

RESEARCH ARTICLE

Deciphering the targets of retroviral protease inhibitors in *Plasmodium berghei*

Noah Machuki Onchieku^{1,2*}, Reagan Mogire^{1,3}, Loise Ndung'u¹, Peter Mwitari², Francis Kimani⁴, Damaris Matoke-Muhia⁴, Daniel Kiboi^{3,5,6*}, Gabriel Magoma^{1,6}

1 Department of Molecular Biology and Biotechnology, Pan African University Institute for Basic Sciences Technology and Innovation (PAUSTI), Nairobi, Kenya, **2** Centre for Traditional Medicine and Drug Research, Kenya Medical Research Institute (KEMRI), Nairobi, Kenya, **3** Kenya Medical Research Institute (KEMRI)/ Wellcome Trust, Collaborative Research Program, Kilifi, Kenya, **4** Centre for Biotechnology Research and Development, Kenya Medical Research Institute (KEMRI), Nairobi, Kenya, **5** West Africa Centre for Cell Biology and Infectious Pathogens, University of Ghana, Accra, Ghana, **6** Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology (JKUAT), Nairobi, Kenya

* noahmachuki@yahoo.com (NMO); dkiboi@kemri-wellcome.org (DK)



OPEN ACCESS

Citation: Onchieku NM, Mogire R, Ndung'u L, Mwitari P, Kimani F, Matoke-Muhia D, et al. (2018) Deciphering the targets of retroviral protease inhibitors in *Plasmodium berghei*. PLoS ONE 13 (8): e0201556. <https://doi.org/10.1371/journal.pone.0201556>

Editor: Georges Snounou, Université Pierre et Marie Curie, FRANCE

Received: March 6, 2018

Accepted: July 17, 2018

Published: August 1, 2018

Copyright: © 2018 Onchieku et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: The raw data for this study are deposited in OSF as follows: Data set 1: Effect of gene Knock out (KO) on the growth rate phenotype of asexual stage *P. berghei* parasite (https://osf.io/rn9e2/?view_only=f39b20791a96421aa676840059b291c7). Data set 2: Four-day (4D) drug sensitivity profiles for both the KO and WT parasites (https://osf.io/zyk5m/?view_only=6531322400384162a1d8122bf217e77d). Data set 3: Molecular docking - Ligand-receptor binding geometries (https://osf.io/b32y6/?view_only=d1e62f0ceb8e47bf98fcdca766b0075a8).

Abstract

Retroviral protease inhibitors (RPIs) such as lopinavir (LP) and saquinavir (SQ) are active against *Plasmodium* parasites. However, the exact molecular target(s) for these RPIs in the *Plasmodium* parasites remains poorly understood. We hypothesised that LP and SQ suppress parasite growth through inhibition of aspartyl proteases. Using reverse genetics approach, we embarked on separately generating knockout (KO) parasite lines lacking Plasmepsin 4 (PM4), PM7, PM8, or DNA damage-inducible protein 1 (Ddi1) in the rodent malaria parasite *Plasmodium berghei* ANKA. We then tested the suppressive profiles of the LP/Ritonavir (LP/RT) and SQ/RT as well as antimalarials; Amodiaquine (AQ) and Piperaquine (PQ) against the KO parasites in the standard 4-day suppressive test. The Ddi1 gene proved refractory to deletion suggesting that the gene is essential for the growth of the asexual blood stage parasites. Our results revealed that deletion of PM4 significantly reduces normal parasite growth rate phenotype ($P = 0.003$). Unlike PM4_KO parasites which were less susceptible to LP and SQ ($P = 0.036$, $P = 0.030$), the suppressive profiles for PM7_KO and PM8_KO parasites were comparable to those for the WT parasites. This finding suggests a potential role of PM4 in the LP and SQ action. On further analysis, modelling and molecular docking studies revealed that both LP and SQ displayed high binding affinities (-6.3 kcal/mol to -10.3 kcal/mol) towards the *Plasmodium* aspartyl proteases. We concluded that PM4 plays a vital role in assuring asexual stage parasite fitness and might be mediating LP and SQ action. The essential nature of the Ddi1 gene warrants further studies to evaluate its role in the parasite asexual blood stage growth as well as a possible target for the RPIs.

Introduction

Notwithstanding the immense investments in malaria control programs to date, it remains to be a significant global health problem in most regions of the world including Africa, Asia and parts of the Eastern Mediterranean Region [1,2]. The sub-Saharan part of Africa continues to

Funding: This work was supported by the African Union Commission under the Pan African University Institute for Basic Sciences, Technology and Innovation (PAUSTI: grant no; PAU/MB300-0004/16); <http://www.jkuat.ac.ke/pauisti/>, and the AFRICA-ai-JAPAN Innovation Research Fund, grant no; iCMoB/08/17(<http://jkuat.ac.ke/projects/africa-aijapan/>). The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript. Daniel Kiboi was supported by a DELTAS Africa grant (DEL-15-007: Awandare). The DELTAS Africa Initiative is an independent funding scheme of the African Academy of Sciences (AAS)'s Alliance for Accelerating Excellence in Science in Africa (AESA) and supported by the New Partnership for Africa's Development Planning and Coordinating Agency (NEPAD Agency) with funding from the Wellcome Trust (107755/Z/15/Z: Awandare) and the UK government

Competing interests: The authors have declared that no competing interests exist.

bear the highest burden of the disease with over 90% of the cases occurring in this region, especially in children under five years of age. In the year 2016 alone, an estimated 285 000 children succumbed to malaria in Africa [2].

