Prefoldins are novel regulators of molecular mechanism associated with unfolded protein response in artemisinin resistant P. falciparum malaria

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Abstract

Emerging Artemisinin (ART) resistance in *Plasmodium* demands novel drugs that can target artemisinin resistance mechanism to manage resistant parasites. ART resistance is attributed by mutations in the *Plasmodium falciparum* (*Pf*) Kelch-13 (*Pf*K13) gene, however a study showed that artemisinin resistance is linked with up-regulated expression of unfolded protein response pathways involving Prefoldins (PFD) of malaria parasite. Here, we functionally characterized all *Pf*Prefoldin subunits, the causative links responsible for providing artemisinin resistance. *Pf*PFD-6 interacts with *Pf*K13 and this phenotype was confirmed in yeast orthologous system to show that Prefoldin decrease the sensitivity of artemisinin in mutant strains. Expression of Prefoldin subunits was upregulated in artemisinin resistant line *Pfk13R539T*, underscoring their significance in providing artemisinin resistance. *Pf*PFD1-6 localize in the cytosol, and these subunits interact in an orchestrated manner (PFD3-PFD2-PFD1-PFD5-PFD6-PFD4) to form a jellyfish like complex. We identified an FDA approved drug ‘Biperiden’ that restricts the formation of Prefoldin complex and inhibits its interaction with key parasite proteins, MSP-1 and α-tubulin-I. Moreover, Biperiden treatment inhibits the parasite growth in *Pf*3D7 artemisinin sensitive and resistant line. Overall, our study provides novel virtues towards understanding the role of *Pf*PFDs in artemisinin resistance mechanism, and opens new avenues for the management of resistant parasite.

Introduction

Malaria is one of the biggest threats to global health, which has existed alongside people for more than 40 centuries. Artemisinin-based combination therapies (ACTs) are the first line of recommended treatment for the malaria burden. However, resistance to artemisinin and ACTs in *Plasmodium falciparum* is spreading rapidly throughout Southeast Asia, posing a threat to control worldwide malaria eradication and elimination ¹. Several mechanisms are proposed to play a role in the development of artemisinin resistance in the *Plasmodium* parasite. Clinical artemisinin resistance is strongly linked to polymorphism (Y493H, R539T, I543T, and C580Y) in the β-propeller domain of the K13 protein (PF3D7_1343700) ². This specific domain is entailed to bind with interacting substrates and allow them for ubiquitination. The function of Kelch-13 is not well understood in *Plasmodium*, however, it shows similarity to Kelch/ BTB/POZ ubiquitination adapters. K13 is hypothesize to function as an ubiquitin-ligase adapter and targets the ubiquitin-mediated degradation of proteins ³,⁴.

A recent study carried out *in-vivo* transcriptomic analysis of 1048 *P. falciparum* isolates from patients with acute malaria and reported that artemisinin resistance is related to enhanced expression of unfolded protein response (UPR) pathways involving the main *Plasmodium* reactive oxidative stress complex (PROSC) and TCP-1 ring complex (TRiC) chaperone complexes. The same report investigated the expression profile of genes linked to artemisinin resistance and found overexpression of one of the genes ‘Prefoldin’ in the artemisinin resistant patient sample ⁵. Another report on the *Leishmania* parasite revealed that FAZP (Prefoldin homolog of *Plasmodium*) is overexpressed by 3.55-fold in ART-resistant parasites in comparison to wild-type parasites ⁶. In light of the above facts, we selected Prefoldin (PFD)
family of proteins to explore their functions in malaria parasite and further attempted to investigate their relevance with respect to artemisinin resistance. Prefoldin is an evolutionarily conserved hetero-hexameric molecular co-chaperone, vital for maintaining cellular homeostasis in both physiological and pathological conditions. PFD complexes are ubiquitously present in all archaea and eukaryotes, and their main function is to capture nascent polypeptides and deliver them to Chaperonin Containing Tailless complex polypeptide 1 (CCT) or Tailless complex polypeptide 1 Ring Complex (TRiC) for proper folding. As of now, mainly Prefoldin roles have been identified in the folding of cytoskeletal protein actin and tubulin. However, our present understanding of Prefoldin's possible function in protein homeostasis maintenance is restricted. Surprisingly, each of the single subunits can bind to independent unique interactors, allowing them to execute diverse tasks.

A complete lack of understanding of the PFD complex in the Plasmodium parasite represents a significant void in our functional understanding of chaperones and co-chaperones, and demands to explore their role in relevance to artemisinin resistance. Here, we delineated the role of Prefoldin in artemisinin-mediated resistance development by interacting with the K13 protein. The functional complementation assay conducted in the orthologous system Saccharomyces cerevisiae demonstrates rescued yeast growth and affirms the reduced sensitivity of artemisinin in the PFD complemented strain. We also identified and characterized the orchestrated assembly of a novel PFD co-chaperone heteroprotein complex in P. falciparum, and elucidate its functional significance in the unerring folding of crucial cytoskeletal and invasion-related parasite proteins, specifically Pfa-tubulin-I and Merozoite Surface Protein (MSP-1), respectively. Through an examination of FDA-approved chemotypes targeting PfPFD, this study unveils 1-(bicyclo[2.2.1]hept-5-en-2-yl)-1-phenyl-3-(piperidin-1-yl)propan-1-ol (commonly referred to as Biperiden or BPD) as a potential inhibitor of the PfPFD complex, exhibiting substantial antiplasmodial efficacy. Overall, our study contribute to understand the role of PfPFD in providing artemisinin resistance, and present BPD as a promising candidate for further exploration as an antimalarial agent. These findings offers a unique opportunity to tackle artemisinin resistance management and development of novel antimalarial chemotherapeutics.

**MATERIAL AND METHODS**

*In vitro culture of P. falciparum*

*P. falciparum* laboratory-adapted strain 3D7 was cultured *in vitro* using the standard protocols, as described previously. Briefly, the parasites were cultured in RPMI 1640 (GibcoTM, USA) medium supplemented with 5.9 gm/L HEPES (Sigma-Aldrich, USA), 50 mg/L hypoxanthine (Sigma-Aldrich, USA), 2 gm/L sodium bicarbonate (Sigma-Aldrich, USA), 5 gm/L AlbuMax I (for 3D7, R539T and Dd2; GibcoTM, USA) and 10 mg/L Gentamicin (Sigma-Aldrich, USA). The culture was maintained in 75 cm2 culture flasks (Corning®, US) using fresh O-positive (O+) human erythrocytes, under an ambient mixed gas environment (5% O2, 5% CO2, and 90% N2) at 37°C. Before every experiment, the parasite culture was tightly synchronized with 5% D-sorbitol for two successive intra-erythrocytic proliferative cycles, followed by enrichment of trophozoites or schizonts parasitized erythrocytes with 65% percoll.
Co-IP assays and mass spectrometry analysis

The co-IP assays were performed using AminoLink Plus Coupling Resin (Pierce Biotechnology, Rockford, USA) as per the manufacturer’s instructions. Briefly, the resin was equilibrated with coupling buffer (0.1M Na 3 PO 4, 0.15M NaCl; pH = 7.2) and incubated with preimmune serum and purified anti- Pfk13 antibodies for 2 hrs, followed by washing with coupling buffer. Post this the resin was incubated with 50 mM sodium cyanoborohydride (NaCNBH 3) overnight at 4°C. The affinity column was incubated with parasite lysate with gentle mixing overnight at 4 °C. followed by washing with wash buffer. Bound proteins were eluted with elution buffer (0.1–0.2M glycine-HCl; pH = 2.5-3.0). The elution fractions were neutralised with neutralising buffer (1M Tris; pH = 9.0) and run on 10% SDS-PAGE. The eluted proteins were digested by sequencing grade trypsin (20 μg/mL). Extracted peptides were acidified to 0.1% formic acid and analysed by Orbitrap VelosPro mass spectrometer coupled with nano-LC 10 0 0 (Thermo Fisher Scientific Inc, USA). Interactome of only those proteins were generated that showed chaperone functions using STRING online tool.

Expression and purification of PfK13

Purification of recombinant PfK13 was performed as described previously 9.

Surface Plasmon Resonance (SPR)-based interaction analysis

The interaction of PfK13 with PfPFD6 was evaluated by Auto LAB ESPRIT SPR instrument (Kinetic Evaluation Instruments BV, The Netherlands). Briefly, recombinant PfK13 (20 μM) was immobilized on a gold sensor chip previously activated through amine coupling. PBS was used as an immobilization and binding buffer. PfPFD6 was injected in increasing concentrations (25 nM, 100 nM, 1 μM, 2.5 μM and 5 μM) over the PfPFD6-immobilized chip surface. 50 mM NaOH was used to regenerate the chip surface. Data were analyzed using Auto Lab ESPRIT kinetic evaluation software.

A similar protocol was followed to assess the interaction of PfPFD1, PfPFD2 and PfPFD3 with Pfa-tubulin-l. Briefly, recombinant PfPFD2 (25 μM) was immobilized on a gold sensor chip and α-tubulin-l was injected in increasing concentrations (100 nM, 250 nM, 500 nM, 750 nM, and 1 μM) over the PfPFD2-immobilized chip surface. Assessing the interaction of PfPFD1 and PfPFD3 with Pfa-tubulin-l served as negative controls. Similarly, the interaction of BPD with PfPFD1-6 was evaluated with SPR, wherein, 25 μM each PfPFD subunit was immobilized on the gold sensor chip followed by injection of BPD in increasing concentrations (5, 25, 50, 75, and 100 uM). Data were analyzed using Auto Lab ESPRIT kinetic evaluation software.

