for X-Pro bonds in peptide substrates. Their active sites also have different architectures and bind to small molecules with totally dissimilar structures (Galat, 2003). Immunophilins (cyclophilins and FKBPs) are grouped together because of their similar roles in the action of the immunosuppressive peptide cyclosporin A (CsA, for which cyclophilins are the major receptors) and the immunosuppressive macrolactones FK506 and rapamycin (both of whose major receptors are FKBPs). The immunosuppressive actions of CsA, FK506 and rapamycin are mediated by drug–immunophilin complexes. CsA–CYP and FK506–FKBP target the phosphoprotein phosphatase calcineurin (PPP3) and rapamycin–FKBP complex inhibits the protein kinase mTOR (mammalian [or mechanistic] target of rapamycin) (Ho *et al.*, 1996). CsA, FK506 and rapamycin are used clinically as immunosuppressants to prevent rejection of transplanted organs. Non-immunosuppressive derivatives of CsA and FK506 have antimalarial activity similar to or better than the parent compounds (Bell *et al.*, 1994; Monaghan *et al.*, 2005). The antimalarial activity of these non-immunosuppressive derivatives suggests that a target or targets exists in the parasite that is distinct from calcineurin.

Are immunophilins required for survival in biological systems? – The answer depends on the species studied. *Caenorhabditis elegans* possesses a number of immunophilin isoforms, many of which have been well characterised. Some of these immunophilins are essential; RNAi experiments have shown some associated phenotypes such as embryonic lethality (Kamath *et al.*, 2003). In bacteria, *Escherichia coli* PPIase SurA is dispensable for growth in culture but required for biogenesis of the pilus that is required for urinary tract invasion (Justice *et al.*, 2005), and mutants of *Bacillus subtilis* with both of the organism's PPIases

deleted had much reduced growth under near-starvation conditions (Gothel *et al.*, 1998). ESS1 is a pin1/parvulin of *Saccharomyces cerevisiae* that is known to be essential (Hanes *et al.*, 1989) but it appears that immunophilins are not required for growth of *S. cerevisiae* under the usual culture conditions (Dolinski *et al.*, 1997). To summarise, with some exceptions immunophilins are only required in response to certain stress conditions or environmental cues. Immunophilins have a number of known roles in disease, including several viral infections and neurodegenerative diseases, and immunophilin ligands are actively being pursued as novel treatments (Frausto *et al.*, 2013; Galat & Bua, 2010; Kang *et al*., 2008).

Plasmodium falciparum, the most prevalent and deadly malaria parasite, possesses thirteen immunophilin or related genes, encoding eleven cyclophilin or cyclophilin-like proteins, an FKBP and an FKBP-like protein (Bell *et al.*, 2006; Krucken *et al.*, 2009; Marin-Menendez & Bell, 2011). Of these thirteen proteins only three, PfCYP19A, PfCYP19B and PfFKBP35, are known to retain the activities characteristic of most immunophilins, i.e. PPIase activity and ability to bind immunosuppressive ligands. All three are also capable of acting as molecular chaperones on model substrates *in vitro*, a feature common to many immunophilins (Marin-Menendez *et al.*, 2012; Monaghan & Bell, 2005). The identities of substrates in the parasite are however unknown. PfCYP19A and PfCYP19B appear to be the most abundant of the blood-stage *P. falciparum* cyclophilins (making up ~1.2% and ~0.5% of total cellular protein respectively) and are located predominantly in the cytosol (Gavigan *et al.*, 2003). Additionally they are the only two proteins that are pulled down from extracts of these stages by cyclosporin-coupled affinity columns (Gavigan *et al.*, 2003). PfCYP19B has also been detected at the surface of infected erythrocytes (Wu & Craig, 2006). PfFKBP35 (Braun *et al.*, 2003) is the only FKBP in *P. falciparum*. It contains an FK506-binding domain (FKBD) linked to a tetratricopeptide repeat (TPR)-containing domain (Kumar *et al.*, 2005; Monaghan & Bell, 2005) and was the only parasite protein detected on affinity columns containing the ethyl FK506 analogue ascomycin (Kumar *et al.*, 2005). During the ring stage, PfFKBP35 is predominantly cytosolic, but as the parasites mature into trophozoites and schizonts, most of it moves to the nucleus (Kumar *et al.*, 2005).

In this study we set out to identify the interacting protein partners of the three major *P. falciparum* immunophilins PfCYP19A, PfCYP19B and PfFKBP35, with a view to elucidating their functions. No previous studies have looked specifically at the protein–protein interactions of immunophilins, though whole proteome yeast 2-hybrid (Y2H) analysis identified one cyclophilin–protein interaction, namely that between PfCYP19A and the product of the gene PF3D7_0604500, a conserved *Plasmodium* protein of unknown function (LaCount *et al.*, 2005). Other studies have shown interactions *in vitro* between PfFKBP35 and heat shock protein 90 (Hsp90) (Kumar *et al.*, 2005) and between PfFKBP35 and calcineurin (Kumar *et al.*, 2005; Monaghan *et al.*, 2005) but in neither case is there evidence that the interaction occurs in intact cells. Therefore, before the present study, almost nothing was known about the immunophilin interactome in *P. falciparum*. We have identified a large cohort of putative interacting partners for the three immunophilins by two separate methods, co-immunoprecipitation (coIP) and Y2H, with significant overlap of interacting partners between all three. Follow-up investigation by a number of different methods revealed a

specific interaction between PfCYP19B and Hsp70, a potential role for PfFKBP35 in regulating histone methylation and a potential role for cyclophilins in chaperoning the rhoptry-associated protein RAP1 to its destination. These data suggest key roles for immunophilins in protein transport and quality control, gene regulation and host cell invasion and may give clues as to the mechanisms of antimalarial action of immunophilin ligands.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents used in this study were purchased from Sigma Aldrich Ireland Ltd. unless otherwise stated. All general chemicals were of analytical grade. All reagents used during electrophoresis were of electrophoresis grade. All chemicals used for cell culture were cell culture tested. $[MeVal]^4$-cyclosporin ($[MeVal]^4$-Cs) was a gift from Sandoz AG, Basle, and BC556 from Biotica, Cambridge, UK. Anti-$RAP1_{1\text{-}14}$ antibody was a kind gift from Prof. G Pluschke, Swiss Tropical and Public Health Institute, Basle.