The emergence and spread of resistance to available drugs including the artemisinin-based combination therapies (ACTs) have aggravated the burden of the malaria disease. Incidences of parasite resistance to the ACTs were first reported in western Cambodia and currently slowly spreading to other parts of Asia. The South East Asia region occupies a historical record as a site of emerging resistance to the previous first-line antimalarial therapies which later rapidly spread across the African countries where malaria transmission is consistently high [3–6]. Since the options of drugs for which the human malaria parasite *Plasmodium falciparum* has not evolved resistance is rapidly diminishing, new and rational approaches to the prevention and treatment of malaria infections are urgently needed.

The burden of malaria is compounded with HIV/AIDS infections which are also concentrated in the malaria-endemic regions, primarily sub-Saharan Africa. This geographical overlap has raised opportunities and concerns for potential immunological, social, therapeutic and clinical interactions [7]. Previous studies have demonstrated that the antiretroviral therapy, especially RPIs exert a potent effect against both the drug-sensitive and drug-resistant *P. falciparum* [8–14], as well as a reduction in the incidence of malaria [15]. For instance, seven RPIs inhibit the development of *P. falciparum* parasites in vitro with lopinavir yielding moderate synergy with lumefantrine [12]. The RPIs are typical examples of drugs that target an aspartyl protease in HIV, HIV-1 aspartyl protease [16,17]. Like in HIV, aspartyl proteases play essential roles in the biology of *Plasmodium* parasites and thus are “druggable” targets [18–21]. The human malaria parasite, *Plasmodium falciparum* expresses a total of ten aspartyl proteases during the asexual blood stage, four of the seven proteases; the PM1, PM2, histoaspartic protease (HAP) and PM4 reside in the digestive vacuole and digest hemoglobin in the red blood cells [22]. In other human malaria species, *P. vivax*, *P. ovalae* and *P. malariae* as well as in the rodent malaria parasite *P. berghei*, Plasmepsin 4 (PM4) is the only aspartic protease localised within the digestive vacuole [22,23]. The PM5, PM9 and PM10 are the other aspartyl proteases expressed within asexual blood stage of the malaria parasite but not localised within the digestive vacuole [24]. The PM5, PM9 and PM10 are responsible for the egress of the parasite from the host erythrocytes [21] as well as processing of effector proteins for export to the infected erythrocytes [25,26]. However, the proteases localized within the digestive vacuole continue to attract enormous attention as potential drug targets [27]. Previous studies geared towards understanding the mode of action of the RPIs in suppressing the growth of *Plasmodium* parasites focused on pepsin-like proteases (PMs) even though *Plasmodium* species express a retropepsin-like protease, referred to as Ddi1 [28].

Using the rodent malaria parasite, *P. berghei*, and highly efficient *Plasmo*GEM genetic modification vectors, we engineered the *Plasmodium* aspartyl proteases; PM4, PM7, PM8 and Ddi1 in our quest to understand the possible mechanisms of action of LP and SQ (the most active RPIs). Here, we report that the PM4 and Ddi1 genes are essential for asexual blood stage parasite, but PM7 and PM8 genes are not. We further discuss the growth rate phenotypes of the KO parasites lacking PM7, PM8 or PM4 genes as well as the *in vivo* susceptibility profiles of the KO parasites to LP and SQ. Finally, using modeling and molecular docking, we predict the *in silico* binding affinities of the LP and SQ towards PM4, PM7, PM8 or Ddi1. The findings reveal that PM4 assures parasite fitness in the asexual stage, mediates the possible mechanism of action of LP and SQ in parasite growth suppression as well as the refractory nature of Ddi1. Here, we show the *in silico* binding profiles of LP and SQ on the Ddi1 protein and provide evidence that the retropepsin-like protease is essential for the asexual stage of the malaria parasite.

Materials and methods

Parasites, vectors, hosts and test compounds

A transgenic *P. berghei* ANKA strain 676m1cl1{PbGFP-LUC(con)} which expresses a fusion protein GFP-Luciferase [29], maintained in cryopreserved stocks in Kenya Medical Research Institute (KEMRI), was used in this study. The KO vectors (PbGEM-039254, PbGEM-086320, PbGEM-057101, and PbGEM-097527) were obtained under a material transfer agreement with the PlasmogEM project at the Wellcome Trust Sanger Institute (PG-MTA-0093). Naive male Swiss albino mice (6–7 weeks old), weighing 18–20g were acquired from the KEMRI animal house and used as models in this study. The animals were kept in the KEMRI animal house in standard polypropylene cages and fed on commercial rodent feed and water *ad libitum*. LP, SQ and RT were purchased from Sigma Aldrich (USA). All the test compounds were prepared by solubilising them in a solution consisting of 70% Tween-80 (density = 1.08gml⁻¹) and 30% ethanol (density = 0.81 gml⁻¹) and were diluted ten times with distilled water. The diluted Tween-80 and ethanol solution was used as a vehicle and control for the drug profile assays.