Co-immunoprecipitation assay to confirm interaction of PfK13 with PfPFD6, and PfPFD with other subunits and its substrates

Co-immunoprecipitation assay was performed using PierceTM Co-Immunoprecipitation (Co-IP) kit to confirm the interaction of PfK13 with PfPFD6. Briefly, the anti-PfPFD6 antibody was cross-linked to
AminoLink plus coupling beads. After extensive washing, beads were incubated with mixed-stage \( Pf3D7 \) and \( Pf\text{Kelch13}^{R539T} \) parasite lysate. Bound protein was eluted in the elution buffer, separated on 12% SDS-PAGE, and subjected to western blotting. Blot was probed with polyclonal anti-K13 antibody (1:1000) followed by secondary anti-rat (1:5000) antibody. Blot was developed by using the ECL substrate.

Similar protocol was followed to confirm the assembly of the \( Pf\text{PFD} \) complex and its interaction with specific protein substrates: \( \alpha\)-tubulin-I and \( Pf\text{MSP-1} \). Briefly, \( Pf\text{PFD6} \) antisera was cross-linked to AminoLink plus coupling beads followed by extensive washing with wash buffer. Mixed-stage culture (~8% parasitemia) was subjected to saponin lysis, followed by RIPA lysis of the purified parasites. Beads cross-linked with the antisera were incubated with the parasite lysate overnight at 4\(^\circ\)C. Elutes were collected, divided into six groups, and resolved (along with appropriate control, r\( Pf\text{PFDs} \)) on 12% SDS-PAGE, and transferred to nitrocellulose membrane. Blots were individually probed with \( Pf\text{PFD1-6} \) antisera (1:1,000 of each) and secondary HRP conjugated anti-mice antibodies (1:5,000; Sigma Aldrich, USA), and developed using diaminobenzidine/\( H_2O_2 \) substrate (Sigma-Aldrich, MA, USA).

**Real-time PCR analysis of \( Pf\text{PFD1-6} \)**

Expression of \( Pf\text{PFD1-6} \) at transcript levels was evaluated in intra-erythrocytic stages of \( Pf3D7 \) using Real-Time PCR (StepOnePlusTM Real-Time PCR system, Applied Biosystems, USA). 18S rRNA served as a positive control. The primer sequences for the real-time PCR analysis of \( Pf\text{PFD1-6} \) and 18S rRNA are mentioned in Table 1.

**Table 1: Primer sequences for the real-time PCR analysis of \( Pf\text{PFD1-6} \).**
The reaction mixture (10 μl) comprised of cDNA, 5 μl SYBR™ Green PCR Master Mix (Applied Biosystems™), and 1 μl (5 μM) PfPFD1-6 specific forward and reverse primers. The PCR conditions included initial denaturation at 95°C for 5 min, followed by amplification for 40 cycles of 15 seconds each at 95°C, 5 seconds at 55°C, and 1 minute at 72°C, with fluorescence acquisition at the end of each extension step. Amplification was immediately followed by a melt program consisting of 15 seconds at 95°C, 1 minute at 60°C, and a stepwise temperature increase of 0.3°C/s until 95°C, with fluorescence acquisition at each temperature transition. All samples were evaluated in duplicates.

**Expression analysis of PfPFD1-6 by immunoblotting**

Mixed-stage asexual cultures of Pf3D7 (5-10% parasitemia) were subjected to saponin lysis, followed by RIPA lysis of the purified parasites. The parasite lysate (10 μg of total protein), recombinant proteins (positive control), haemoglobin from the cytosolic fraction of parasitized RBCs, unparasitized RBCs, and crude extract of *E. coli* (negative control) were resolved on 12% SDS-PAGE and transferred to

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences (5’-3’)</th>
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<tr>
<td>PFD1</td>
<td>F: TTACAAGATCTTTTTTGATATG</td>
</tr>
<tr>
<td></td>
<td>R: ATCCAGTAGGACAATTTCTC</td>
</tr>
<tr>
<td>PFD2</td>
<td>F: AGAACAGTGCGGGGAATTAAGC</td>
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<tr>
<td></td>
<td>R: TTGTTTTTGACATTCAGCTATAACC</td>
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<td>PFD3</td>
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<tr>
<td></td>
<td>R: AAAGGGAAACTCAACCATTACG</td>
</tr>
<tr>
<td>PFD4</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>18srRNA</td>
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</tr>
<tr>
<td></td>
<td>5’-CTTGTACGACTTCTCCTCC-3’</td>
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</tbody>
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nitrocellulose membrane. The transferred blots were blocked with 5% BSA in PBS overnight at 4 °C, and probed with mice PfPFD1-6 antisera (1:5000 of each) followed by incubation with Horseradish Peroxidase (HRP)-conjugated goat anti-mice IgG (1:2000). Blots were developed by using diaminobenzidine/H$_2$O$_2$ substrate (Sigma-Aldrich, MA, USA).

To check for the stage-specific expression of PfPFD1-6, synchronized ring, trophozoite, and schizont stages of the parasite were harvested separately and subjected to saponin lysis. Parasite pellets, thus obtained, were lysed with RIPA buffer for 30 minutes at 4ºC to rupture the parasite's membrane and release its cytosolic content. Supernatants of the parasite lysate (10 µg of the total protein) prepared for all three stages were resolved on 12% SDS-PAGE and transferred to the nitrocellulose membrane. The blot was blocked with 5% BSA in PBS overnight at 4°C, and probed with PfPFD1-6 antisera (1:5,000 of each) followed by incubation with HRP-conjugated goat anti-rabbit IgG (1:2,000). Blots were developed using diaminobenzidine/H$_2$O$_2$ substrate (Sigma-Aldrich, MA, USA).

Similar protocol was followed to check the expression of PfPFDs in Plasmodium falciparum 3D7 sensitive and ART-resistant PfKelch13R539T parasites. Synchronized ring, trophozoite, and schizont stage parasite lysate were prepared in RIPA buffer and equal amount of protein was loaded on 12% SDS-PAGE and transferred to the nitrocellulose membrane. The membrane was blocked in 5% skimmed milk, followed by probing with primary PFDs anti-sera (1:1000) and secondary anti-mice (1:1000) antibodies.

Immuno-Fluorescence Assay (IFA)

Thin smears of mixed-stage parasite culture were fixed in ice-cold methanol for 30 minutes at -20ºC. Fixed smears were permeabilized with PBS/Tween-20, and blocked with 5% BSA (w/v) in PBS for 2 h at RT. For localization studies, PfPFD1-6 antisera (1:200 of each) were added followed by incubation at RT for 2 h. Alexa Fluor 488 conjugated anti-mice (1:250; green; Molecular Probes, Invitrogen, Carlsbad, CA, USA) was used as a secondary antibody. For co-localization studies, anti-mice PfNapL (1:250), anti-rabbit PfMSP1 (1:250), and anti-rabbit α-tubulin-l (1:250) were used. Alexa Fluor 488 conjugated anti-mice and Alexa Fluor 546 conjugated anti-rabbit (1:250; red; Molecular Probes) were used as secondary antibodies. DAPI-antifade (Invitrogen, Life Technologies corporation, Eugene, OR, USA) was used to counterstain parasite nuclei followed by mounting the slides with coverslips. The slides were viewed under a confocal microscope at 100X magnification (Olympus Corporation, Tokyo, Japan).

Cloning, expression, and purification of PfPFD1-6

PfPFD1-6 were PCR amplified from the cDNA of Pf3D7 using gene-specific primers. CDS (coding sequence) encoding for PfPFD1-6 (PF3D7_1107500: PfPFD1, PF3D7_1416900: PfPFD2, PF3D7_0718500: PfPFD3, PF3D7_0904500: PfPFD4, PF3D7_1128100: PfPFD5, and PF3D7_0512000: PfPFD6) were cloned in pET-28a(+) vector (Novagen, Merck KGaA Madison, WI, USA) at BamHI and XhoI restriction sites, and over-expressed in E. coli BL21 ( DE3). Ni-NTA (Qiagen, Hilden, Germany) affinity
purification of \( PfPFD1-6 \) was done in lysis buffer (50 mM Tris/HCl, 300 mM NaCl, and 0.02% Na-azide, pH 8.0).

**Generation of antisera against \( PfPFDs \)**

To raise antibodies against \( PfPFD1-6 \), three male Balb/c mice (6 to 8 weeks old) were each administered (i.p.) with 50 \( \mu \)g of the recombinant \( PfPFDs \) (in 0.9% saline), in a prime and boost regimen. A formulation for the priming dose (day 0) was prepared by thoroughly combining equal parts of Freund’s complete adjuvant and saline containing the protein. Freund’s incomplete adjuvant was used to make formulations for the following booster doses (days 21 and 42). After primary immunization, blood samples were collected from the retro-orbital sinus of the mice on days 31 and 52 (terminal bleed). Extracted blood samples were incubated at 37°C for 30 minutes before being centrifuged at 1,200g for 15 minutes at 4°C, and serum samples were collected and stored at -80°C until further analysis. The raised antisera were checked for specificity by performing western blotting.

**MicroScale Thermophoresis (MST) assays**

Binding affinities among the \( PfPFD \) subunits were evaluated by MST analyses, using Monolith NT.115 instrument (NanoTemper Technologies, Munich, Germany). MST relies on binding-induced changes in the thermophoretic mobility of a given macromolecule, which depends on several molecular properties including particle charge, size, conformation, hydration state, and solvation entropy. Thus, under constant buffer conditions, the thermophoresis of unbound proteins typically differs from that of proteins bound to their interacting partners, and the thermophoretic movement of a fluorescently labelled protein is measured by monitoring the fluorescence distribution. Briefly, 20 \( \mu \)M each of \( PfPFD3 \) and \( PfPFD5 \) was labelled using NanoTemper’s Protein Labelling Kit RED-NHS (L001, NanoTemper technologies, Germany)\(^{10}\). The labelled \( PfPFD3 \) and \( PfPFD5 \) were titrated with increasing concentrations of other \( PfPFD \) subunits in 1XPBS (pH 7.5) with 0.01% tween-20. The samples were pre-mixed and incubated for 10 minutes at RT in the dark before being loaded into standard treated capillaries (K002 Monolith NT.115). For the interaction analysis, the change in thermophoresis was expressed as the fluorescence change in MST signal, defined as \( F_{hot}/F_{cold} \) (\( F_{hot} \) as the hot region after IR laser heating and \( F_{cold} \) as the cold region at 0 s). Titration of the non-fluorescent ligand results in a gradual change in thermophoresis, which was plotted as \( \Delta F_{norm} \) to yield a dose-response (binding) curve, which can be fitted to derive binding constants. Data evaluation was done with the Monolith software (Nano Temper, Munich, Germany).