Culture, harvesting and lysis of parasites

P. falciparum line 3D7 was cultured in human erythrocytes as previously described (Fennell *et al.*, 2006). Free parasites were generated from parasite cultures with high parasitaemia by standard methods (Zuckerman *et al.*, 1967). Free parasites were lysed by incubation with parasite lysis buffer (PBS containing 10% w/v glycerol, 1 x Complete mini protease inhibitor [Roche Diagnostics, Mannheim, Germany] and 0.5% [v/v] Triton X-100) on ice for 30 min with agitation every 5 min to enhance lysis. The lysate was clarified by centrifugation at 18,000 x *g* for 10 min at 4 °C, and the supernatant was carefully removed to a fresh microcentrifuge tube, leaving behind the unwanted cellular debris. This process was repeated twice more to ensure removal of cellular debris and insoluble material.

Generation of anti-immunophilin antibodies

E. coli strains previously generated in our laboratory harbouring plasmids pMAL-PfFKBD-His_6 (Monaghan *et al.*, 2005) and pET22b-PfCYP19A (Marin-Menendez *et al.*, 2012) were grown and the proteins encoded by these plasmids were overproduced and purified as described (Marin-Menendez & Bell, 2011; Monaghan *et al.*, 2005). These proteins were used as antigens for generation of custom polyclonal antibodies by CovalAb (St John's Innovation Centre, Cowley Road, Cambridge, UK). Briefly, immunisation was performed on two female New Zealand white rabbits for each protein by the following method: Day 0, rabbits were bled (4–5 ml) to harvest pre-immune serum which was stored at -20 °C, 1 ml injection consisting of 0.5 ml antigen (between 0.5 and 1 mg/ml) and 0.5 ml incomplete Freund's adjuvant was administered. Injections were repeated on days 14, 28 and 42. Test bleeds were performed on day 39 (4–5 ml) and day 53 (10–15 ml) with storage of the sera at 4 °C, with

final bleed performed on day 67. Antibodies were purified on a protein-A column by standard methods (Phizicky & Fields, 1995).

Co-immunoprecipitation (co-IP)

A preparation of 9.62 x 10^8 parasites was harvested as described in section 2.2. Parasites prepared in this manner formed the "bait and prey" fraction for use in co-IP. Co-IP was performed using the Pierce co-IP Kit (Product #26149) according to the manufacturer's instructions with the following modifications. Columns were prepared using 200 µl of 50% (v/v) resin slurry and approximately 500 µg of the relevant antibody. During co-IP all wash steps were increased to 400 µl, the 500 µl of "bait and prey" prepared as above were diluted in 400 µl of IP lysis/wash buffer and mixed with the prepared column resin suspended in 200 µl of IP lysis/wash buffer. This mixture was incubated with gentle shaking at 4 °C in a 1.5-ml microcentrifuge tube. The procedure was then completed as per the manufacturer's instructions. Concentration of the eluted co-immunoprecipitates was performed using 0.5-ml Amicon Ultra 10-kDa centrifugal filter units (Millipore), in a benchtop centrifuge at 14,000 x g for 25 min at 4 °C. This was followed by a buffer exchange (by re-diluting the concentrated eluate in desired buffer and centrifuging at 14,000 x g for 25 min at 4 °C in the same centrifugal filter unit, repeated 4 times) to reduce background staining in subsequent electrophoretic analysis. The concentrated immunoprecipitates were analysed by sodium dodecyl sulphate–10% polyacrylamide gel electrophoresis (SDS-PAGE), with component solutions filtered through a 0.2-µm filter to ensure removal of contaminating particles such as keratin, and bands corresponding to immunoprecipitating partners were cut out with clean scalpels and analysed by liquid chromatography/mass spectrometry (LC/MS) at the University College Dublin Conway Institute mass spectrometry facility on either a Thermo Fisher Q-exactive LC/MS or a Thermo Fisher Orbitrap LC/MS. Details of LC/MS methodology and database searching are given in Supplementary Methods 1. Two control columns were also prepared, one using 100 µl pre-immune serum from the same rabbit in which the anti-immunophilin serum was produced, the second using Pierce control agarose resin (crosslinked 4% (v/v) beaded agarose) and the co-IP procedure was repeated as above and analysed by SDS-15% PAGE in the same manner.

Yeast two-hybrid (Y2H) screening

A pLexA-N bait construct containing the FK506-binding domain of PfFKBP35 was generated from pMal-FKBP-His_6 (Monaghan *et al.*, 2005) as follows. Primers PfFKBP35fw and PfFKBDrev (5'-GACGAATTCATGACTACCGAACAAG-3' & 5'-GTCCTGCAGTCATCTAAAGCTTAATAATTC-3' respectively) were used to amplify the coding sequence for the FKBD of PfFKBP35 with an *Eco*RI site and a *Pst*I site at the 5' and 3' ends, respectively, to facilitate subsequent cloning into the pLexA-N expression vector. PCR was performed using ~100 ng of pMal-PfFKBP35-His_6 template, 0.3 µM primers and 1X KAPA HiFi HotStart® ReadyMix (KAPA Biosystems) in a Techne TC-3000 thermocycler (95°C for 5 min; followed by 35 cycles of 98°C for 20 s, 65°C for 15 s, 72°C for 30 s; followed by 72°C for 5 min.)

pLexA-N and the PCR-amplified FKBD coding sequence purified from agarose gel slices were digested with *Eco*RI and *Pst*I (Roche). Briefly, 3-µl reactions were set up in microcentrifuge tubes containing 0.02–1 µg DNA, 10 units each of *Pst*I and *Eco*RI, 3 µl of 10X buffer 'H' (Roche), 0.3 µl of 100X (10 mg/ml) bovine serum albumin (BSA) and 20.7 µl of deionised water. Tubes were incubated at 37 °C for 3 h in a water bath. Ligation of pLexA-N-PfFKBP35 and pLexA-N-FKBD was performed using a total of ~100 ng DNA in 1:1 and 1:3 ratios of vector:insert with 1 unit of T4 DNA ligase (Roche) and the reaction incubated overnight at 4 °C. The ligation mixture was transformed by the heat-shock method (Maniatis & Sambrook, 1982) into competent *E. coli* XL1-Blue cells and plated onto L-agar supplemented with 100 µg tetracycline/ml. Resulting colonies were screened for presence of the desired constructs by restriction digestion using *Eco*RI and *Pst*I endonucleases and agarose gel electrophoresis. Y2H screening was performed commercially by Dualsystems Biotech AG, Zurich, Switzerland. Details of the methodologies involved can be found in Supplementary Methods 2.