Ethics statement

All protocols were conducted in accordance with prior approvals obtained from the Kenya Medical Research Institute (KEMRI)'s Scientific Ethical Review Unit (SERU; 3572) and the KEMRI Animal Care and Use Committee (ACUC). The KEMRI ACUC adheres to national regulations on the care and use of animals in research in Kenya enforced by the National Commission for Science, Technology and Innovation (NACOSTI). The Institute has a Public Health Service (PHS)-approved assurance, number F16-00211 (A5879-01) from the Office of Laboratory Animal Welfare (OLAW) and commits to the International Guidelines for Bio-medical Research Involving Animals.

Generation of the knockout parasite lines

Isolation, digestion, and purification of vector DNA. Plasmid DNAs were isolated from overnight cultures of *E. coli* (vector hosts) in terrific broth (TB) supplemented with kanamycin using QIAfilter Plasmid Midi Kit. The isolated plasmid was prepared for transfection by NotI restriction digestion to liberate the vector backbone and was purified using standard ethanol precipitation. Each isolated construct was dried at 65°C for 5 min, dissolved in 10 µl of PCR water and the plasmid DNA concentration determined in a 1µl sample volume using a Nano-Drop. Both restriction digestion and diagnostic polymerase chain reaction (PCR), using QCR2/GW2 primer pair, were used as vector verification strategies.

Collection of the *P. berghei* schizonts for transfection. Collection of the *P. berghei* schizonts for transfection was done using standard protocols as described by [30]. Briefly, for each of the vectors, at least three mice were used for schizont culture. *P. berghei* parasites were propagated by intraperitoneal (IP) injection into the mice and were harvested for schizont culture at 3% parasitaemia using cardiac puncture. The asexual stage parasites (rings) were cultured *in vitro* at 37°C in tightly sealed and gassed flasks containing 100ml of schizont culture medium (72 ml RPMI1640, 25 ml freshly thawed FBS, 1ml of antibiotic Pen/Strep (1:100 Penicillin/streptomycin) and 2ml 0.5M NaHCO₃). Each flask contained 2ml of blood and was incubated overnight, in a shaker incubator. The schizonts were harvested after 22 hours, purified by Nycodenz density gradient centrifugation and each pellet re-suspended in schizont culture media, ready for transfection.

Transfection, selection and genotype analysis of mutant parasites. For every transfection, 20µl of schizont pellet was used. Parasite pellet was re-suspended in 100µl AMAXA

Table 1. Primer pairs used in PCR amplification of gDNA isolated from PM4_KO parasites.

Purpose	Primer pair	Primer Sequence
Vector integration	GT	AGACAAACTTTGCCCAACA
	GW1	CATACTAGCCATTTTATGTG
Selectable marker cassette	QCR2	AAGGGCCGCAACAACATTCA
	GW2	CTTTGGTGACAGATACTAC
WT locus	QCR1	TGAACTTGACCCAGTCGGCGG
	QCR2	AAGGGCCGCAACAACATTCA

The primer pairs used to genotype the PM4_KO parasites to evaluate the presence of the selectable marker cassette, correct integration of the vector in the parasite genome and the absence of WT gene locus.

<https://doi.org/10.1371/journal.pone.0201556.t001>

supplemented nucleofector solution containing purified vector DNA. Exactly 100µl of the solution (vector DNA /nucleofector solution and schizonts) were pipetted into the Amaxa cuvette. The electroporation was done using program U33 of the Nucleofector 2B Device (Lonza). The electroporated parasites were injected intravenously (IV) into mice (two mice per transfection). Twenty-four hours post parasite inoculation, pyrimethamine drug (7 mg/mL), in drinking water, was provided to the mice for nine days. To generate a genetically homogenous parasite, the KO parasites were diluted to approximately 0.1%, passaged successively three times in naïve mice each time under the Pyrimethamine selection pressure. After cloning the recovered KO parasites, the genomic DNA (gDNA) was extracted and purified using QIAamp DNA Mini Kit following instructions from the manufacturer. The diagnostic and genotyping PCR analysis was used to genotype the KO lines using three primer pairs; QCR2/GW2, GT/GW1 or GW2 and QCR1/QCR2. QCR1 and QCR2 anneal to the genomic sequence of the targeted locus, GT anneals to the genomic sequence outside of but preceding the region covered by the gDNA clone, while GW1/2 anneals to the sequence of the selectable marker cassette (human dihydrofolate reductase; hDHFR), (Tables 1–3). The PCR amplification was done using 2xGoTag Green master mix for thirty cycles with an annealing temperature of 50°C and an extension temperature of 62°C. The PCR amplicons were analysed on a 1% agarose gel electrophoresis.