Competitive MST analysis was done to check whether BPD hinders the interaction among the PFD subunits. Towards this, the labelled \( PfPFD3 \) was mixed with \( PfPFD1 \) (7.5 \( \mu \)M), and \( PfPFD2 \) (28 \( \mu \)M), and incubated for 10-15 minutes. The \( PfPFD3-PfPFD1 \), \( PfPFD3- PfPFD2 \) complexes, thus formed, were
titrated with serial dilutions of BPD in PBS (with 0.01% tween-20) starting from 100 μM, and the interaction analysis was done under the same conditions as described above.

**Generating the 3D-structure model of the PfPFD complex**

Amino acid sequences of the PFD subunits 1-6 from P. falciparum strain 3D7 (1: PF3D7_1107500; 2: PF3D7_1416900, 3: PF3D7_0718500, 4: PF3D7_0904500, 5: PF3D7_1128100, and 6: PF3D7_0512000) were retrieved from the PlasmoDB database (https://plasmodb.org/plasmo/app) 11. A multiple threading approach, which is one of the most common structure prediction methods in structural genomics and proteomics, was employed to generate 3D-structural coordinates of the PfPFD subunits. To accomplish this feat, individual structural models of each subunit were generated using I-TASSER (Iterative Threading ASSEmbly Refinement), a web server that uses a hierarchical approach to protein structure prediction and structure-based function annotation (https://zhanggroup.org/I-TASSER/) 12, as described previously 13. Structural models of PfPFD subunits 1-6, thus generated, with higher values of Confidence-score (C-score) were selected and subjected to structural refinement by using ModRener (https://zhanglab.ccmb.med.umich.edu/ModRener/) which is an algorithm-based approach for atomic-level, high-resolution protein structure refinement 14. The refined structural models of PfPFD subunits were rendered with PyMOL Molecular Graphics System, v2.1 by Schrödinger, LLC (http://pymol.org/2/) 15, and set to submit to generate PfPFDhexamer structure.

The X-Ray diffraction-based structural model of the human TRiC (T-complex protein Ring Complex, also known as Chaperonin Containing TCP-1 (CCT))-PFD complex (PDB ID: 6NR8; resolution: 7.80 Å) 16 was used as a suitable template to generate a 3D structural model of the PfPFDhexamer, as described previously 17. The reliability of the PfPFD structural model was assessed by examining backbone dihedral (torsion) angles: phi (Ø) and psi (Ψ) of the amino acid residues lying in the energetically favourable regions of Ramachandran space 18. This was done by using PROCHECK v.3.5 which checks the stereochemical quality of a protein structure, producing several PostScript plots analyzing its overall and residue-by-residue geometry (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/) 19. Percent quality measurement of the protein structures was evaluated by using four sorts of occupancies called ‘core’, ‘additional allowed’, ‘generously allowed’, and ‘disallowed’ regions. The 3D structural model of PfPFD, thus generated, was subsequently used for in silico and in vitro interaction analysis, and inhibition studies.

**LOPAC®1280 library screening for a possible inhibitor of PfPFD-mediated protein folding**

In a recent investigation by Aline Bamia et al., novel small molecules with Prion (PrPSc) propagation-inhibitory activities were identified, which interfered with the Protein Folding Activity of the Ribosome (PFAR), and significantly prolonged the survival of prion-infected mice 20. Using an in silico therapeutic repositioning approach, we screened LOPAC®1280 Library of 1,280 Pharmacologically Active Compounds (Sigma-Aldrich) based on similarities with one of the potent PFAR inhibitors identified in the study, Metixene (https://www.sigmaaldrich.com/IN/en/product/sigma/lo1280). Metixene is a member of
piperidines and has a role as an antiparkinson drug and a muscarinic antagonist. Structural Data Format (SDF) files of Metixene and LOPAC®1280 library were retrieved from PubChem, a database of freely accessible chemical information of chemical molecules and their activities against biological assays (https://pubchem.ncbi.nlm.nih.gov/), and Sigma-Aldrich, respectively. Structural superimposition of LOPAC®1280 ligands with Metixene was done by using Discovery Studio Visualizer v20.1.0.19295, developed by Dassault Systèmes Biovia Corp. (https://www.3ds.com/products-services/biovia/), and overlay structural similarity for each of the 1,280 compounds were evaluated, taking Metixene as a reference compound. Structural superimposition analysis was done by using ChemDraw Ultra v12.0.2.1076, one of the CambridgeSoft products for producing a nearly unlimited variety of biological and chemical drawings (https://perkinelmerinformatics.com/products/research/chemdraw).

*In silico* interaction analysis of BPD with *PfPFD*

Structural Data Format (SDF) file of BPD.HCl was retrieved from PubChem, converted to standard PDB format, followed by the generation of its energy-minimized 3D-structural model by using Chem3D Pro 12.0, as described previously. Molecular docking studies were performed by using Autodock Vina Tools 1.5.6 to rationalize the inhibitory activity of BPD against *PfPFD*. We ensured that the entire *PfPFD* complex was covered while constructing a virtual 3D grid for the in silico interaction analysis. A grid of 80 × 100 × 80 with x, y, and z coordinates of the center of energy, 209.514, 147.278, and 209.119, respectively was constructed through the Autogrid module of AutoDock Tools, with default spacing. Top scoring docked conformations of BPD were selected based on their most negative free binding energies and visualized for polar contacts (H-bonds; if any) with the amino acid residues of *PfPFD* complex using PyMOL Molecular Graphics System.

Parasite growth inhibition assay

To evaluate the effect of BPD on the intra-erythrocytic proliferation of the parasite, a growth inhibition assay was performed. Briefly, *Pf3D7* culture synchronized at ring stage (0.8% parasitemia and 2% hematocrit) was treated with BPD (250 nM, 500 nM, 750 nM, 1 μM, 2.5 μM, and 5 μM). Dilutions of BPD were prepared in iRPMI. The assay plate was maintained at 37°C in a controlled atmosphere (5% O2, 5% CO2, and 90% N2) for 72 h. Post-incubation, Giemsa-stained thin blood smears of *P. falciparum* were prepared and ~3,000 RBCs were counted in each smear. The experiment was performed in triplicates. Percent growth inhibition was calculated by using the formula: % Inhibition = [1 - % Parasitemia\text{treatment} / % Parasitemia\text{control}] * 100. The graph was plotted using GraphPad PRISM software.

Cytotoxic evaluation of BPD on the parasite

To evaluate the cytotoxic effect of BPD on the parasite, BPD (1 and 5 μM)-treated trophozoite-parasitized RBCs were co-stained with Propidium Iodide (PI) and SYTO9 fluorescent dyes. Untreated parasitized RBCs served as a negative control. 8 h post-treatment, cells were washed with iRPMI and stained with PI and SYTO9 (100 μM of each; InvitrogenTM Thermo Fisher Scientific) in a 1:1 ratio. Cells were incubated in the dark at RT for 15 minutes, washed with iRPMI, and transferred onto a glass slide for visualization.
under an Olympus fluorescence microscope. The fluorescence intensities of PI and SYTO9 in the untreated and treated parasites were also spectrophotometrically measured using the Varioskan LUX Multimode Microplate Reader (ThermoFisher Scientific), at excitation/emission wavelengths of 490/635 nm (PI) and 480/500 nm (SYTO9).

**Enzyme-Linked Immune Sorbent Assay (ELISA) to evaluate the interaction of PfPD1-6 with α-tubulin-I**

To assess the interaction of PfPD1-6 with α-tubulin-I, a 96-well ELISA plate was coated with purified PfPD1-6 (bait; 100 ng of each) in PBS at RT for 5 h, and blocked overnight at 4°C with 5% BSA in PBS, followed by incubation at RT for 2 h with increasing concentrations (0-100 ng) of α-tubulin-I (prey). After washing with PBS, antisera (1:10,000) against the respective PfPFD subunits was added to each well, followed by incubation with secondary anti-mice HRP conjugated antibody at RT for 2 h. After washing with PBS, a detection reagent (TMB; HIMEDIA) was added, followed by the addition of 3 M HCl to stop the HRP reaction. Absorbance was measured in a microplate reader at 450 nm. The graph was plotted using GraphPad PRISM software.

**Effect of BPD on the expression levels of PfPFD substrates**

To evaluate the effect of BPD on the expression levels of α-tubulin-I, trophozoite-parasitized RBCs were treated with BPD (1 and 5 μM). After 6 h of treatment, parasitized erythrocytes were harvested and lysed with 0.05% saponin. The purified parasites, thus obtained, were lysed with RIPA buffer. The parasite lysate was used for western blotting with α-tubulin-I anti-serum to check for the expression levels. Similarly, to assess the effect of BPD on the expression levels of PfMSP-1, trophozoite-parasitized RBCs (at 0.8% parasitemia and 3% hematocrit) were treated with BPD at a concentration equivalent to its IC50 (i.e., 1 μM) for 6 and 12 h; whereas, ring parasitized RBCs were treated for 48 h. Thin smears of the BPD-treated cultures were prepared on glass slides for IFA. Similarly, BPD-treated parasites were harvested for western blotting with PfMSP-1 antiserum to check for the expression levels of PfMSP-1.

