



Plasmodium bromodomain PfBDP4



A Target Enabling Package (TEP)

Gene ID / UniProt ID / EC	PF3D7_0110500 (<i>Pf</i> BDP4) Homologues: PF3D7_1033700 (<i>Pf</i> BDP1), PF3D7_1475600 (<i>Pf</i> BDP3), PF3D7_0823300 (<i>Pf</i> GCN5)
Target Nominator	SGC Internal Nomination
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Target PI	Raymond Hui (SGC Toronto)
Therapeutic Area(s)	Malaria
Disease Relevance	<i>Plasmodium</i> bromodomains may be a new class of druggable anti-malarial targets
Date approved by TEP Evaluation Group	17 th June 2016
Document version	Version 5
Document version date	October 2018
Citation	Hui D, Fonesca M, Josling G, Tallant C, Wernimont A, Fedorov O, Hutchinson A, Loppnau P, Duffy M, Knapp S, and Hui Raymond (2016) <i>Plasmodium</i> bromodomain <i>Pf</i> BDP4; A Target Enabling Package. 10.5281/zenodo.1209237 .
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SUMMARY OF PROJECT

There are 8 bromodomain-containing proteins in each annotated *Plasmodium* genome, with only one containing dual bromodomains (BRDs) – i.e. a total of 9 BRDs. In collaboration with SGC Oxford, we have expressed, purified and crystallized the BRDs in *Pf*BDP1, *Pf*BDP3, *Pf*BDP4 (namely *Pf*BDP4B) and *Pf*GCN5. By screening all four against a focused BRD inhibitor library (SGC Oxford) using the DSF assay, we identified a small number of hits that were subsequently validated and quantitatively assessed using ITC. Two similar compounds with the dihydropteridinone scaffold, namely BI2536 and BI6727, stood out as potent inhibitors of *Pf*BDP4B (both with K_d below 200 nM). We further collaborated with U of Melbourne to test them against *Pf*3D7 and found them capable of inhibiting blood stage development at sub-micromolar concentrations. Analysis of the co-structure of *Pf*BDP4B+BI2536 suggests the possibility of increasing the potency and selectivity of inhibition.

SCIENTIFIC BACKGROUND

Plasmodium parasites are responsible for malaria, an infectious disease responsible for nearly half a million deaths and over 200M new infections in 2015 (1). Their life cycle spans transmission from the mosquito vector animal hosts, invasion of liver hepatocytes, subsequent release into the bloodstream, invasion of red blood cells (RBCs), replication, egress and reinvasion of RBCs, gametogenesis, return transmission to the vector and sexual reproduction. Spurred by breakthroughs in study of human epigenetics, emergent research on parasite epigenetics is beginning to produce new valuable insights into the regulation of these stages as well as evasion of host immune response (2,3). For example, stage-specific histone marks, including acetylated residues, have been identified using MS analysis (4-10). Notably, lysine acetylation on H3 and H4 are more prominent in comparison to mammals (10), with H3K9_{ac} linked to both immune evasion and parasite growth (9). Accordingly, we have taken an interest in bromodomains, with the dual objectives of learning their biological function and identifying novel anti-parasitic drug targets.

Objectives of this TEP:

- Express and purify *Plasmodium* bromodomains as recombinant proteins in order to characterize them.
- Generate crystal structures to study their acetyl-histone binding properties and to aid identification of potent and selective inhibitors.
- Identify chemical starting points for at least one *Plasmodium* bromodomain.

RESULTS – THE TEP

The *Plasmodium* BRD family

In examining the annotated *Plasmodium* genomes, we found 8 bromodomain-containing proteins in each, including one with dual BRDs.