Histone purification and far western blotting

Histones were harvested by the method of Longhurst and Holder (Longhurst & Holder, 1997). Far-western blotting was performed essentially by the method of Wu *et al.* (Wu *et al.*, 2007). Briefly, after SDS-PAGE, and transfer to polyvinylidenedifluoride (PVDF) membrane, the membrane was incubated with 1 µg of the protein probe/ml in 5% (v/v) skimmed milk in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) with gentle shaking overnight at 4 °C. Western blotting was then continued from primary antibody step by standard methods.

Thermal melt and stability shift assay

The protein being assessed (1 µM) was prepared in a final volume of 50 µl into 0.2-ml thin-walled PCR tubes (VWR, Dublin, Ireland) with one of eleven buffers. The buffers were as follows: Buffer 1: 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 150 mM NaCl, pH 7.5. Buffer 2: 100 mM potassium phosphate, pH 7.0. Buffer 3: 100 mM sodium phosphate, pH 7.5. Buffer 4: 100 mM sodium citrate, pH 5.5. Buffer 5: PBS. Buffers 6–11 consisted of 100 mM NaCl and 50 mM HEPES at pH values 6.2, 6.6, 7.0, 7.4, 7.8, and 8.2 respectively.

The fluorescent dye used in this assay was SYPRO® Orange (Invitro-gen™ Molecular Probes™). Triplicates of each sample were heated from 30°C to 80°C at a rate of 2°C per minute. Fluorescence readings were taken for each sample at 0.2°C increments at 470 nm excitation wavelength and 585 nm emission wavelength in a Rotor Gene-3000 thermal cycler (Corbett Research, Sydney, Australia). The melting temperature (T_m) was determined by obtaining the first derivative of the curve and identifying the curve's maximal point.

Immunofluorescence microscopy

Eight-well multitest immunofluorescence microscopy slides (Thermo Scientific) were pre-treated with 0.1% (w/v) poly-L-lysine overnight at room temperature in a humid chamber. They were then washed five times for 10 minutes with wash medium (RPMI 1640 supplemented with 25 mM HEPES, 0.18% w/v sodium bicarbonate, 50 µg hypoxanthine/ml, 0.16% w/v glucose). Infected erythrocytes from cultures of *P. falciparum* at about 10% parasitaemia or treated for 14–16 h overnight with relevant inhibitors were washed two times in wash medium at room temperature. Twenty µl of a solution of 4% (w/v) paraformaldehyde and 0.1% (v/v) Triton X-100 were pipetted into each window of the slide and 30 µl of cells resuspended in wash medium were added. This was incubated at room temperature for 3 h. Wells were washed five times for 10 min with PBS and blocked with 30 µl 5% (v/v) normal goat serum for 30 min at room temperature. Immunostaining was started by incubating the cells with 30 µl of the relevant antibody (0.2 mg $PfRAP1_{1\text{-}14}$/ml, or a 1:40 dilution of PfCYP19B serum) for 1 h at room temperature. After five washes with 5% (v/v) goat serum, 30 µl of a 1:500 dilution of the relevant secondary antibody (donkey anti-mouse conjugated Alexafluor®-488, donkey anti-rabbit conjugated Alexafluor®-546 [Invitrogen], or goat anti-mouse conjugated fluorescein isothiocyanate [FITC, DakoCytomation]) were pipetted onto each window and incubated for 1 h at room temperature. Afterwards slides were washed five times for 10 min each with PBS, incubated for 2 min with 0.2 µg 4',6-diamidino-2-phenylindole (DAPI)/ml, and washed again three times for 10 min with PBS. Slides were mounted with 2 µl per window Prolong Gold antifade reagent (Bio-Sciences, Dun Laoghaire, Ireland) and covered with a coverslip. The coverslip was sealed to the slide using a clear nail varnish and left to set overnight. Antibody binding and DNA staining were assessed by confocal fluorescence microscopy (on an Olympus FV1200 Biological Laser Scanning Confocal Microscope).

RESULTS

Identification of interacting partners by co-IP

Co-IPs of PfCY19A, PfCYP19B and PfFKBP35 were analysed by SDS-PAGE (Fig. 1). Bands of interest were excised from the gels and analysis by mass spectrometry revealed 161, 11 and 113 high-confidence (PEAKS analysis score >95%)[1] protein identifications for PfCYP19A, PfCYP19B and PfFKBP35 respectively. A number of putative interactions of interest have been highlighted in Table 1. A full list of the putative interactions is available in Supplementary Table S1. The difference between the numbers of high-confidence protein identifications may be largely due to the lower sensitivity of the Thermo Fisher Orbitrap LC/MS used for PfCYP19B.

[1] Confidence is defined by PEAKS score as follows: the PEAKS score is a composite score that takes into account results of the database search and *de novo* sequencing: as a rule of thumb, proteins with a PEAKS score higher than 95% can be considered confidently identified, but below this and down to approximately 70% there is a certain linear correspondence between PEAKS score and percentage probability that the identification is correct.

Identification of interacting partners of PfFKBP35 by Y2H

Y2H screening ultimately revealed eleven putative interacting partners for the FKBD of PfFKBP35 (Table 2). Three of these proteins were identified twice in the screen (Class B) and the remainder were found only once (Class C). Of particular note in view of the co-IP results reported above was the identification of the histone subunits H2B and CenH3 (an H3 variant) by this method.

Interaction between PfCYP19B and PfHsp70

In order to confirm that PfCYP19B interacts with PfHsp70 we attempted to pull down the cyclophilin using co-IP of the heat shock protein. Antibodies to heat shock protein 70 from other organisms are readily available. We sourced a polyclonal antibody which had been generated against a recombinant full length Hsp70 from *Homo sapiens* to increase the possibility of cross-reactivity, due to the high level of sequence similarity between Hsp70s, and confirmed that it was able to detect a band of apparent molecular mass 70 kDa on a western blot of a crude parasite lysate (Supplementary figure S1).