**Parasite egress and invasion assay in vitro**

To determine the effect of BPD on the invasion and egress rate of the parasite, mature schizonts (45-47 h post-invasion, hpi) were diluted to ~4% parasitemia and 2% hematocrit. Parasites were treated with varying concentrations of BPD (5, 2.5, 1.25, and 0.625 μM). Untreated parasites were taken as control. After 8 h of treatment, thin smears were prepared on glass slides and stained with Giemsa. Approximately 3,000 RBCs were counted under a light microscope at 100X magnification. Percent egress was calculated by using the formula: (No. of schizonts at 0 h - No. of schizonts in treated sample) / (No. of schizonts at 0 h - No. of schizonts in untreated sample) * 100. The initial number of schizonts was taken as 100%. Percent egress inhibition was calculated as 100 - percent egress. The number of rings formed per schizont egress: No. of rings / (No. of schizonts pre-treatment - No. of schizonts post-treatment)²⁵,²⁶.

**Generation of PfPFD6 complementation strain in yeast mutant**
Full-length sequence of \( PfPFD6 \) (1-360 bp) was amplified using \( P. falciparum \) cDNA as a template, and gene-specific primers. The purified insert and p416 GPD vector, with a host range in bacteria and yeast, harbors GPD promotor and bears the Uracil-encoding gene (ura+) for selection, were digested with BamHI/SalI (New England Biolabs, UK) and ligated overnight at 4°C using T4 DNA ligase (New England Biolabs, UK). The ligation mix was transformed into \( E. coli \) DH5-\( \alpha \) competent cells and positive clones were screened by colony PCR.

The \( PfPFD6 \)-p416-GPD and p416-GPD constructs were transformed in \( S. cerevisiae \) mutants YTM1304::pf6\( \Delta \) (gim1\( \Delta \)), using Frozen-EZ Yeast Transformation IITM kit (Zymo Research) as per the manufacturer's protocol and plated on YNB (yeast nitrogen base) agar plate (supplemented with 2% glucose and 1X amino acid mix without uracil) as selective media. Cells were allowed to grow at 30°C. The transformed colonies were confirmed by colony PCR using gene-specific primers. \( S. cerevisiae \) BY4742 (MAT\( \alpha \); his3\( \Delta \); leu2\( \Delta \); lys2\( \Delta \); ura3\( \Delta \)) was used as the wild-type control wherever applicable. YTM1304-p416-GPD was also used as a control in experiments.

**Growth curve analysis in complemented mutant yeast strain**

To assess the growth pattern of mutant yeast strain complemented with \( P. falciparum \) \( PfPFD6 \), primary culture of wild type (BY4742), YTM1304 (pf6\( \Delta \)), YTM1304-\( PfPFD6 \), YTM1304-p416-GPD were inoculated in YPD media and incubated at 300°C for overnight. Subsequently, the secondary culture was inoculated and after every 1.5 hours, absorbance at 600 nm was observed spectrophotometrically. Growth curve was plotted using GraphPad Prism 8.0 software. Additionally, the initial \( A_{600} \) of 0.1 cell suspension was 10-fold serially diluted, and each dilution was carefully spotted onto YPD-agar media plates, followed by incubating the culture plates for two days at 30°C.

**Assessment of Biperiden Selectivity for \( PfPFD6 \)**

To confirm the selectivity of BPD towards \( PfPFDs \), YTM 1304-p416-GPD and YTM 1304-\( Pfpd6 \) were cultured in their respective media overnight until sufficient growth was achieved. Subsequently, the cultures were diluted to an optical density, \( A_{600} \) of 0.1 using sterile fresh media and further incubated in the absence and presence of 20 \( \mu M \) BPD at 30°C for 12 hours. The resulting cell suspension was then serially diluted to 10-folds, and each dilution was spotted onto a YPD agar plate. The culture plate was incubated for two days at 30°C.

**Assessment of Artemisinin effect on growth of complemented mutant yeast strains**
To elucidate the impact of Artemisinin (ART) treatment on the yeast cell growth pattern, YTM1304-
PfPFD6, and YTM1304-p416-GPD were grown in YPD media and used as a pre-culture. Once the culture
reached appropriate growth, it was diluted to an initial $A_{600}$ value of 0.1 and subjected to further
incubation at 30°C for 12 hours, with and without the addition of artemisinin (8 µM). Post incubation
cultures were harvested and adjusted to $A_{600}$ value of 0.1. The cultures were then subjected to ten-fold
serial dilution, spotted on the YPD agar plate and incubated at 30°C for the next two days.

**Parasite growth inhibition in vivo**

BALB/c female mice (6 weeks old) were divided into four groups and each group consisted of four
animals. At day 0, mice were infected intra-peritoneally with $1 \times 10^6$ infected RBCs (100 µl, diluted in PBS),
obtained from P. berghei ANKA-infected donor mice. Group 1 mice were treated with artesunate at a
concentration of 6 mg/kg (positive control), Group 2 was treated with 12.5 mg/kg of BPD, while Group 3
was left untreated (negative control). Blood samples from the tail end of the infected mice were taken
daily. Percent parasitemia was determined by observing the Giemsa-stained smears of the blood samples
under a microscope at 100× magnification (Olympus Corporation, Tokyo, Japan).

**Statistical analysis**

All graphs were generated by the software GraphPad Prism (version 8; La Jolla, CA, USA)) and statistical
analysis was calculated by using unpaired two-tailed Student's t-tests. The level of significance was
established at $nsp > 0.05$, $*p < 0.05$, $**p<0.01$

**RESULTS**

**Prefoldins identified as interacting partners of PfKeltch-13**

A previous study reported that artemisinin resistance is linked with up-regulated expression of unfolded
protein response pathways involving Prefoldins of malaria parasite. Also, artemisinin resistance is
strongly associated with the polymorphism in the β-propeller domain of the K13 protein. In light of the
above facts, we performed Co-IP studies where anti-K13 antibody was crosslinked to AminoLink plus
Coupling Resin and pull down the interacting partners from parasite lysate. The eluted fractions were
subjected to mass spectrometry analysis. The hits obtained were used to generate protein-protein
interaction network using STRING which clearly demonstrate the possibility of interaction between
PfPFDs with PfK13 (Fig. 1 A).

To confirm the direct interaction between prefoldins and PfK13, we performed SPR-based interaction
analysis. We used recombinant PfPFD6 and PfK13 to quantify the interaction strength by SPR. PfK13
was purified using Nickel NTA based affinity chromatography (Fig. 1 B). In SPR, immobilized PfPFD6 was
titrated with increasing concentrations of PfK13. A $K_D$ value of 174 nM was observed for PfPFD6 - PfK13
The interaction of PfPFD6 with PfK13 was further validated using co-immunoprecipitation assays in Pf3D7 and artemisinin resistant strain PfR539T. Here, PfPFD6 antiserum was cross-linked to the AminoLink plus Coupling Resin, followed by incubation with the parasite lysate prepared from the mix-stage parasite population. Bound protein fractions were eluted and subjected to western blotting by probing with anti-PfK13 antibodies. The desired protein band of PfK13 was observed in the eluted fraction (Fig. 1D). Overall, these results suggest interaction between PfPFD6 and PfK13.

### Expression of PfPFD1-6 in the intra-erythrocytic stages of the parasite

Reverse Transcriptase-quantitative Polymerase Chain Reaction (RT-qPCR) stands as a precise and convenient technique for quantifying target gene expression levels. RT-qPCR revealed that the transcripts encoding for PfPFD1-6 are expressed throughout the intra-erythrocytic life cycle of *P. falciparum* (Fig. 1E). As a positive control, transcripts encoding for 18S rRNA were amplified using *Pf18s*-specific primers. The numbers on the graph show the Ct-values of each gene. Our result demonstrates that the Prefoldin subunits express more at the trophozoite and schizont stages of the parasite.

Western blot analysis of the native PfPFD1-6 in the parasite lysate prepared from all three intra-erythrocytic stages corroborated the expression of PfPFD1-6 in the parasite (Fig. 1F). At the trophozoite and schizont stages, distinct bands of PfPFD1-6 were detected with their anticipated molecular weights (18.4 kDa: PfPFD1, 16.6 kDa: PfPFD2, 22.6 kDa: PfPFD3, 15.3 kDa: PfPFD4, 29.1 kDa: PfPFD5, and 13.6 kDa PfPFD6). However, at the ring stage, the expression of PfPFD1-6 was not detected. PfTubulin served as a loading control (Fig. 1F). Full uncropped blots of these results are represented in fig. S1. Furthermore, expression of PFDs was not detected in the *E. coli* lysate or uninfected RBCs (both insoluble and soluble fractions) which served as negative controls (Fig. S2). We also tested the expression of PfPFD1-6 in artemisinin resistant line PfR539T and found that Prefoldins expression are upregulated in PfR539T as compared to artemisinin sensitive line Pf3D7 (Fig. 1G). RT-PCR and Western-based expression analysis was validated by Immunofluorescence Assays (IFAs), which depicted that PfPFD1-6 are expressed at the trophozoite and schizont stages of the parasite (Fig. 2A). Altogether, these findings suggest that PfPFD1-6 are expressed at both the transcript and protein levels in the intra-erythrocytic stages of the parasite. Furthermore, PfPFD1-6 is mostly confined to the cytoplasm of schizonts, as confirmed by its co-localization with a cytosolic protein marker, PfNapL (Fig. 2B).