Drug sensitivity profiles

Drug sensitivity profiles were determined using the quantitative standard 4-Day suppressive test [31], after four consecutive drug dosages. Briefly, for LP/RT and SQ/RT, groups of eight mice were used (four mice for each mutant parasite line and the other four for the WT parasite). For AQ or PQ, groups of sixteen mice were used (eight mice for mutant parasite line and

Table 2. Primer pairs used in PCR amplification of gDNA isolated from PM7_KO parasites.

Purpose	Primer pair	Primer Sequence
Vector integration	GT	AGCTGCCTATGAAGGAGGGA
	GW2	CTTTGGTGACAGATACTAC
Selectable marker cassette	QCR2	AAGGGCCGCAACAACATTCA
	GW2	CTTTGGTGACAGATACTAC
WT locus	QCR1	TGGATGGACGCTAGGACAAGT
	QCR2	ATGTGCATGGAAAACAGGGTT

The primer pairs used to genotype the PM7_KO parasites to evaluate the presence of the selectable marker cassette, correct integration of the vector in the parasite genome and the absence of WT gene locus.

<https://doi.org/10.1371/journal.pone.0201556.t002>

Table 3. Primer pairs used in PCR amplification of gDNA isolated from PM8_KO parasites.

Purpose	Primer pair	Primer Sequence
Vector integration	GT	TCCAATGGCCCAACAACCTTTTCGA
	GW1	CATACTAGCCATTTTATGTG
Selectable marker cassette	QCR2	AGGTGCACTTATACCGATTCTCA
	GW2	CTTTGGTGACAGATACTAC
WT locus	QCR1	TCGAGTATTGCAGGACCCCA
	QCR2	AGGTGCACTTATACCGATTCTCA

The primer pairs used to genotype the PM8_KO parasites to evaluate the presence of the selectable marker cassette, correct integration of the vector in the parasite genome and the absence of WT gene locus.

<https://doi.org/10.1371/journal.pone.0201556.t003>

the other eight for the WT parasites for two different AQ and PQ dosages). Each control group had four mice. Each mouse was IP inoculated with 200µl of 1×10^5 parasitised erythrocytes from infected donor mice, on the first day (day 0; D0). Two hours after parasite inoculation, four mice from the groups received 0.2ml of LP/RT; 40mg/kg, 10mg/kg, SQ/RT; 50mg/kg, 5mg/kg, AQ; 2.5 mg/kg, AQ; 1.25mg/kg, PQ; 2.5mg/kg and PQ; 1.25mg/kg, once daily oral gavage. The drug treatment proceeded for three consecutive days. On the fifth day (day 4: D4) Giemsa stained blood smears were prepared for microscopic examination. Percentage (%) activity (parasite reduction) of each drug dose tested was determined using the following equation:

$$\% \text{activity} = \{(\text{Parasitaemia in negative control} - \text{Parasitaemia in study group}) / \text{Parasitaemia in negative control}\} \times 100.$$

Data presentation and statistical analysis

The parasitaemia data from microscopy were analysed using R software. Boxplots were generated to portray the KO growth trends and parasite densities in presence and absence of the test drugs. Using the Student's t-test in the Stata version 15.1 software, we computed means of percentage drug activity/percentage parasitaemia from each mouse in the treated group and compared them to that from mice in the control group.

Molecular docking

Homology modelling and structure validation. The sequences of *P. berghei* PM4, PM7, PM8, and Ddi1, referred to as receptors, were obtained from PlasmoDB (<http://plasmodb.org/plasmo/>) and their three dimensional (3D) structures predicted using SWISS-MODEL [32] available at <https://swissmodel.expasy.org/>. The best structures were downloaded, saved in PDB format and visualized using PyMOL [33]. The quality of the modelled structures was validated using PROCHECK, for stereochemical evaluation [34], PROSA-web, for detection of potential errors in the 3D structures [35] and verify_3D, for compatibility of the 3D structures with their respective amino acid sequences [36].

Ligand preparation. The structures of LP and SQ were downloaded from the ChemSpider available at <http://www.chemspider.com/>, an online database that provides access to unique chemical compounds [37]. The CADD Group's Chemoinformatics Tools and User Services, available at <https://cactus.nci.nih.gov/translate/>, was used to convert the chemical structures from Mol2 format into PDB format for compatibility with the docking software.

Docking with autoDock vina. Optimization of the grid box parameters of the receptors and the ligands was executed using ADT. The files were saved in the PDBQT format; their corresponding coordinates rewrote into a configuration file used for the docking strategy.

Docking was carried out using Autodock Vina [38,39], for docking of a single ligand with a single receptor. The binding geometries (binding energies and the positional root-mean-square deviation; RMSD) of the ligands and the proteins were displayed on an output file. A ligand orientation with low binding energy indicates better affinity towards a receptor.