After confirming cross-reactivity of the HsHsp70 antibody we used it to generate a co-IP column. The co-IP eluate from this column contained PfCYP19B when analysed by western blotting with an anti-PfCYP19B antibody (Fig. 2, lanes 1 & 2). We can conclude that this ability to pull down PfCYP19B is specific to the anti-Hsp70 column since neither co-IPs performed using a column made with an irrelevant antibody (Fig. 2, lane 3 & 4) nor a non-reactive column that is unable to bind antibody (Fig. 2, lane 5) were able to pull down PfCYP19B. Similarly the co-IP eluate from the anti-Hsp70 column was negative for an irrelevant protein (Figure 2, lane 6 & 7). Taken together, these results lead to the conclusion that PfCYP19B specifically interacts with PfHsp70, at least under the conditions used for co-IP.

Interaction between PfFKBP35 and histones

As mentioned above a putative interaction between PfFKBP35 and histones was identified by both co-IP and Y2H in the initial screening. To investigate this interaction we first employed the method of Longhurst & Holder (Longhurst & Holder, 1997) to purify histones from *P. falciparum* cultures and demonstrated an interaction between recombinant PfFKBP35 and 2–3 *P. falciparum* histones by far-western blotting (Fig. 3, A). This interaction was specifically between PfFKBP35 and histones because loading with no histones (BSA lane) did not reveal any bands. In the same way PfCYP19B (as an unrelated protein control) did not bind histones since no PfCYP19B was detected in the corresponding lane. Additionally, we investigated whether the action of FK506 had an effect on the methylation of the lysine residue at position 36 of histone H3 (H3K36). Previously, H3K36 methylation was shown to be controlled by the PPIase action of the yeast FKBP Fpr4p. This regulation is governed by *cis-trans* isomerisation of the prolines P30 and P38 on histone H3 by Fpr4p (Nelson *et al.*, 2006). In this experiment we incubated parasites in culture with FK506 or chloroquine for 14

h. As shown (Fig. 3, B), there was an increase in H3K36 methylation with increasing concentrations of FK506, which is known to inhibit the PPIase activity of PfFKBP35. The standard antimalarial drug chloroquine, whose primary action is to disrupt haemozoin formation, had a much less significant effect; bands for H3K36me3 disappeared at high chloroquine concentration possibly due to the toxicity of the drug.

Interaction between cyclophilin and RAP1

Immuno-staining schizonts for RAP1 under normal conditions resulted in the bi-punctate staining characteristic of rhoptry proteins, while PfCYP19B was located in the cytoplasm as expected (Figure 4, **1**). After 14–16 h of incubation with CsA at 5x IC_{50} the characteristic staining of PfRAP1 was disrupted and RAP1 and PfCYP19B co-located (Figure 4, **2**). The disruption was still evident at the IC_{50} (Figure 4, **3**) and somewhat evident at 0.2x IC_{50} and 0.04x IC_{50} while the characteristic bi-punctate staining for RAP1 was restored after reduction below this concentration (data not shown). At none of the concentrations tested was there evidence of an effect on the location of PfCYP19B.

In order to exclude the possibility that this effect was due to calcineurin inhibition by the ligand–cyclophilin complex we tested the effects of the non-calcineurin binding immunophilin ligands $[MeVal]^4$-Cs (Bell *et al.*, 1994) and BC556 (Fischer *et al.*, 2010) on RAP1. These ligands were both able to disrupt RAP1 location in schizonts at similar relative concentrations to CsA, i.e. 5x IC_{50} (Figure 4, **4**&**5**) and IC_{50} (not shown). Also tested were the classic antimalarial drugs chloroquine and artemisinin, which had no effect on RAP1 location after 14–16 h at 5x IC_{50} (Figure 4, **6**&**7**). Taken together these results suggested that the disruption of proper RAP1 location was not associated in a non-specific way with parasite damage or growth inhibition but was likely mediated by interference with the action of one or more cyclophilins. It is known that BC556 binds to other cyclophilins (Fischer *et al.*, 2010) but in order to confirm ligand binding to *Plasmodium* cyclophilin we utilised the thermal stability shift assay. Briefly, an increase in the peak of the first derivative of the melting curve indicates binding of a ligand to a protein. BC556 was able to bind to recombinant PfCYP19B (Fig. 5).

DISCUSSION

This study has investigated the protein–protein interactome of the major immunophilins PfCYP19A, PfCYP19B and PfFKBP35 of *P. falciparum* with a view to understanding better the cellular functions of these immunophilins. Two methodologies, co-IP and Y2H, were used. Co-IP specifically and reproducibly pulled down a number of protein bands and identified a large cohort of putative immunophilin–protein interactions. Below we have highlighted a number of these putative interactions that may be important to the biology of the parasite, for which there are similar data from other organisms, or for which we have confirmation of the interaction from a second experimental source.

Specifically we believe all three immunophilins to interact with large portions of *P. falciparum*'s heat-shock machinery: all co-IPs pulled down Hsp90 and four Hsp70 isoforms (Hsp70, Hsp70-2, Hsp70-3 and Hsp70-x). PfFKBP35 pulled down a putative Hsp90 and a putative DnaJ (Hsp40) protein, though with low peptide coverage; DnaJ was also indicated as a putative interaction by our Y2H study. PfCYP19A pulled down Hsp60 with 21 peptides identified by mass spectrometry covering 41% of the protein. Additionally both PfCYP19A and PfFKBP35 pulled down another Hsp70 isoform (Hsp70-z) and Hsp70/Hsp90-organising protein. We believe these putative interactions potentially to be important because of analogous interactions in other organisms such as the steroid receptor complex in humans (Ratajczak *et al.*, 2003), as well as the general importance of heat-shock proteins for parasite biology (Acharya *et al.*, 2007).

We subsequently demonstrated that PfCYP19B was specifically pulled down with Hsp70 in co-IP experiments using whole parasite lysate *in vitro*. These data, along with the known interaction between PfFKBP35 and Hsp90 (Kumar *et al.*, 2005), lend confidence to the idea that *P. falciparum* possesses a chaperone complex similar to the high molecular weight chaperone machinery known to exist in other organisms. This machinery, usually consisting of immunophilins (CYPs and FKBPs), Hsp90 and p23 along with accessory proteins Hsp70, Hsp40, Hip and Hop, appears to be present in most eukaryotes. In *P. falciparum* such machinery might be involved in chaperoning correct folding and regulating activities of various proteins. In other organisms different immunophilins are associated with this complex depending on the substrate which is chaperoned, for example FKBP51 and 52 are associated with the complex during steroid receptor assembly, while cyclophilin 40 is associated during oestrogen receptor chaperoning (Galat, 2003). This may explain in part why the parasite requires a large repertoire of immunophilins.