### Orchestrated assembly of PfPFD_{hexamer}

CDS for *pfpd1, pfpd2, pfpd3, pfpd4, pfpd5, and pfpd6* were cloned in pET-28a(+), over-expressed, and recombinant PfPFD subunits (6x-His-PfPFD1-6; or, rPfPFD1-6) were purified. rPfPFD1-6 resolved on SDS-PAGE as protein species of 20, 19, 24, 17, 30, and 16 kDa, respectively (Fig. 3 A). Polyclonal antibodies were raised against rPfPFD1-6 in male BALB/c mice. PfPFD complexation was investigated using co-Immunoprecipitation assay (co-IP), in which PfPFD6 antiserum was cross-linked to AminoLink plus Coupling Resin followed by incubation with the parasite lysate. Eluted fractions were resolved on SDS-PAGE, followed by western blotting with the respective PfPFD1-6 antisera. Each blot showed a
respective band of the PfPFD subunits (Fig. 3B), suggesting that PfPFD1-6 form a complex similar to their counterparts in other species. Full uncropped blots are represented in fig. S1.

In archaea and eukaryotes, PFD2 and PFD6 interact with PFD3 and PFD5, respectively, to generate sub-complexes. This is followed by the recruitment of PFD1 and PFD4, which are then assembled by these sub-complexes to form a complex 27. In a similar manner, we labelled PfPFD3 and PfPFD5, and demonstrated their interaction with other PfPFD subunits by MST analyses. Binding affinity (K_D) determined by steady-state analysis, by plotting response at equilibrium as a function of unlabelled protein concentration, are as follows: 81.6 nM (PfPFD3-PfPFD1), 682 nM (PfPFD3-PfPFD2), 11.3 μM (PfPFD3-PfPFD4), 4.6 μM (PfPFD3-PfPFD5), and 1.88 μM (PfPFD3-PfPFD6), which indicates higher binding affinity of PfPFD3 with PfPFD1 and PfPFD2 (Fig. 3C (i-iv, ix)). Similarly, K_D values were determined to be 851 nM (PfPFD5-PfPFD1), 628 nM (PfPFD5-PfPFD2), 104 nM (PfPFD5-PfPFD4), and 324 nM (PfPFD5-PfPFD6), which indicates higher binding affinity of PfPFD5 with PfPFD4 and PfPFD6 (Fig. 3C (v-viii)).

We next attempted to generate a reliable structural model of PfPFD complex using in silico approach. The top threading templates used by I-TASSER to generate individual structural models of PfPFD subunits 1-6 are as follows: PFD from Pyrococcus horikoshii OT3, chain C (PDB ID: 2ZDI; for PfPFD1, PfPFD3, and PfPFD5); and, PFD-beta subunit from Thermococcus strain KS-1, chain A (PDB ID: 2ZQM; for PfPFD2, PfPFD4, and PfPFD6) 28,29, and set to submit to generate PfPFD_hexamer complex structure by using X-Ray diffraction-based structural model of human TRiC-PFD complex as a suitable template 16. After optimal rigid-body superimposition of the generated structural model of PfPFD_hexamer with HsPFD, the overall Root-Mean-Square Deviation (RMSD) value of the C-alpha atomic co-ordinates was found to be 1.45 Å, suggesting a reliable 3D structure. Assessment of the stereochemical quality and accuracy of the generated structural model of PfPFD_hexamer displayed 91.8% of amino acid residues lying in the most favored (core) regions, with 6.8%, 0.9%, and 0.5% residues in additional allowed, generously allowed, and disallowed regions of Ramachandran plot, respectively.

PfPFD_hexamer structural model revealed a strong resemblance with its counterparts from other eukaryotes, forming a hetero-hexamer with a jellyfish-like architecture composed of two canonical classes of subunits: α (PFD subunits: 3 and 5) and β (subunits: 1, 2, 4, and 6), arranged in the following manner - PFD3-PFD2-PFD1-PFD5-PFD6-PFD4. 16,30 (Fig. 3D). The core body of PfPFD_hexamer was found to consist of a double beta-barrel assembly, with six long tentacle-like coiled coils protruding from it in a regularly structured fashion. The comparable RMSD value and Ramachandran plot characteristics confirmed the reliability of the PfPFD_hexamer complex to be taken further for in silico and in vitro interaction analysis, and inhibition studies.

Identification of BPD as a potential binder of PfPFD_hexamer
The *in silico* therapeutic repositioning approach which we adopted to screen the LOPAC® library based on similarities with one of the potent PFAR inhibitors, Metixene, generated BPD, with an overlay structural similarity index of 0.75 (Fig. 4 A(i)). Similar to Metixene, BPD (IUPAC name: 1-{bicyclo[2.2.1]hept-5-en-2-yl}-1-phenyl-3-(piperidin-1-yl)propan-1-ol) is a member of piperidines, and has a role as an antiparkinson drug and a muscarinic antagonist (Fig. 4A (ii)). In a recent investigation, BPD along with 16 other FDA-approved drugs, were identified to harbor anti-prion activity. In the study, seven out of the 17 compounds with lower IC₅₀ values were further examined for their ability to inhibit PFAR. However, because of its high IC₅₀ value, the PFAR activity of BPD was not investigated further. Given the fact that the structural features of BPD are similar to those of the PFAR-active compound, Metixene, we hypothesized that BPD would interact with additional genes whose activity is associated with protein folding and show an inhibitory effect, which prompted us to investigate the direct interaction of BPD with PfPFD.

A plausible architecture of the PfPFD-BPD complex was constructed using the generated structural model of the PfPFDhexamer. A schematic representation of complex formation between PfPFDhexamer and BPD is shown in Fig. 4B. BPD was found to interact with PfPFDhexamer via two different conformations. In conformation 1, BPD was found to engage at the interface of PfPFD subunits 2 and 6, with free binding energy (ΔG_bind) of -8.4 kCal/mol, via polar contact (H-bonds) with Glu⁶⁸ PfPFD6 with a bond length of 3.5 Å (Fig. 4B (i)). Contrastingly, in conformation 2, BPD was found to interact with the core body of the PfPFD complex consisting of a double beta-barrel assembly, although with a lower ΔG_bind of -8.0 kCal/mol (Fig. 4B (ii)). It was, therefore, hypothesized that PfPFD complexation with BPD in either of the conformations, would result in a diminished ability to bind and stabilize newly synthesized proteins, thereby impairing the correct folding of the nascent polypeptides. Structural representation of BPD is depicted in Fig. 4C.

We performed Surface Plasmon Resonance-based analysis to investigate the direct interaction of PfPFD1-6 with BPD. With increasing concentration of BPD, a gradual increase in sensor signal was observed which linearly correlated with a corresponding change in the refractive index of the medium immediately adjacent to the PfPFD1-6-immobilized sensor surface. The binding affinities of BPD for each PFD subunit differed significantly. Notably, K_D values for the interaction of BPD with PfPFD-1, PfPFD-2, PfPFD-3, PfPFD-4, and PfPFD-6 were determined to be 57, 200, 100, 40, and 100 µM respectively (Fig. 4D). However, BPD showed no interaction with PfPFD5.

To further evaluate the binding strength between PfPFD3 and BPD, we performed an MST-based competition experiment, in which PfPFD3 was used as a labelled protein; and, BPD, PfPFD1, and PfPFD2 as the unlabelled competing ligands. The interaction of PfPFD3 with PfPFD1 and PfPFD2 exhibited increasing MST signals (F_norm [%]) starting at 886.1 to 892.1 units (for PfPFD1), and 886.4 to 893.8 units (for PfPFD2), resulting in the K_D values of 81.6 nM and 682 nM, respectively (Fig. 3C (i) and (ii)). Competing the interactions with BPD resulted in a decrease in the MST signal starting at 893 to 888.1 units (for PfPFD1), and 894.5 to 887.6 units (for PfPFD2), thus enhancing the K_D values to 1.99 µM and
9.6 nM, respectively (Fig. 4E (i) and (ii)). The difference in the MST signal in the absence and presence of BPD indicated that it competes with PfPFD1 and PfPFD2 for binding to PfPFD3.

**BPD treatment inhibits in vitro growth of *P. falciparum***

BPD was evaluated for its growth-inhibitory effect on Pf3D7 and PfR539T using an *in vitro* intra-erythrocytic Growth Inhibition Assay (GIA), wherein, parasitemia levels were determined at 72 h post-treatment. BPD inhibited the growth of the malaria parasite and displayed a potent anti-plasmodial effect with an IC$_{50}$ value of ~1 μM in Pf3D7 and PfR539T (Fig. 4F (i) and (ii)). Artesunate served as a control, which is a well-known anti-malarial drug and killed ~49% of the parasites at a concentration of 8 nM. Giemsa stained images of BPD treated and untreated parasites are shown in fig. 4F iii. Viability of the BPD-treated parasites was further assessed by co-staining the parasites with Propidium Iodide (PI) and SYTO9. PI is a nuclear counterstain that fluoresces red and is used to identify dead cells in a cell population; and, SYTO9 is a green fluorescent nucleic acid that stains both live and dead cells. A significant difference was observed in the mean fluorescence intensities of SYTO9 and PI in the untreated and BPD-treated parasites, demonstrating that the parasites are susceptible to death due to BPD exposure (Fig. 4G (i) and (ii)). Even at 100 μM, BPD displayed negligible cytotoxicity in the HepG2 cell line upon 48 h of treatment (Data not shown).

**PfPFD2 interacts with Pfα-tubulin-I**

Previous reports in eukaryotes indicate that PFD interacts with cytoskeletal proteins $^{31-33}$. Protein-Protein Interaction (PPI) data available in the PlasmoDB database also indicate the interaction of PfPFD2 with Pfα-tubulin-I. To validate the interaction, preliminary screening was carried out with semi-quantitative ELISA, wherein, PfPFD2 upon titration with Pfα-tubulin-I depicted the interaction in a concentration-dependent manner (Fig. 5A). To quantify the interaction strength, SPR-based interaction analysis was performed in which upon titrating the immobilized PfPFD2 with Pfα-tubulin-I, K$_D$ value was determined to be 1.9 μM (Fig. 5B). SPR analysis was also performed by titrating the immobilized PfPFD1 and PfPFD3 with Pfα-tubulin-I, which demonstrated no interaction between the proteins (Fig. S3), demonstrating that Pfα-tubulin-I specifically interacts with PfPFD2.