Results

Generation of the PM4, PM7, PM8 or Ddi1 knockout parasites: The Ddi1 gene was refractory to deletion thus essential for the growth of the asexual blood stage parasites

Overall, the transfection experiments yielded a total of three KO parasite lines. Of the three mice infected with schizonts transfected with PbGEM-039254 targeting the PM4, two mice had developed parasitaemia of >4% by day eight post parasite inoculation (7 days of pyrimethamine treatment). All the three mice infected with schizonts transfected with PbGEM-086320 and PbGEM-057101 targeting the PM7 and PM8 were parasite positive by day six post parasite inoculation. Three successive attempts to delete the Ddi1 failed to recover parasite twenty days post infection suggesting that the Ddi1 gene was refractory to deletion and thus essential for the asexual blood stage parasite growth. We then proceeded to genotype the PM4, PM7 and PM8 KO parasite lines using three sets of primers; QCR2/GW2, GT/GW1 or GT/GW2 and QCR1/QCR2. Both the WT and KO parasite genomic DNA (gDNA) was isolated and diagnostic polymerase chain reaction (PCR) amplification conducted. As expected, using the standard GW2 and the vector specific QCR2 primers, we obtained a fragment of 1kb, 0.8kb, and 1kb in PM4, PM7 and PM8 parasite lines (Fig 1) indicating the presence of the vector within the parasite. The PCR amplification using vector specific quality control primers, the QCR1 and QCR2 only amplified in the WT parasite line but failed to amplify in the KO lines confirming successful deletion of the respective genes. To further confirm correct integration of the specific vector into the correct chromosome and position, we utilized the standard primers GW1 or GW2 and the vector-specific primers GT. As expected, we obtained a PCR product of 2.9kb, 2.1kb and 2.6kb for PM4, PM7 and PM8 respectively. These results affirmed the successful generation of the KO parasite lines here referred to as PM4_KO, PM7_KO and PM8_KO. We used these parasite lines to assay drug response profiles with WT parasites as the reference line.

The PM7 and PM8 genes are dispensable, deletion of the PM4 gene results in significant attenuation of growth rate of the asexual blood stage parasites

We evaluated the effect of the gene KO on the growth rate phenotypes by measuring the *in vivo* asexual stage parasite density (parasitaemia) for all the KO and WT parasites. The phenotype growth rate results revealed that deletion of PM4 significantly reduces normal parasite growth rate phenotype by 58% ($P = 0.003$), suggesting a substantial contribution to the fitness of the asexual blood stage parasites. On the contrary, PM7_KO and PM8_KO exhibited significantly increased growth rate phenotypes compared to the WT parasites ($P = 0.007$ and $P = 0.0007$ respectively) D4 PI (Fig 2).

The PM7_KO and PM8 KO lines remained sensitive to LP and SQ while the PM4_KO line lost significant susceptibility to both LP and SQ

We assayed whether the deletion of the PM4, PM7 or PM8 affected susceptibility to LP, SQ, PQ and AQ. We reasoned that if the PM4, PM7 or PM8 are targets for the LP and SQ drugs, the KO parasite lines would lose susceptibility to the drugs. We determined the parasite