We also highlighted the putative interaction of PfFKBP35 with the nucleosome complex of *P. falciparum*. Our Y2H study indicated a putative interaction between PfFKBP35 and the histones H2B and CenH3. Co-IP with PfFKBP35 also pulled down H2B and H3, along with the other histones H2A and H4 and the nucleosome assembly protein (NAPS). We believe that the direct interactions may be with H2B and H3 and since these proteins exist as heterodimers of H2A–H2B and H3–H4 they may pull down H2A and H4 by that association. These interactions have a precedent in the literature in that nuclear FKBPs in *S. cerevisiae* and *Schizosaccharomyces pombe* were shown to possess histone chaperone activity (Kuzuhara & Horikoshi, 2004) and the nuclear FKBP Fpr4p in *S. cerevisiae* regulates methylation of amino acid lysine-36 on histone H3 (Nelson *et al.*, 2006). We demonstrated by far-western blotting that recombinant PfFKBD bound to purified histones immobilised on PVDF membrane and appeared to bind with higher affinity to bands corresponding to the molecular weights of PfH2B and PfH3. Treatment of parasites with the FKBP ligand FK506 increased H3K36 methylation. In *S. cerevisiae* H3K36 methylation is regulated by the PPIase activity of the FKBP Fpr4p, and inhibition of this protein leads to increased H3K36 methylation. It appears that PfFKBP35 via its PPIase activity is also involved in regulation of H3K36 methylation, which in *P. falciparum* is known to affect expression of *var* genes

encoding clonally variant antigens that are exported to the surface of the parasitized erythrocyte (Jiang *et al.*, 2013).

The co-IP study also indicated putative interactions between all three immunophilins and RAP1, as well as between PfFKBP35 and PfCYP19A and a number of other rhoptry proteins. RAP1 is known to be critical for invasion of erythrocytes by *P. falciparum* merozoites (Cowman *et al.*, 2012). When analysed by immunofluorescent microscopy with antibodies directed against it, RAP1 exhibits a characteristic bi-punctate staining in parasite schizonts, indicative of location in the rhoptry body (Moreno *et al.*, 2001). We demonstrated that when parasites were grown in the presence of the cyclophilin ligands CsA, [MeVal]^4-Cs or BC556, RAP1 lost its bi-punctate pattern and instead appeared in the cytosol of immature merozoites within the schizont. There was no detectable effect of ligand treatment on PfCYP19B location but given that this is an abundant protein its presence in a compartment other than the cytosol cannot be excluded. Short treatments (2 h) of parasite schizonts with the same ligands indicated that they had little effect on merozoite invasion, consistent with the hypothesis that the action of these ligands occurs at some point before arrival of RAP1 at the rhoptry (data not shown).

Among the large number of other putative interacting partners that were identified from the co-IP and Y2H studies, there was a significant representation of proteins involved in protein translation, chaperoning and digestion. From these data it appears that these major *P. falciparum* cytosolic immunophilins may be involved in a wide variety of cellular functions in the parasite. Some of these interactions may be analogous to immunophilin–protein interactions in other organisms, like the known role of immunophilins in cytoskeletal architecture, molecular chaperone machinery, and nucleosome assembly and modification, while some may represent novel immunophilin–protein interactions specific to *P. falciparum* and/or critical for its life cycle.

In summary, with the results from our co-IP experiments and our Y2H screen we have been able to generate an interaction map which provides a body of evidence not only to support predictions of protein–protein interactions inferred from other organisms but also as a starting point for further research. Our own follow-up work confirmed a number of these putative interactions, namely immunophilin interactions with Hsp70 and histones. We were also able to demonstrate a potential novel role for immunophilins in parasite biology, that of chaperoning RAP1. These results may also have relevance for the mechanisms of antimalarial action of cyclosporins, macrolactones, and other immunophilin ligands that have shown promise as antimalarial agents (Bell *et al.*, 2006; Harikishore *et al.*, 2013a; Harikishore *et al.*, 2013b).

ACKNOWLEDGEMENTS

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436 FIGURE LEGENDS
437

438 Fig. 1. Sypro Ruby® stained SDS-4–20% polyacrylamide gel electrophoretograms showing
439 concentrated co-IP eluates from anti-immunophilin (A) PfCYP19A, (B) PfCYP19B, (C)
440 PfFKBP35) columns. Molecular weight marker positions are indicated to the left of each
441 image: numbers indicate mass in kDa. Co-IP eluates were concentrated using a 9-kDa cut-off
442 protein concentrator: 100% of the fraction was loaded, containing ~80–100 ng of protein.
443 Concentration was performed at 5000 x g for 30 minutes. Red arrow = co-precipitating
444 protein also present in pre-immune serum control. White arrow = PfCyp19B. Black arrows =
445 putative interacting partners. Bracket = ~25 to ~70 kDa section excised as a whole from gel.

446 Fig. 2. Co-IP and western blot investigation of PfCYP19B–Hsp70 interaction. Co-IP was
447 performed using anti-Hsp70 to pull down PfCYP19B by its affinity for Hsp70 (Lanes 1 & 2).
448 Controls comprising an Hsp70 co-IP western blot probed with an antibody to an irrelevant
449 protein (Lanes 3 & 4), an irrelevant antibody column (anti-His_6: Lanes 5 & 6) and a non-
450 reactive column (Lane 7) are also shown. In the cases of the unbound fraction approximately
451 20 µl of a 400-µl fraction were loaded, and in the cases of the bound fractions the eluate was
452 concentrated and a volume equalling the total fraction was loaded onto the gel.

453 Fig. 3. (A) Far-western blotting analysis of *P. falciparum* histone interactions. Thirty µg of *P.*
454 *falciparum* histones or 10 µg of BSA were separated by SDS-15% PAGE and transferred to
455 PVDF membrane, which was probed with 1 µg/µl recombinant PfFKBD-His_6 or 1 µg/µl
456 recombinant PfCYP19B-His_6, extensively washed and the interaction was detected by
457 standard western blot using an antibody for PfFKBD-His_6 or PfCYP19B as appropriate.
458 Arrows (A&B: ~15.5 kDa & ~13.1 kDa) indicate bands corresponding to the apparent masses
459 of histones H3 and H2B. Numbers and lines to the left and right indicate the positions and
460 sizes of molecular mass markers in kDa. (B) Representative western blot of extracts from
461 triplicate experiments on parasites incubated with decreasing concentrations of either FK506
462 or chloroquine for ~14 h. Parasites were lysed by incubation on ice with Triton X-100 for 30
463 min and the clarified lysate was separated by SDS-12.5% PAGE. The blot was probed with
464 either anti-H3K36me3 (Abcam®, ab9050) or anti-PfCYP19B as a loading control. Numbers
465 underneath the anti-H3K36me3 panel indicate the band intensity relative to the control lane
466 as estimated by densitometry.