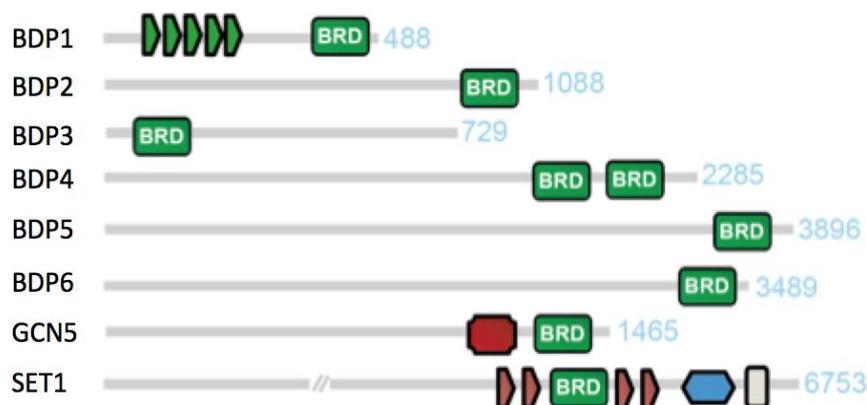


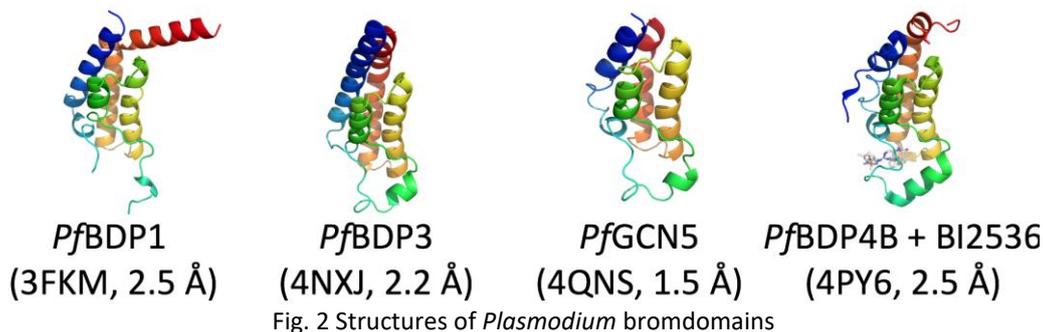
Fig. 1 The *Plasmodium* bromodomain family

Purified proteins

Using *E. coli* as the expression host, we expressed and purified pure and stable his-tagged samples of the bromodomains of *PfBDP1*, *PfBDP3*, *PfGCN5* and *PfBDP4B*.

Structures

We crystallized and obtained high resolution diffraction structures of all four purified *Plasmodium* bromodomains.



The four structures conserve the canonical BRD fold of a four-helix bundle (αZ , αA , αB and αC) linked by the ZA and BC loops to form a hydrophobic receptor of acetylated lysine. The K_{ac} -binding asparagine is also present in all of them.

Binding assays

DSF (differential scanning fluorimetry): As previously reported for human bromodomains (11,12), parasite BRDs showing detectable shift in T_m in the presence of stabilizing conditions. This is an effective medium throughput assay for screening focused libraries.

ITC: This is an effective secondary assay for validating DSF hits, including determination of binding affinity (K_d).

Chemical starting points

Using ITC (Fig. 3), we found BI2536, a known dual inhibitor human PLK1 and BRD4(1) with a dihydropteridinone scaffold, to bind *Pf*BDP4B with $K_d \sim 100$ nM. These compounds were tested against *Pf*3D7 in blood stage and found to inhibit the parasite at relatively low sub-micromolar concentrations (Fig. 4). (Ligand Efficiency approximately 0.25, Ligand Lipophilicity Efficiency approximately 2.6).

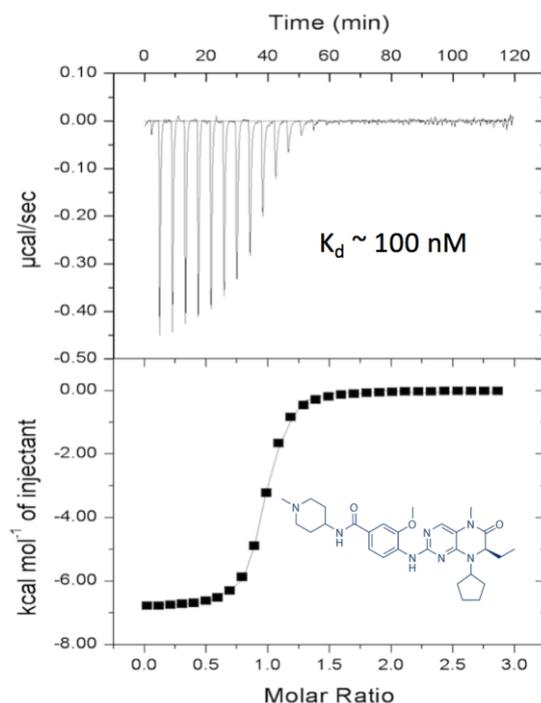


Fig. 3 BI2536 binds *Pf*BDP4B at low sub-micromolar concentrations

For more information regarding any aspect of TEPs and the TEP programme, please contact teps@thesgc.org