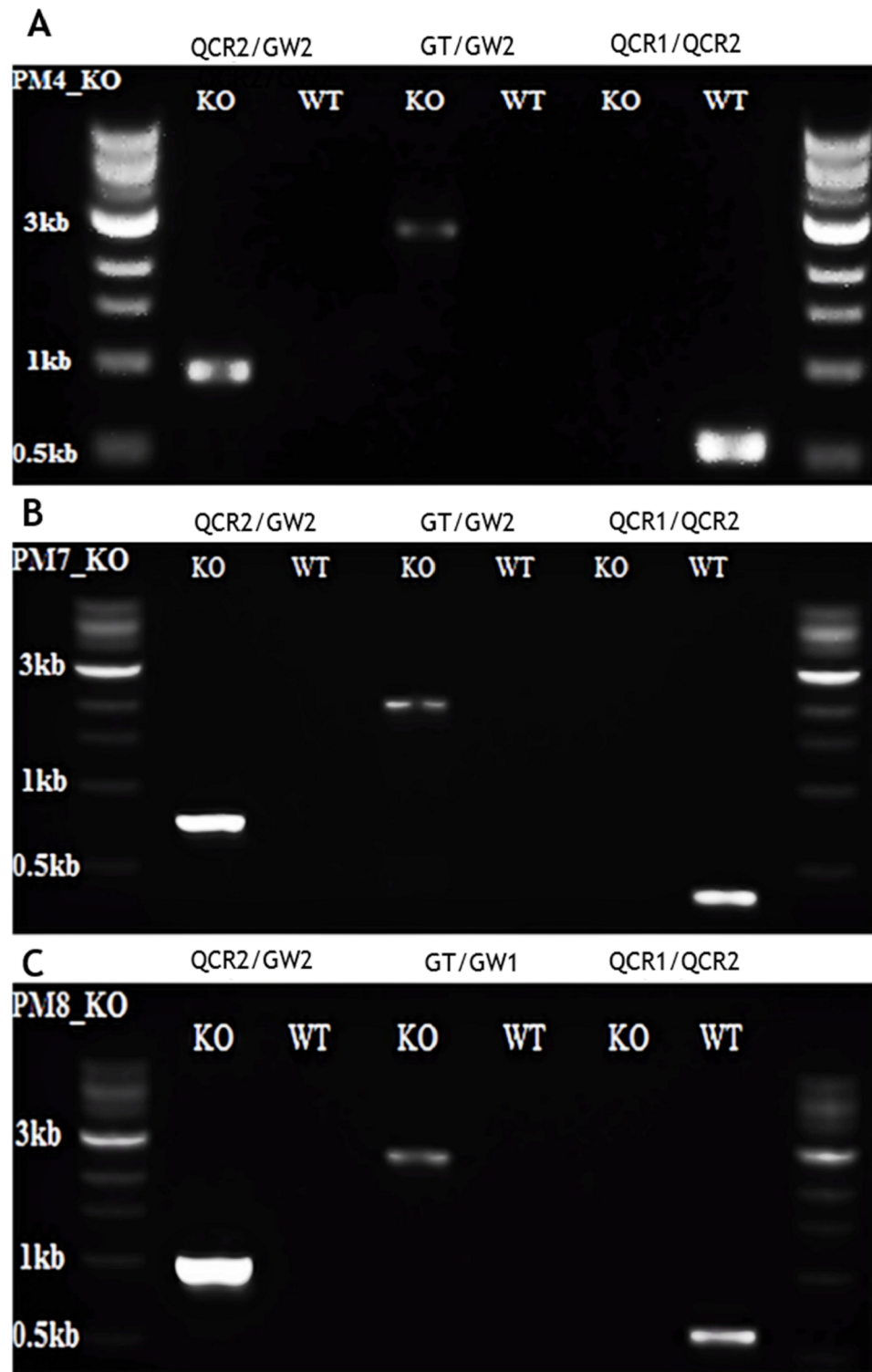


Fig 1. PCR genotyping of the knockout (KO) parasites; (A) PM4_KO, (B) PM7_KO, (C) PM8_KO, and the wild-type (WT) control lines. The PCR amplification used three sets of primer pairs; QCR2/GW2 to confirm the presence of the vector, GT/GW1 or GT/GW2 to verify integration of the vector into the parasite genome and QCR1/QCR2 to confirm deletion of the specific genes. The QCR1, QCR2 and GT1 are vector specific primers while GW2 and GW1 primers are standard primers.