467 Fig. 4. Confocal immunofluorescence microscopic images of *P. falciparum* schizonts treated
468 with: (1) vehicle only control, (2) 5x IC_{50} cyclosporin A, (3) IC_{50} cyclosporin A, (4) 5x IC_{50}
469 BC556, (5) 5x IC_{50} $[MeVal]^4$-Cs, (6) 5x IC_{50} chloroquine and (7) 5x IC_{50} artemisinin.
470 Schizonts were stained with DAPI (nuclear stain), Alexafluor-488 (PfRAP1) and Alexafluor-
471 546 (PfCYP19B). White scale bars indicate 5 µM. White arrows show characteristic bi-
472 punctate rhoptry staining.

473 Fig. 5. Binding of BC556 to PfCYP19B assessed by thermal stability shift assay. Binding
474 was indicated by an increase in the peak of the first derivative of the melting curves of a
475 protein in the presence of a ligand. T_m values are given above the peaks.

476 Fig S1. Supplementary figure S1. Western blot of crude parasite lysate probed with an
477 antibody generated against full length recombinant HsHsp70 demonstrating cross-reactivity
478 of anti-HsHsp70 with parasite proteins. Bars and numbers on the right of the image indicate
479 the position and size in kDa of molecular mass markers. The black arrow indicates the
480 predominant reactive band, likely to be parasite Hsp70.

481

481 Table 1. Examples of putative immunophilin–protein interactions identified by co-IP.

Gene_ID	PEAKS Score (%)[a]	Coverage (%)	No. of Peptides	Description
Triple Interactors				
PF3D7_0708400	99.2	18	13	Heat shock protein 90 (Hsp90)
PF3D7_0818900	99.2	24	17	Heat shock protein 70 (Hsp70)
PF3D7_0917900	99.2	42	33	Heat shock protein 70 (Hsp70-2)
PF3D7_1134000	99.2	23	16	Heat shock protein 70 (Hsp70-3)
PF3D7_0831700	99.2	9	7	Heat shock protein 70, putative (Hsp70
PF3D7_1410400	99	9	8	Rhoptry-associated protein 1 (RAP1)
PF3D7_1357000	99.2	43	22	Elongation factor 1-alpha
PfFKBP35 Interactors				
PF3D7_1246200	97.7	6	2	Actin I (ACT1)
PF3D7_0903700	98.9	11	4	Alpha tubulin 1
PF3D7_1008700	99.1	10	4	Tubulin beta chain
PF3D7_0617800	98.9	32	5	Histone H2A (H2A)
PF3D7_1105100	99.2	57	8	Histone H2B (H2B)
PF3D7_0610400	96.2	5	1	Histone H3 (H3)
PF3D7_1105000	98.9	39	4	Histone H4 (H4)
PF3D7_0919000	99	14	4	Nucleosome assembly protein (NAPS)
PF3D7_0501600	98.3	7	3	Rhoptry-associated protein 2 (RAP2)
PF3D7_0322000	99	22	4	Peptidyl-prolyl cis-trans isomerase (CYP
PF3D7_1115600	98.3	14	3	Peptidyl-prolyl cis-trans isomerase (CYP
PF3D7_0708800	95.1	2	2	Heat shock protein 70 (Hsp70-z)
PF3D7_1118200	97	1	1	Heat shock protein 90, putative
PF3D7_1434300	97.6	5	3	Hsp70/Hsp90 organizing protein (HOP)
PF3D7_1473200	98.2	3	2	DnaJ protein, putative
PfCYP19A Interactors				
PF3D7_1015600	99.2	41	21	Heat shock protein 60 (Hsp60)

PF3D7_0708800	99.2	14	11	Heat shock protein 70 (Hsp70-z)
PF3D7_1434300	99	15	9	Hsp70/Hsp90 organizing protein (HOP)
PF3D7_0929400	99.2	13	20	High molecular weight rhoptry protein
PF3D7_0905400	99.2	9	8	High molecular weight rhoptry protein
PF3D7_1252100	97.9	2	4	Rhoptry neck protein 3 (RON3)
PF3D7_1116000	97.7	1	1	Rhoptry neck protein 4 (RON4)
PF3D7_0501600	98.5	6	3	Rhoptry-associated protein 2 (RAP2)
PfCYP19B Interactors				
PF3D7_1246200	99.1	18	5	Actin I (ACT1)

[a] See footnote in text.

Table 2. Putative PfFKBD–protein interactions identified through Y2H screening.

Class*	Gene ID	Product Description
B	PF3D7_1473200	DnaJ protein, putative
B	PF3D7_0519800	conserved protein, unknown function
B	PF3D7_0731300	Plasmodium exported protein (PHISTb), unknown function (PfG174)
C	PF3D7_0408400	conserved Plasmodium protein, unknown function
C	PF3D7_0206500	conserved Plasmodium protein, unknown function
C	PF3D7_1035200	S-antigen
C	PF3D7_0730300	transcription factor with AP2 domain(s) (ApiAP2)
C	PF3D7_1333700	histone H3 variant, putative (CenH3)
C	PF3D7_1105100	histone H2B (H2B)
C	PF3D7_1025100	glucosamine-fructose-6-phosphate aminotransferase, putative
C	PF3D7_1013800	conserved Plasmodium protein, unknown function

* “Class B” interactors were identified two times (out of three possible) and represent highly likely interactors with the bait; “Class C” interactors were found only once in the screen.