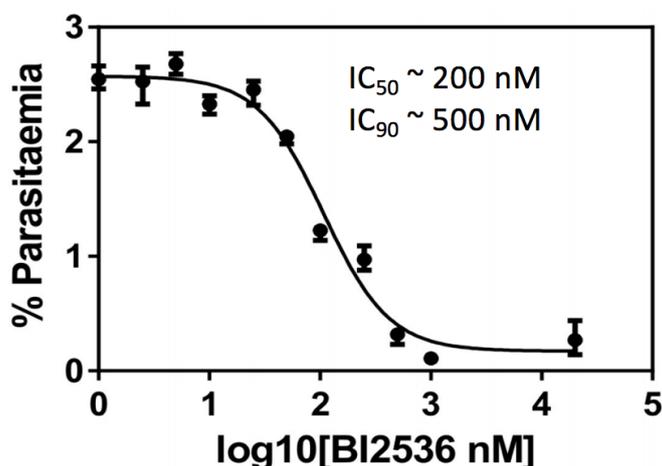


Fig. 4 BI2536 inhibits blood stage *Pf3D7* at sub-micromolar concentrations

BI6727, another dihydropteridinone analogue known to inhibit PLK1 and human BRD4(1) potently, also demonstrated similar potency as BI2536 in binding *PfBDP4B* and inhibiting *Plasmodium* parasites. Interestingly, neither compound binds *PfBDP1*, *PfBDP3* or *PfGCN5*. Furthermore, there is no PLK homologue in *Plasmodium*. In fact, the PLK motifs that bind these compounds are not conserved in any *Plasmodium* kinase.

Structural evidence (PDB: 4O74) (12) shows that BI2536 at two key interfaces (C1 and C2 in Fig. 5). The C2 interaction is not conserved in *PfBDP4B* in which Y1220 and T1223 replace P82 and Q85, the two residues that interact with the methoxy moiety in the ligand. This may be an opportunity for parasite-specific modifications.

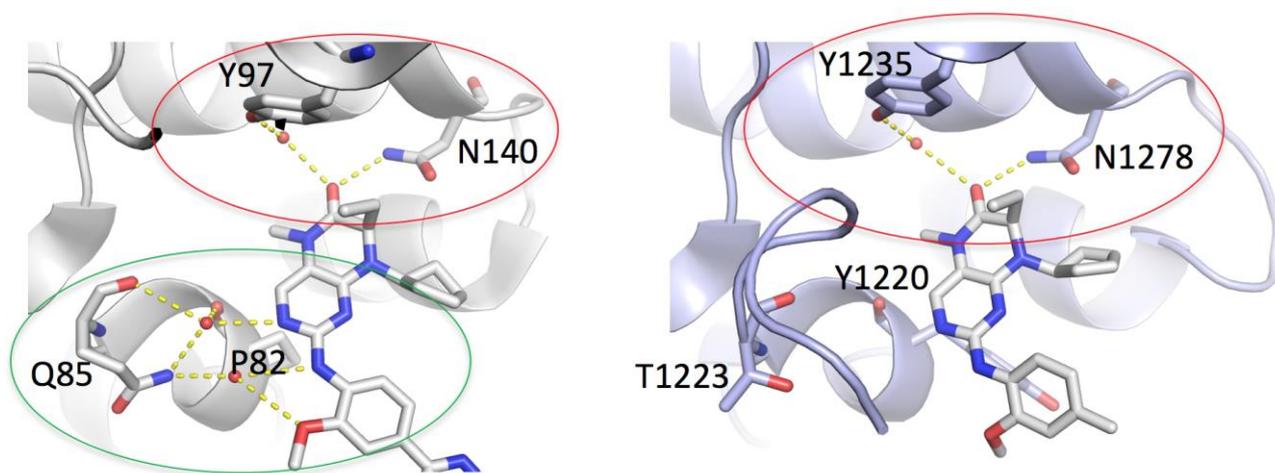


Fig. 5 Left: BI2536 makes key water-mediated contacts with human BRD4(1) at C1 (red) and C2 (green). Right: Only C1 is conserved in the co-structure of *PfBDP4B* and BI2536. At C2, P82 and Q85, which interact with the methoxy moiety, are replaced by Y1220 and T1223 in *PfBDP4B*.

IMPORTANT: Please note that the existence of small molecules within this TEP only indicates that chemical matter can bind to the protein in a functionally relevant pocket. As such these molecules should not be used as tools for functional studies of the protein unless otherwise stated as they are not potent enough and not characterised enough to be used in cellular studies. A TEP's small molecule ligands are intended to be used as the basis for future chemistry optimisation to increase potency and selectivity and yield a chemical probe or lead series.

Future work

- Identify selective inhibitors of other *Plasmodium* bromodomains.
- Identify histone peptide substrates of purified *Plasmodium* bromodomains.
- Validate that the target of BI2536 is PfBDP4B.
- Functional studies.