The interaction of PfPFD2 with Pfα-tubulin-I was confirmed with co-IP, in which PfPFD2 antiserum was cross-linked to the AminoLink plus Coupling Resin, followed by incubation with the parasite lysate prepared from the mix-stage parasite population. The desired protein band of Pfα-tubulin-I was observed in the eluted fraction (Fig. 5C (i)). Similarly, reverse co-IP, in which Pfα-tubulin-I antisera was cross-linked to the resin, followed by incubation with the parasite lysate, confirmed the interaction between the two proteins (Fig. 5C (ii)). Collectively, these findings point to a possible interaction between PfPFD2 and Pfα-tubulin-I. Further, western blot analysis demonstrated that the Pfα-tubulin-I levels get markedly reduced upon the treatment of the parasites with BPD (Fig. 5D), suggesting the inhibitory effect of BPD in blocking PfPFD2 interaction with its substrate protein Pfα-tubulin-I. Full uncropped blot is represented in fig. S1.
**BPD destabilizes PfMSP-1, a substrate of PfPFD**

We previously reported that PfMSP-1, a critical protein involved in the egress and invasion of the parasite, is a substrate of PfPFD. To evaluate how BPD affects the function of PfPFD subunits, the expression, and localization of PfMSP-1 were assessed in the BPD-treated parasites. Western blot analysis revealed that the expression of PfMSP-1 gets reduced in the treated parasites as compared to the untreated ones (Fig. 5E i, ii). GAPDH served as a loading control (Fig. 5E iii). We also evaluated the effect of BPD on the expression of PfMSP-1 at two different time points of drug treatment and found reduced PfMSP-1 expression with prolonged drug treatment (Fig. 5E iv; Full uncropped blots are represented in fig. S1). Confocal microscopy validated the western-based analysis, wherein, reduced expression of PfMSP-1 was observed in the BPD-treated schizonts (Fig. 5F); NapL served as a control (Fig. S4). These findings imply that BPD destabilizes PfMSP-1 expression by interacting with PfPFD.

We next evaluated the effect of BPD on the egress and invasion of the parasite. BPD significantly inhibited the egress of the parasite by ~60% at 1.25 µM, and ~75% at 2.5 µM and 5 µM concentrations (Fig. 5G (i)). Similarly, the number of rings formed per egress of schizont was significantly reduced in the presence of BPD, as compared to the control (Fig. 5G (ii)). This indicates that disturbing the PfPFD-mediated proteostasis by BPD inhibits the egress and invasion processes of the parasite.

Based on these findings, we hypothesized that PfPFD2 interaction with α-tubulin-I is responsible for the transfer of nascent α-tubulin-I polypeptide to the TRiC/CCT complex, ensuring the accurate folding and subsequent polymerization (Fig. 5H i). However, if biperiden interacts with the PFD complex, it disrupts the interaction between PfPFD2 and α-tubulin-I, leading to the degradation of the α-tubulin-I and inhibition of microtubule polymerization ((Fig. 5H i). Similarly, PfPFD6 interacts with PfMSP-1 and helps in facilitating the proper functioning of MSP-1. However, when BPD interacts with PfPFD6, it interferes with interaction of PfPFD6 with PfMSP-1, resulting in impaired parasite invasion and egress (Fig. 5H ii).

**Complementation of Prefoldin-6 in yeast orthologous system and effect of BPD and artemisinin in complemented yeast strains.**

To ascertain the identity and function of *P. falciparum* Prefoldins, we exploited *Saccharomyces cerevisiae* orthologous model. Functional characterization was done in yeast orthologous model by expressing PfPFD6 in cells harboring mutants. We cloned PfPfd6 in p416-GPD expression vector, followed by transformation of the resulting plasmid (p416-GPD-PfPfd6) in Scpfd6-deleted strain of *S. cerevisiae* (YTM 1304). Following transformation, spot assay analysis was performed that revealed restoration of cell growth in YTM 1304 complemented with PfPfd6 (YTM 1304-PfPfd6), as depicted in (Fig. 6A). WT (BY4742) was used as positive control in the assay. Similarly, our growth curve analysis corroborated with the results of Spot assay, where the complemented PfPFD6 (YTM 1304-PfPfd6) displayed enhanced growth in comparison to the Scpfd6-deleted strain (Fig. 6B).

Having demonstrated the restored growth of YTM 1304 upon complementation with PfPfd6, we next investigated BPD specificity for PfPFD6. Two sets of yeast cultures (Wild type and YTM 1304-PfPfd6)
were used in the assay and were treated with 20 μM BPD to assess their growth patterns through spot assay analysis. Interestingly, YTM 1304-Pfpfd6 exhibited reduced growth in the presence of BPD as compared to the control (untreated) (Fig. 6C). However, no significant difference was observed in BPD treated and untreated wild-type S. cerevisiae (Fig. 6C). These results suggest that the decline in growth is primarily attributed to the targeted effect of BPD on PfPFDs. Further, to elucidate the effect of artemisinin, two batches of yeast cultures were cultivated: one comprising YTM 1304-p416-GPD and the other consisting of YTM 1304-Pfpfd6. These cultures were grown in the presence and absence of ART. Our Spot assay analysis showed the reduced growth of YTM1304-p416-GPD in the presence of ART, while, complemented strain YTM 1304-Pfpfd6 exhibited enhanced growth (Fig. 6D). These data points that Pfpfd6 has a significant role in the development of resistance against artemisinin.

**BPD inhibits parasite growth in vivo**

The anti-plasmodial activity of BPD was also evaluated in vivo, in mice infected with P. berghei ANKA. Schematic representation of methodology followed for the experiment is shown in Fig. 7A. On the tenth day post-infection, parasitemia in the untreated mice reached over 23%, and all mice died (Fig. 7B (i)). However, the parasitemia was around 7% in infected mice treated with BPD or artesunate. On the twenty-third day post-infection, survival of the infected mice treated with BPD or artesunate was found to be 50% (Fig. 7B (ii)). These findings suggest that BPD-treated mice possess reduced parasitemia and a higher survival rate than the untreated ones.

**DISCUSSION**

Artemisinin resistance has become a threat to the efficacy of artemisinin-based combination therapies as its spread from Southeast Asia to Africa. Although artemisinin resistance has become prevalent, there is less knowledge about the molecular mechanisms that makes *P. falciparum* insensitive to artemisinins. A study by Mok et. al. carried out transcriptome analyses of 1043 clinical *P. falciparum* isolates and identified the underlined mechanisms on the transcriptional level that mediate artemisinin resistance. The same study reported that artemisinin resistance is linked with the coordinated transcription of several chaperone partners including the unexplored Prefoldins of *P. falciparum*. In light of these facts, the present study attempted to elucidate the functions of Pf Prefoldins in malaria parasite and their relevance to artemisinin resistance. This piece of knowledge can fill the keys gaps to malaria biology, and can help to combat artemisinin resistance.

Since PfK13 protein is reported to be responsible in mediating ART resistance, we performed CO-IP studies coupled with mass spectrometry analysis to identify the possibility of interaction between PfK13 and Pf Prefoldin. The protein-protein interaction network generated using STRING suggest the interaction of PfK13 with Prefoldins. This interaction was further confirmed by SPR and pull down studies.

Abnormalities in protein synthesis, folding, or clearance disrupt cellular processes, leading to pathological consequences. To maintain a functional proteome, cells rely on a complex network of surveillance mechanisms directed by molecular chaperones, which fold newly synthesized polypeptides, refold
misfolded proteins, and guides protein degradation\textsuperscript{35}. Genomic cataloging reveals that approximately 2\% of the \textit{P. falciparum} genes encodes for an extensive array of molecular chaperones that are believed to play a crucial role in assisting the parasite to adapt to infection-induced stress\textsuperscript{36}. Investigating chaperones in the parasitic system, therefore, may not only provide potential therapeutic targets but also offers insights into novel principles of chaperone function in the parasite biology. Among the chaperones, Prefoldin, a highly acclaimed co-chaperone protein, aids in the proper folding of essential proteins. Prefoldin has garnered considerable attention for its significance in emerging domains such as nanoparticles, biomaterials, and tumour biology. Furthermore, each subunit of Prefoldin possesses distinct and independent roles apart from its involvement in the Prefoldin complex\textsuperscript{27}. Although archaea and eukaryotes have been well-characterized in terms of Prefoldin function, \textit{Plasmodium} species lack sufficient studies investigating the potential importance of Prefoldin subunits in managing the proteostasis of critical plasmodial proteins. The transcriptome data obtained from PlasmoDB indicates the presence of all six Prefoldin subunits in \textit{Plasmodium} (PF3D7\_1107500, PF3D7\_1416900, PF3D7\_0718500, PF3D7\_0904500, PF3D7\_1128100, and PF3D7\_0512000). CDS for the full-length Prefoldin subunits was cloned into a bacterial expression system and subsequently overexpressed and purified. Mice were immunized to generate polyclonal antibodies against each Prefoldin subunit. These antibodies exhibited specific binding to the corresponding subunits, enabling their detection and analysis. qRT-PCR and western blot analysis confirmed the expression of Prefoldin subunits at both transcript and protein levels during the intra-erythrocytic stages of the malaria parasite. Also, expression of Prefoldin subunits was observed to be upregulated in artemisinin resistant strain as compared to sensitive parasites, underlining their importance during resistant mechanism. Furthermore, our immunofluorescence-based experiments illustrate the localization of Prefoldin subunits within the cytosol of the parasite. Notably, the co-localization of Prefoldin subunits with the \textit{Plasmodium} cytosolic marker protein ‘\textit{Pf}NapL’ reveals a substantial overlap of localization signals, indicating their coexistence within the parasite cytosol. These findings align with previous reports in archaea and eukaryotes, suggesting the presence of the Prefoldin complex in the cytoplasm\textsuperscript{37}. Moreover, this suggests that the role and localization of Prefoldin subunits may be conserved across different organisms.