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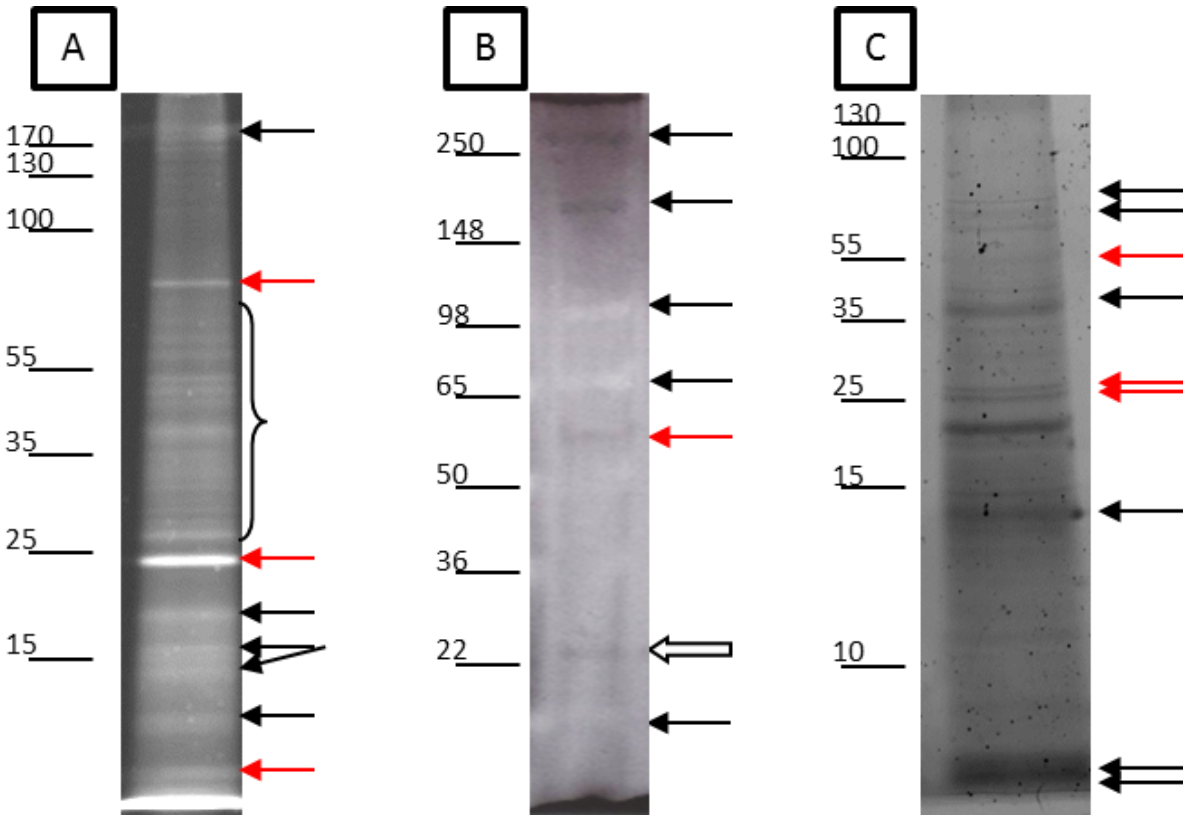
605 Fig. 1



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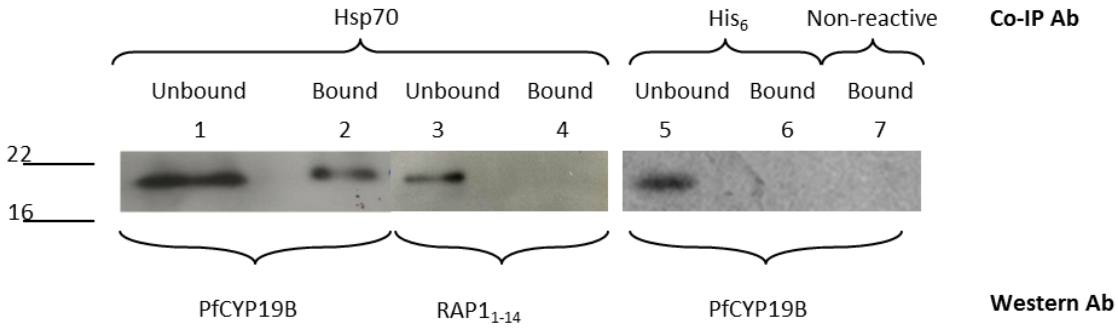

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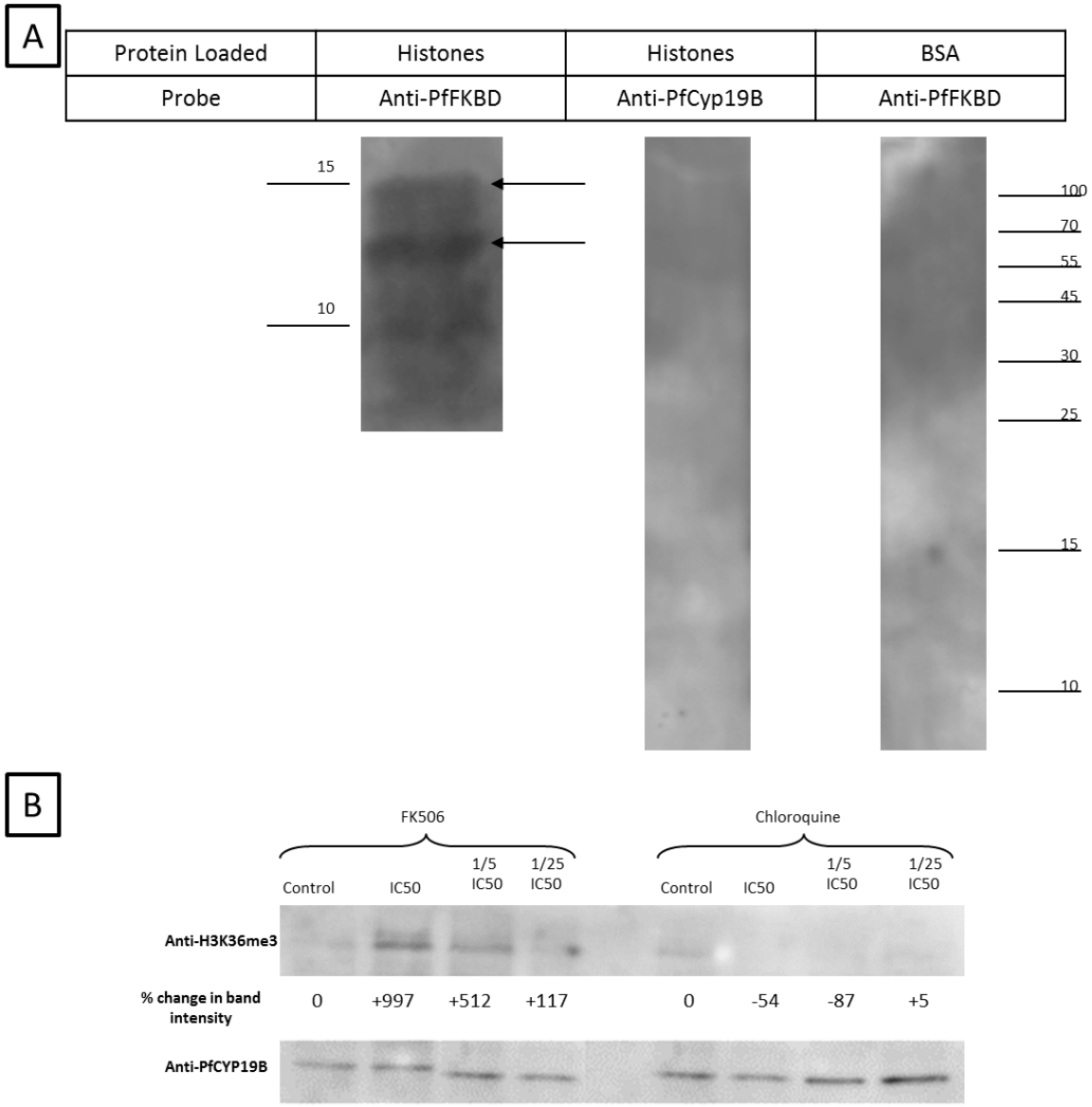