Key SGC-Toronto contributors

- David Hou
- Linda Lin

Collaborations

- Chemistry: Paul Brennan, SGC Oxford
- Screening: Stefan Knapp, Marbella Fonesca, Oleg Fedorov, SGC Oxford
- Biology, target validation and parasite assays: Gabrielle Josling, Michael Duffy, U of Melbourne
- Drug discovery: Chris Walpole, SDDC
- Parasite assays: Ian Crandall, U of Toronto

CONCLUSION

We have achieved all three objectives. In the process, we have learned that *Plasmodium* bromodomains conserve the canonical four-helix structure of mammalian bromodomains. As found with human bromodomains, the K_{ac}-binding pocket interacts with small molecules in a potentially novel anti-malarial mechanism. A manuscript on the work done so far is being prepared.

FUNDING INFORMATION

The SGC is a registered charity (number 1097737) that receives funds from AbbVie, Bayer Pharma AG, Boehringer Ingelheim, Canada Foundation for Innovation, Eshelman Institute for Innovation, Genome Canada through Ontario Genomics Institute [OGI-055], Innovative Medicines Initiative (EU/EFPIA) [ULTRA-DD grant no. 115766], Janssen, Merck KGaA, Darmstadt, Germany, MSD, Novartis Pharma AG, Ontario Ministry of Research, Innovation and Science (MRIS), Pfizer, São Paulo Research Foundation-FAPESP, Takeda, and Wellcome.

ADDITIONAL INFORMATION

Structure Files

PDB ID	Structure Details
3KFM	<i>Apo PfBDP1</i> ; 2.5 Å
4NXJ	<i>Apo PfBDP3</i> ; 2.2 Å
4PY6	<i>PfBDP4B</i> + BI2536; 2.5 Å
4QNS	<i>Apo PfGCN5</i> ; 2.2 Å

Materials and Methods

Procedure for protein expression and purification

E. coli BL21 rosetta cells transformed with a plasmid containing the clone of interest and pET15-MHL were grown in 1.0 liter Terrific Broth (Merck) medium at 37 °C until reaching OD₆₀₀ of approximately 0.8. Protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18 °C for 16h. Cells were collected at 6500 rpm in JLA 8.1000 rotor (Beckman) for 20 min and re-suspended in lysis buffer (20 mM HEPES pH 7.5, 500 mM NaCl, 10 mM Imidazole) supplemented with 1 mM tris(2-carboxyethyl) phosphine (TCEP), and 1:1000 (v/v) Protease Inhibitor Cocktail set III (Carbiochem). Cells were lysed by sonication, and clarified lysates were obtained by centrifugation at 15,000 x g in JA 16.250 (Beckman) for 60 min. Lysates were loaded onto Ni-NTA column (GE Healthcare), washed with 50 mL washing buffer (20 mM HEPES pH 7.5, 500 mM NaCl, 30 mM Imidazole, 1mM TCEP) and gradient eluted from 60-300 mM Imidazole. All fractions were collected and loaded in SDS-polyacrylamide gel electrophoresis (Bio-Rad). The eluted proteins were treated overnight with TEV (Tobacco Etch Virus) protease at 4 °C to remove the His₆-tag. Selected fractions were pooled together and concentrated for further purification by size-exclusion chromatograph using a HiLoad Superdex S75 16/60 column (GE Healthcare) buffered in 10 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol and 1 mM TCEP. Purified proteins were finally concentrated on Amicon® Ultra (Millipore) employing 10 kDa cut-offs. The intact mass of the proteins was confirmed by electrospray ionization/time-of-flight mass spectrometry (Agilent Technologies).

PfBDP1

Growth

The solutions were prepared according to the instructions of the M9 SeMET High-Yield growth media kit package (MD045004-50L, Orion enterprise inc, Northbrook, IL, USA). Amendments (final amounts) per liter, added in a sterile manner to 900 - 950 ml autoclaved water:

"A": MD045004A (Na/K/P/C/N sources + non-inhibitory amino acid cocktail: EDRHAPGSQNW), one pouch for 1 Liter cell culture, added just before growth: 6 g Na₂HPO₄, 3 g KH₂PO₄, 1 g NH₄Cl, 0.5 g NaCl, 4.4 g glucose, non-inhibitory amino acids 200 mg each (1-glutamate, 1-aspartate, 1-arginine, 1-histidine, 1-alanine, 1-proline, 1-glycine, 1-serine, 1-glutamine, 1-asparagine, 1-tryptophane)