The Prefoldin complex, which functions as a cytoplasmic chaperone protein, is assembled through the interaction of its six distinct subunits, resulting in the formation of a hybrid oligomer. Based upon previous research\textsuperscript{27}, we conducted Nano temper-based interaction analysis, revealing the high affinity of PFD3 for PFD2 and PFD2, while PFD5 exhibited greater affinity towards PFD4 and PFD6. To validate the binding of these sub-complexes, we also examined the binding of PFD3 and PFD5, further confirming their strong affinity.

To gain insights into the role of the \textit{Pf}PFD complex and explore potential antimalarial small molecules, we conducted an analysis of various chemotypes approved by the FDA and identified biperiden as a probable \textit{Pf}PFD-binding molecule. \textit{In silico} analysis suggested that BPD binds to the PFD complex, which was further confirmed by SPR-based interaction analysis, demonstrating a significant one-to-one interaction of \textit{Pf}PFD subunits with BPD. MST analysis revealed disruption of protein-protein interactions
involving the Prefoldin subunits. Further, through parasite growth inhibition assay, we evaluated the effect of BPD treatment on the *in-vitro* growth of *P. falciparum*, and observed an IC$_{50}$ of 1 μM, indicating its efficacy in inhibiting parasite growth without inducing cytotoxic effects.

Actin and tubulins are highly abundant cytoskeletal proteins that play crucial roles in numerous cellular functions, including cellular mobility, morphogenesis, cellular mobility, polarity establishment, cell division, and intracellular transport. Considering the importance of these functions, we observed that *Plasmodium* Prefoldin also interacts with α-tubulin-I, thus supporting the crucial role of Prefoldin abundance in microtubule function. This is consistent with the previous findings in *Arabidopsis*, wherein, *Pfd1-6* mutant showed considerable microtubule defects, including oryzalin hypersensitivity, impaired cell division, cortical array disorganization, and reduced microtubule dynamics.$^{38}$

Previously, we reported the interaction of *PfPFD6* with MSP-1.$^{34}$ Moreover, treatment with BPD efficiently impeded the interaction between the PFD complex and its substrate, resulting in the degradation of α-tubulin-I and MSP-1. These proteins are known to be essential for the growth and proliferation of the parasite, prompting us to investigate the impact of Prefoldin inhibition on parasite growth and proliferation. Our results demonstrated that treatment with BPD led to the inhibition of parasite egress and reduced the formation of rings per schizont egress. Understanding the processes involved in *Plasmodium* egress inhibition could pave the way for the development of new pharmacological targets and more effective antimalarial therapies.

We next used yeast orthologous system to show that *PfPFD-6* complementation in yeast mutants restores the growth of mutant strain. The yeast model system was also used to show the selectivity of the compound BPD for *Pf* Prefoldins. Interestingly, we observed that Prefoldin decrease the sensitivity of artemisinin in yeast, providing evidence for the role of PFDs in providing resistance to *Pf*.

Assessing the *in vivo* efficacy of a drug is crucial for identifying promising leads in drug development. Therefore, we evaluated the *in vivo* efficacy of BPD in a rodent malaria model. The results showed that the drug efficiently inhibited parasite development and increased the survival rate of mice at a dosage of 12.5 mg/kg.

Altogether, this study is the first to shed light on the unexplored Prefoldin subunits during the asexual stage of *P. falciparum* with special relevance to understand their role in providing artemisinin resistance, and identifies them as potential pharmacological targets. Complementation of prefoldin in yeast mutants provide evidence for the role of prefoldins in providing resistance to artemisinin. Additionally, we identified the interaction between the drug molecule, BPD and the Prefoldin subunit complex, and demonstrated its impact on the proteostasis of key interacting proteins, namely α-tubulin-I and MSP-1. Furthermore, our *in vitro* and *in vivo* results establish BPD as an anti-plasmodial inhibitor. This research contributes to ongoing efforts in combating resistance management and reducing the burden of this deadly disease worldwide.
Declarations

Author’s contribution

RS, NP, VK: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, review and editing. AB, SG: Formal analysis, Investigation, Writing – review and editing. PG, DR: Methodology, Formal analysis. MS, PM, NG, JK, SB: Methodology. R. Jain: Formal analysis, Methodology, Writing – review and editing. KP: Formal analysis, Writing – review and editing. MA: Formal analysis, Supervision, Writing – review and editing. SS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review and editing.

Declaration of Competing Interest

None

Data availability

Data will be made available on request.

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Ethics Approval

The mice-based studies were conducted at Animal House, Jawaharlal Nehru University. The mice were handled following the rules and regulations set by the Institutional Animal Ethics Committee (IAEC) of Jawaharlal Nehru University, New Delhi, India (code no. 35/2019); and, the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Mice-based in vivo experiments are also reported following the guidelines of “Animal Research: Reporting of In Vivo Experiments” (ARRIVE) guidelines (https://arriveguidelines.org/).
References


**Figures**
**Figure 1**

**Interaction studies of Prefoldin subunits with Pfk-13.** (A) Protein-protein interaction network generated using STRING showing the interaction between Pfk-13 and Prefoldin subunits. (B) Overexpression and purification of Pfk-13. Coomassie-stained gel showing the purified recombinant protein Pfk-13 (~43 kDa). (C) Evaluation of the interaction strength between Pfk-13 and PpPFD6. SPR-based interaction analysis upon immobilizing Pfk-13 and titrating PpPFD6 displayed a good binding strength with a
K_{D} value to be 174 nM. (D) Co-immunoprecipitation assays validating the binding of PfPFD6 and PfK-13. Anti-PfPFD6 antisera was cross-linked to amino coupling plus resin followed by incubation with *P. falciparum* 3D7 and R539T lysate. Eluted fractions were resolved on 12% SDS-PAGE and subjected to Western blotting using anti PfK-13 (1:1,000) antibodies and their respective HRP-conjugated anti-rat antibodies (1:5,000). (E) RT-qPCR analysis. Heat map illustrating Ct-values of PfPFD1-6 in different asexual stages of the parasite. Blue and red shadings represent lower and higher relative expression levels, respectively. (F) Stage-specific expression of PfPFD1-6 at protein levels. (i) Parasite lysates were prepared for different asexual stages of the parasite and subjected to western blotting. Immunoblotting with PfPFD1-6 antisera (1:1,000 of each) and HRP-conjugated anti-mice antibodies (1:5,000) showed differential expression of PfPFD1-6 across different asexual stages of the parasite. (ii) Heat map shows differential expression of PfPFD at different intraerythrocytic stage. Shading of blue and red depict lower and higher expression levels, respectively. (G) Upregulated expression of PfPFD1-6 at protein levels in *P. falciparum* R539T. Parasite lysates (*Pf*3D7 and R539T strain) were prepared for different asexual stages and subjected to western blotting using PfPFD1-6 antisera (1:1,000) and HRP-conjugated anti-mice antibodies (1:5,000).
Localization of PfPFDs in asexual blood stages of *P. falciparum*. (A) Cellular localization of PfPFD1-6. Smears of methanol-fixed *Pf3D7*-infected erythrocytes were stained with anti-PfPFD1-6 antibodies (1:200) followed by incubation with Alexa Fluor-conjugated secondary antibodies (Alexa Fluor 488, green). DIC: differential interference contrast image, DAPI: nuclear staining 40, 6-diamidino-2-phenylindole (blue); *PfPFD1*-6: mouse anti-*PfPFD1*-6 (green) (Scale bar: 2 µm). IFA depicted that *PfPFD1-*
6 are expressed at the trophozoite and schizont stage of the parasite. (B) *PfPFD1-6 are mostly confined to the cytoplasm of schizonts*, as confirmed by its co-localization with a cytosolic protein marker, *PfNapL*. DIC: differential interference contrast image, DAPI: nuclear staining 4, 6-diamidino-2-phenylindole (blue); *PfPFD1-6: mouse anti- PfPFD1-6 (green); PfNapL: anti-PfNapL antibody (red); merge 1: overlay of *PfPFD1-6 with PfNapL;* merge 2: overlay of DAPI, *PfPFD1-6 and PfNapL* (Scale bar: 2 µm).