<https://doi.org/10.1371/journal.pone.0201556.g001>

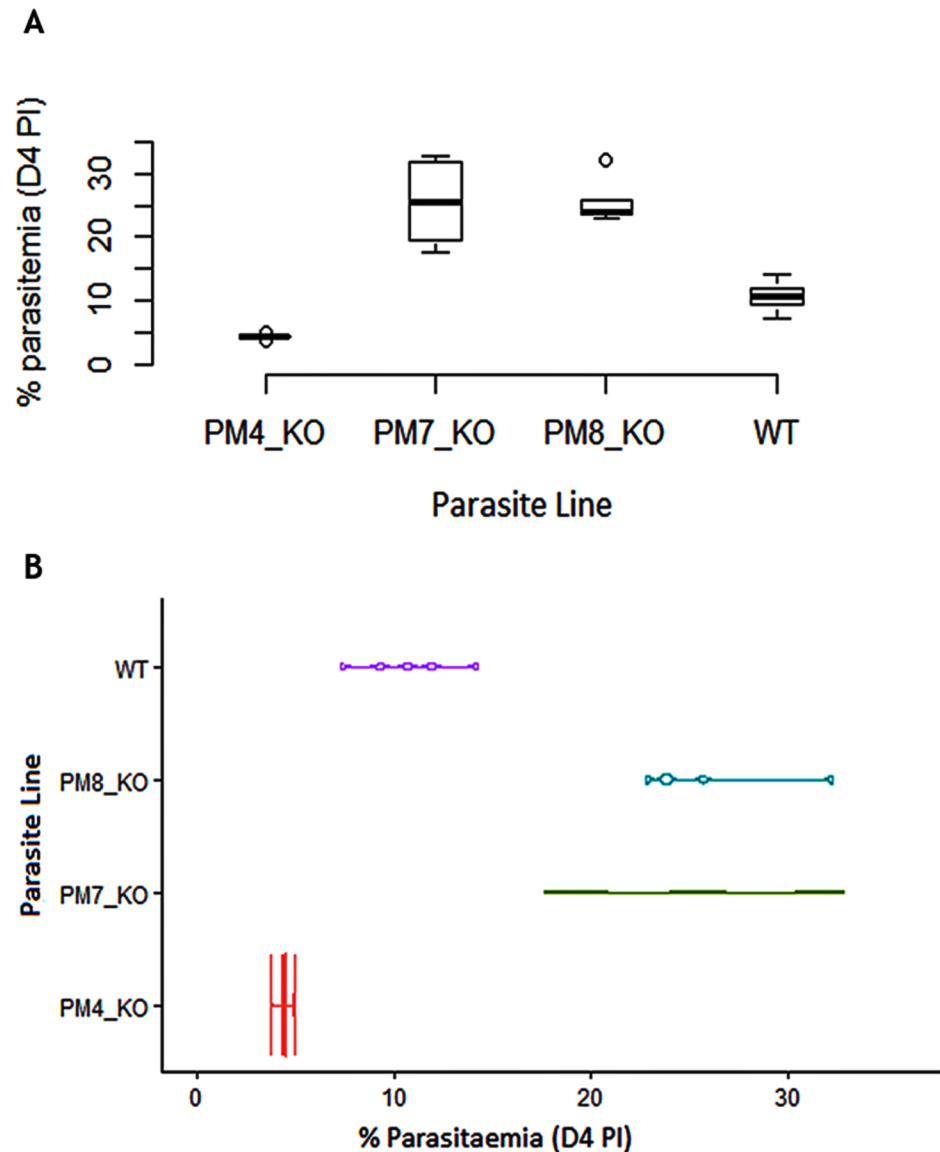


Fig 2. Deletion of the PM4 gene significantly attenuates the growth of asexual parasites. (A). Box plots are showing the percentage parasitaemia in mice infected with the PM4_KO, PM7_KO or PM8_KO parasite line relative to the wild-type (WT) parasite line as measured on day four post parasite inoculation in the standard 4-day suppressive test. (B). Violin plots are showing the distribution of the parasites in mice infected with the PM4_KO, PM7_KO or PM8_KO parasite line relative to the wild-type (WT) parasite line as measured in the standard 4-day suppressive test. The PM4_KO parasites acquired a reduced growth rate phenotype ($P = 0.0003$), while the PM7_KO and PM8_KO parasites lines attained an increased growth rate phenotype.

<https://doi.org/10.1371/journal.pone.0201556.g002>

susceptibility by calculating the activity of LP/RT at 40mg/kg, 10mg/kg, SQ/RT at 50mg/kg, 5mg/kg, AQ at 2.5 mg/kg and PQ at 2.5mg/kg on day four post parasite infection. The LP/RT reduced the PM4_KO mutants' parasitemia by 5.01% compared to a 15.71% in the WT parasites ($P = 0.036$). Similarly, the SQ/RT reduced PM4_KO mutants' parasitemia by 5.29% compared to a 14.12% against the WT parasites ($P = 0.030$). However, the activity of both the LP/RT and SQ/RT against the PM7_KO and PM8_KO parasites were comparable to the percentage activity of WT parasites (Fig 3). As expected, AQ and PQ were active against the WT parasites. At the 2.5mg/kg of AQ or PQ, the mutant parasite remained equally susceptible.

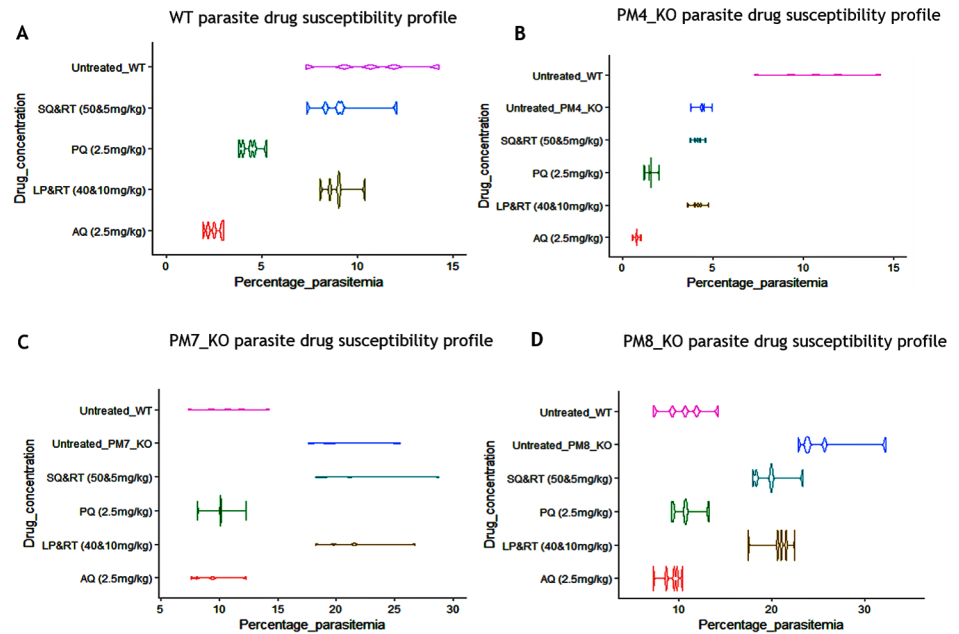


Fig 3. The PM4_KO parasites lost significant susceptibility to both LP and SQ. Violin plots are showing growth profiles and the distribution of the asexual parasites. (A) The wild-type (WT) parasite line displayed expected growth pattern and the susceptibility level to amodiaquine (AQ), piperaquine (PQ), LP/RT and SQ/RT. (B) The PM4_KO parasite line exhibited a significant reduction in the growth outline and a substantial loss of susceptibility to LP/RT ($P = 0.036$) and SQ/RT ($P = 0.030$) but not to AQ or PQ drugs. (C) The PM7_KO and (D) PM8_KO parasite lines displayed rapid growth pattern as compared to the WT parasite line but retained susceptibility to AQ and PQ as well as to the RPIs; LP/RT and SQ/RT.