"B": MD045004B (Mineral supplements), stored at 4°C, light avoided. 10 ml for 1 Liter cell culture: 5 mg EDTA, 430 mg MgCl₂*6 H₂O, 5 mg MnSO₄, 10 mg NaCl, 1 mg FeSO₄*7H₂O, 1 mg Co(NO₃)₂*6H₂O, 11 mg CaCl₂, 1 mg ZnSO₄*7H₂O, 0.1 mg CuSO₄*5H₂O, 0.1mg AlK(SO₄)₂, 0.1mg H₃BO₃, 0.1 mg Na₂MoO₄*2H₂O, 0.01 mg Na₂SeO₃, 0.1 mg Na₂WO₄*2H₂O, 0.2 mg NiCl₂*6H₂O.

"C": MD045004C (Vitamins), added to 50 ml of water and mixed, stored at 4°C for short term, at -20°C for long time use, light avoided after dissolution. 1 ml for 1 Liter cell culture: 1 ug thiamine (vitamin B1), 2.7 ug vitamin B12.

"D": MD045004D (inhibitory amino acid cocktail: VILKTF + SeMet). One pouch dissolved in 250 ml. 20 ml for 1 Liter cell culture: 25 mg each of 1-valine, 1-isoleucine, 1-leucine, 1-lysine, 1-threonine, 1-phenylalanine and 15 mg of selenomethionine.

"E": For MD045004E (IPTG). One pouch dissolved in 50 ml of water. 1 ml for 1 Liter cell culture. Final concentration: 1 mM isopropylthio-beta;-d-galactoside (IPTG).

Selenomethionyl proteins were produced in BL21-(D3) - a strain not auxotrophic for methionine- using feedback inhibition of methionine biosynthesis. On day 1, one colony of the transformed E. coli BL21-(DE3)-V2R-pRARE2 was transferred to 5 mL LB medium supplemented with carbenicillin (100 µg/mL) and chloroamphenicol (34 µg/mL) in a 10 ml tube and grown at 37 °C, 220 rpm overnight. On day 2, 100 ml of freshly prepared "A" solution, supplemented with "B", "C", glycerol and antibiotics were inoculated with 0.5 ml of the LB culture and grown overnight in a 200 ml flask at 37°C, 220 rpm. On day 3, 1.8 L/ 2 L bottle of freshly prepared "A" solution, supplemented with "B", "C", glycerol, antibiotics and 0.5 ml antifoam A204 (Sigma) were inoculated with 20 ml of the flask cultures and grown in the LEX system at 37°C for ~ 4 h until OD600 ≈ 1.2. Then "D" was added, immediately the cultures were cooled down to 20°C (the cooler was set to 18°C). After 15 min IPTG was added and induced overnight. In the morning of day 4, the OD was taken and the cells harvested.

Purification

The cleared lysate was loaded onto a 1.0-2.5 mL Ni-NTA (Qiagen) column (pre-equilibrated with Binding Buffer) at approximately 1.5-2.0 mL/min. The Ni-NTA column was then washed with 150 mL of Wash Buffer at 2-2.5 mL/min. After washing, the protein was eluted with Elution Buffer. The eluted sample was applied to a Sephadex S200 26/60 gel filtration column pre-equilibrated with Gelfiltration Buffer. The fractions corresponding to the eluted protein peak were collected and further concentrated using a 15 mL Amicon Ultra centrifugal filter device (Millipore). The protein sample identity and purity were evaluated by mass spectroscopy and SDS-PAGE gel. The concentrated protein (23.37 mg/ml) was stored at 4°C. For long term storage, the protein was flash frozen and stored at -80°C.

Extraction

The culture was harvested by centrifugation. Pellets from 4 L of culture were resuspended to approximately 30 mL/L of cell culture in Binding Buffer with the addition of protease inhibitors (1 mM benzamide and 1 mM phenylmethyl sulfonyl fluoride (PMSF)). Resuspended pellets stored at -80 degC were thawed overnight at 4 °C on the day before purification. Prior to sonication, each pellet from 1 L of culture was pretreated with 0.5 % CHAPS and 500 units of benzonase for 40 minutes at room temperature. After 6 minutes sonication, the cell lysate was centrifuged using a Beckman JLA-16.250 rotor at 16000 rpm for 1 h at 4 degC.
Concentration: 23.37 mg/ml

Crystallization

Alpha-chymotrypsin (1 mg/ml) was added to protein at 1:100 (V:V) ratio prior to crystallization, final concentration: 10 µg/ml.

Crystal condition: 20% glycerol plus 8% PEG8000, 0.1M Tris-HCl, pH8.5, sitting drop vapor diffusion, 293K.

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