**Figure 3**
Orchestrated assembly of \( P.f.PFD_{\text{hexamer}} \) \textit{in vitro} and \textit{in vivo}. (A) SDS-PAGE of purified recombinant prefoldin subunits. \( P.f.PFD \) (20 kDa), \( P.f.PFD \) (19 kDa), \( P.f.PFD3 \) (24 kDa), \( P.f.PFD4 \) (17 kDa), \( P.f.PFD5 \) (30 kDa), \( P.f.PFD6 \) (16 kDa). (B) Co-immunoprecipitation assay. Anti-\( P.f.PFD6 \) antiserum was cross-linked to amino coupling plus resin followed by incubation with parasite lysate. Eluted fractions were resolved on 12% SDS-PAGE and subjected to Western blotting and immunoblotting using in-house generated \( P.f.PFD1-6 \) antisera (1:1,000 of each) and their respective HRP-conjugated anti-mice antibodies (1:5,000). Co-IP suggested that \( P.f.PFD1-6 \) form a complex similar to their counterparts in other species. (C) Microscale thermophoresis. \( P.f.PFD3 \) and \( P.f.PFD5 \) (20 \( \mu \)M of each) were labeled and titrated with increasing concentrations of the other \( P.f.PFD \) subunits. Binding affinity \( (K_D) \) as determined by steady-state analysis, are as follows: 81.6 nM \( (P.f.PFD3-P.f.PFD1) \), 682 nM \( (P.f.PFD3-P.f.PFD2) \), 11.3 \( \mu \)M \( (P.f.PFD3-P.f.PFD4) \), 4.6 \( \mu \)M \( (P.f.PFD3-P.f.PFD5) \), and 1.88 \( \mu \)M \( (P.f.PFD3-P.f.PFD6) \), indicating higher binding affinity of \( P.f.PFD3 \) with \( P.f.PFD1 \) and \( P.f.PFD2 \) (i-iv, ix). Similarly, \( K_D \) values were determined to be 851 nM \( (P.f.PFD5-P.f.PFD1) \), 628 nM \( (P.f.PFD5-P.f.PFD2) \), 104 nM \( (P.f.PFD5-P.f.PFD4) \), and 324 nM \( (P.f.PFD5-P.f.PFD6) \), indicating higher binding affinity of \( P.f.PFD5 \) with \( P.f.PFD4 \) and \( P.f.PFD6 \) (v-viii). (D) Overall architecture of the \( P.f.PFD_{\text{hexamer}} \) Structural models of \( P.f.PFD1-6 \) were generated using i-TASSER, and \( P.f.PFD_{\text{hexamer}} \) structure was generated using human TRiC-PFD complex (PDB ID: 6NR8) as a template. Upon rigid-body superimposition of both the complexes, the overall RMSD value of the C-alpha atomic co-ordinates was found to be 1.45 Å, suggesting a reliable 3D structure to be taken further for \textit{in silico} and \textit{in vitro} interaction analysis, and inhibition studies. \( P.f.PFD_{\text{hexamer}} \) structural model revealed a strong resemblance with its counterparts from other eukaryotes, forming a hetero-hexamer with a jellyfish-like architecture.
Identification of a drug 'Biperiden' targeting prefoldins and its effect on artemisinin sensitive (Pf3D7) and resistant parasite (PfKelchR539T). (A i, ii) In silico therapeutic repositioning approach. LOPAC®1280 library was screened based on structural similarities with the Protein Folding Activity of Ribosomes (PFAR) inhibitor, Metixene. BPD was identified with an overlay structural similarity index of 0.75. (B) Possible architecture of the PfPFD-BPD. The complex was generated using the modeled structure of the PfPFD.
complex. BPD was found to interact with PfPFD complex via two different conformations. (i) In conformation 1, BPD engaged at the interface of PfPFD subunits 2 and 6, with free binding energy ($\Delta G_{bind}$) of -8.4 kCal/mol. (ii) In conformation 2, BPD was found to interact with the double beta-barrel assembly of the PfPFD complex, with $\Delta G_{bind}$ of -8.0 kCal/mol. (C) Schematic diagram of the BPD molecule. (D) BPD interacts with PfPFD in vitro. Interaction of BPD with PfPFD1-6 was investigated by Surface Plasmon Resonance. rPfPFD1-6 (25 µM of each) were immobilized on gold sensor chip followed by titration with varying concentrations of BPD. Dissociation constant curves generated K$_D$ values of 57, 200, 100, 40, and 100 for the interaction of BPD with PfPFD1, PfPFD2, PfPFD3, PfPFD, PfPFD4, and PfPFD6, respectively. (E) BPD competes with PfPFD1 and PfPFD2 for binding to PfPFD3. Competing the PfPFD3-PfPFD1 and PfPFD3-PfPFD2 interactions with BPD resulted in a decrease in the MST signal starting at 893 to 888.1 units (for PfPFD3-PfPFD1), and 894.5 to 887.6 units (for PfPFD3-PfPFD2), thus enhancing the K$_D$ values to 1.99 µM and 9.6 nM, respectively (i, ii). (F) In vitro anti-plasmodial activity of BPD in artemisinin sensitive (Pf3D7) (i) and resistant parasite (PfKelch$^{R539T}$) (ii). Ring stage Pf3D7 was treated with varying concentrations (250 nM to 5 µM) of BPD for 72 h. BPD inhibited the growth of the malaria parasite and displayed a potent anti-plasmodial effect with an IC$_{50}$ value of ~1 µM in artemisinin sensitive and resistant parasite. The experiments were performed in triplicates and ± SD value was calculated for each data point (iii) Light microscopy-based Giemsa-stained images of Pf3D7 in artemisinin sensitive (Pf3D7) and resistant parasite (PfKelch$^{R539T}$) pre- and post-72 h, showing formation of pyknotic bodies in BPD treated parasite. (G) Effect of BPD on the parasite viability. Trophozoite-parasitized RBCs were treated with BPD (1 and 5 µM) for 6 h, untreated parasite served as the negative control. Post-treatment, parasites were subjected to PI/SYTO9 (Red/Green) co-staining. (i) Microscopic imaging data revealed that the BPD-treated parasites were SYTO9-positive and PI-negative, suggesting that the viability of the parasites was not compromised upon BPD-treatment. (ii) Mean fluorescence intensity of cells was measured for both stain and plotted.
**Figure 5**

**PfPFD2** interacts with *Pfα*-tubulin-1 and BPD destabilizes *Pfα*-tubulin-1. **(A)** PfPFD2 interacts with *Pfα*-tubulin-1. Semi-quantitative ELISA was done in which PfPFD2 upon titration with *Pfα*-tubulin-1 depicted the interaction in a concentration-dependent manner. **(B)** Quantification of the interaction strength. SPR-based interaction analysis upon titrating the immobilized PfPFD2 with *Pfα*-tubulin-1, depicted $K_D$ value to be 1.9 μM. **(C)** Co-IP-based interaction analysis of PfPFD2 and *Pfα*-tubulin-1. (i) PfPFD2 antiserum was
cross-linked to the AminoLink plus Coupling Resin, followed by incubation with the parasite lysate prepared from the mix-stage parasite population. The desired protein band of Pfα-tubulin-I was observed in the eluted fraction. (ii) Similarly, reverse co-IP confirmed the interaction between the two proteins. (D) BPD destabilizes Pfα-tubulin-I. Western blot analysis demonstrated that the Pfα-tubulin-I levels get markedly reduced upon the treatment of the parasites with BPD, suggesting the significance of PfPFD in the proteostasis of Pfα-tubulin-I. (E) BPD destabilizes PfMSP-1. (i) Western blot analysis revealed that the expression of PfMSP-1 gets reduced in the BPD-treated parasites as compared to the untreated ones. (ii) Intensity plot of PfMSP-1 levels in the parasite treated with BPD. (iii) GAPDH served as loading control in the assay. (iv) Western blot analysis demonstrating reduction in PfMSP-1 expression level in BPD-treated parasites at two different time points of treatment (6 h and 12 h). (F) Confocal microscopy-based analysis demonstrated reduced expression of PfMSP-1 in the BPD-treated schizonts. (G) BPD inhibits egress and invasion of the parasite. (i) BPD significantly inhibited the egress of the parasite by ~60% at 1.25 µM, and ~75% at 2.5 µM and 5 µM concentrations. (ii) The number of rings formed per egress of schizont was also significantly reduced in the presence of BPD, as compared to the control. These findings suggest that BPD disturbs the PfPFD-mediated proteostasis of PfMSP-1. (H) (i) Schematic representation depicting the interaction of PfPFD2 with α-tubulin-I as required for fundamental cellular processes including cell growth and movement, which gets hampered in the presence of BPD. (ii) Similarly, PfPFD6 interacts with PfMSP-1, a protein essential for the erythrocyte invasion and egress of merozoites; BPD interferes with the interaction which results in impaired invasion and egress.

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Figure 6
Complementation of Prefoldin-6 in yeast orthologous system and effect of BPD and artemisinin in mutant yeast strains. (A) Complementation with PfPfd6 rescues YTM 1304 cell growth as depicted using Spot assays. Cultures of wild-type (BY4742), pfΔ6, and pfΔ6 complemented with PfPfd6 were harvested and adjusted to an optical density, A600 of 0.1 using sterile YPD media. Serial 10-fold dilutions of each culture were spotted onto YPD agar plates. Upon complementing YTM 1304 with PfPfd6, partial restoration of cell growth was observed. (B) Line graph depicting growth curve of S. cerevisiae BY4742 wild type, YTM1304 (PFD6Δ) yeast mutants, and complemented YTM1304-PfPFD6-p416 GPD strains. The yeast cells were grown in YPD media and diluted to initial A600 = 0.1. After every 1.5 hours, absorbance at 600 nm was measured spectrophotometrically. (C) PfPFD6-targeting effect of BPD. The growth pattern of YTM1304 and YTM1304 complemented with PfPfd6 was analyzed in the presence of BPD. Cultures were harvested and adjusted to A600 of 0.1 using sterile YPD media. Serial 10-times dilutions of each culture were spotted onto solid YPD-agar plates and incubated for two days at 30°C. Interestingly, the PfPfd6-complemented yeast exhibited reduced growth in the presence of BPD as compared to the control (untreated). (D) Effect of artemisinin on the growth pattern of YTM1304-PfPFD6. The yeast cells YTM1304-p416GPD, and YTM1304-p416GPD-PfPFD6 were grown overnight and cultures were diluted to initial A600 = 0.1. Cultures were allowed to grow for 12 hours in the presence and absence of artemisinin (8 μM). Each culture was harvested and adjusted to an A600 value of 0.1. Subsequently, it was 10-fold serially diluted and was spotted onto a YPD agar plate followed by incubation at 30°C for two days. PfPFD6 renders the mutant cells less sensitive to artemisinin.

Figure 7
BPD inhibits growth of the rodent malaria parasite and increases the survival rate. (A) Schematic representation of the methodology used for dose administration of BPD in mice. (B) In vivo anti-malarial activity of BPD. (i) Graph showing percent parasitemia in five experimental groups. On day 9, parasite load in the untreated group was ~24%, whereas, in BPD and artesunate treated group, ~5% parasitemia was observed. (ii) Graph showing percent survival of *P. berghei* infected mice. On day 9, all mice of untreated group died while more than 50% mice survived for 20 days in the treated group.

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