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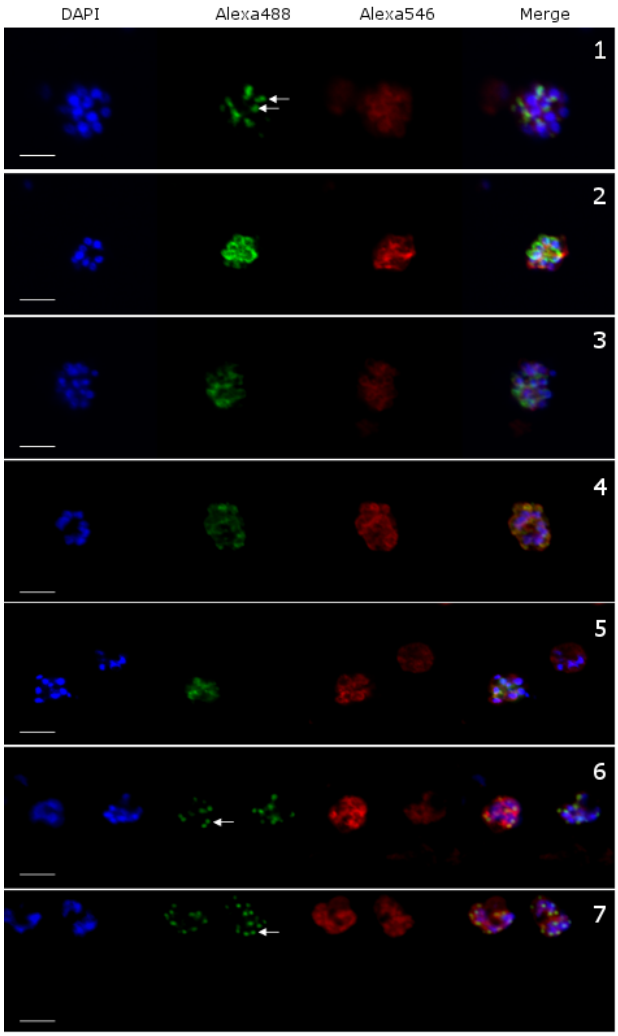

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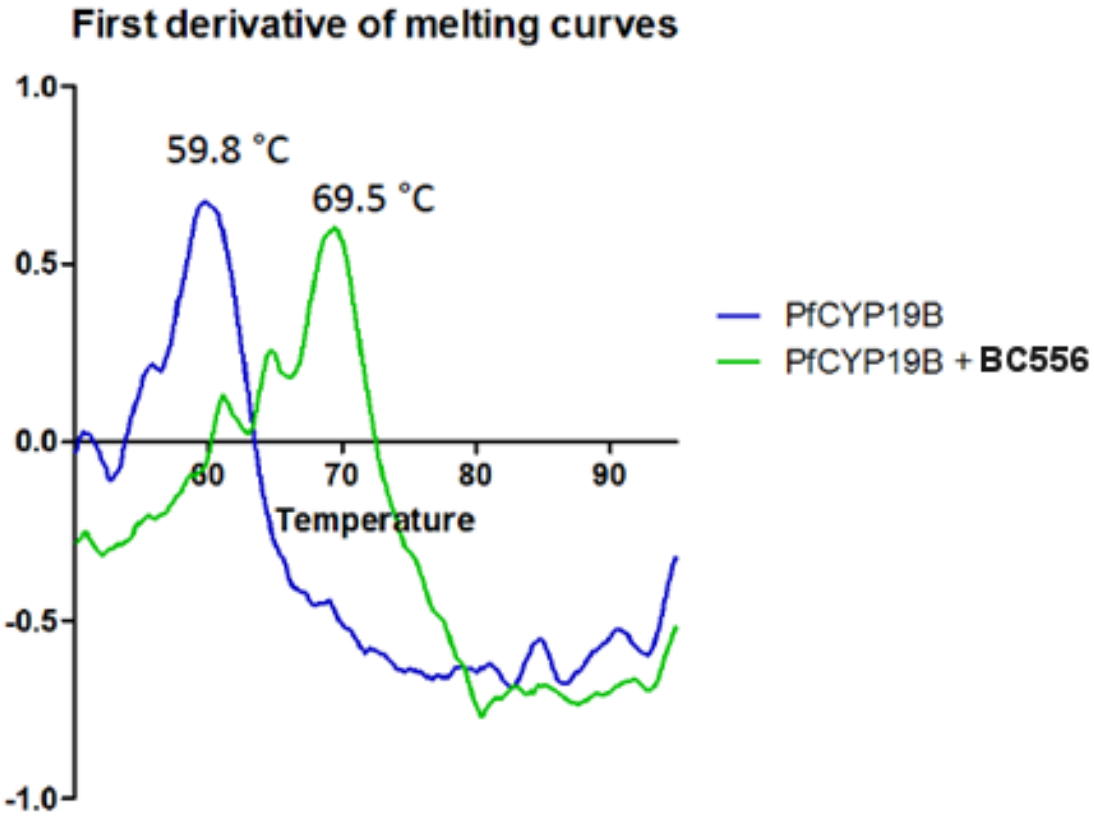
613 Fig. 5



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