<https://doi.org/10.1371/journal.pone.0201556.g003>

Both the LP and SQ exhibited higher binding affinity for PM4 protein than PM7, PM8 or Ddi1

To estimate the degree of correctness for all the modeled structures, the Z-scores from PROSA-web server were determined and the proteins scored -5.09, -9.11, -7.94 and -7.52 for Ddi1, PM4, PM7 and PM8 respectively. The Z-scores implied that the structures of the modeled proteins were within the range of scores typically found with experimentally defined proteins. The averaged 3D-ID values were obtained from VERIFY 3D while PROCHECK confirmed residue positioning in the 3D structures and the values confirmed the integrity of the modeled structures (Table 4).

Table 4. Validation of the modeled 3D structures of the *P. berghei* aspartyl proteases.

Protein	Gene ID	Z-score	Averaged 3D-ID score > = 0.2 (%)	Residues in most favored regions (%)	Residues in additional allowed regions (%)	Residues in disallowed regions (%)
Ddi1	PBANKA_103320	-5.09	84.17	90.5	8.7	0.0
PMIV	PBANKA_103440	-9.11	97.59	90.9	8.8	0.0
PMVII	PBANKA_051760	-7.94	82.24	85.8	11.7	0.9
PMVIII	PBANKA_132910	-7.52	87.40	86.3	10.9	0.6

The 3D structures of the proteins were modelled using SWISS-MODEL, and their quality validated using PROCHECK for stereochemical evaluation; PROSA-web, for detection of potential errors in the 3D structures; and verify_3D, for compatibility of the 3D structures with their respective amino acid sequences. All the modelled structures have features characteristic of native structures.

<https://doi.org/10.1371/journal.pone.0201556.t004>

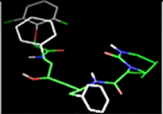
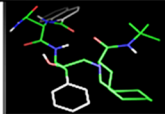
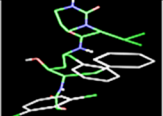
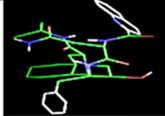
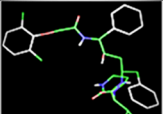
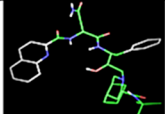
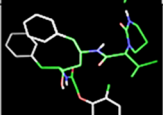
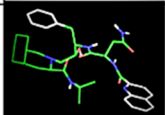
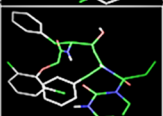
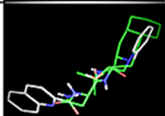
PROTEIN	LIGAND			
	Lopinavir		Saquinavir	
	Ligand Mode	Binding Affinity (kcal/mol)	Ligand Mode	Binding Affinity (kcal/mol)
PM4	1 	-9.3	1 	-10.6
PM7	1 	-8.4	1 	-8.5
PM8	1 	-9.1	1 	-9.4
DDI1	1 	-6.3	1 	-7.0
HIV_Asp	1 	-6.3	1 	-7.9

Fig 4. Both the LP and SQ exhibited high binding affinity to the PM4 protein. The binding geometries for docking of a single ligand with a single receptor as modelled using the Autodock Vina. The binding energies for LP and SQ to the PM4 were the lowest suggesting better affinity to the protein. The LP and SQ yielded higher binding affinity to both PM7 and PM8 as compared to the known RPIs target; the HIV Aspartic protease (HIV Asp). Both LP and SQ exhibited equal or low binding energy to the essential Ddi1 protein as compared to the HIV Asp.

<https://doi.org/10.1371/journal.pone.0201556.g004>

When we evaluated the protein binding affinity, we determined the ligand conformations using the AutoDock Tools (ADT; Fig 4), and the best binding modes (mode 1). All the protein binding complexes showed high binding affinities, with the highest affinity at -10.6 kcal/mol and the lowest at -6.3 kcal/mol. Unlike the Ddi1 that had an equal binding affinity as HIV-1 asp protease for LP (-6.3 kcal/mol), the binding affinities of LP or SQ to PM4, PM7 or PM8 were higher than the affinity of the two drugs to the HIV-1 aspartyl protease (-6.3 kcal/mol). The PM4 had the lowest binding energy; -9.3 and -10.6 kcal/mol for LP or SQ respectively, indicating better binding with the LP and SQ (Figs 4 and 5).

The Ddi1 has a pepsin_retropepsin-like domain conserved across all *Plasmodium* species

We then reasoned that since deletion of the Ddi1 seemed toxic to the growth of the asexual blood stage parasites, the protein might have unique and conserved motifs across the different malaria parasites. We searched and analysed conserved motifs in the Ddi1 gene sequence using the Batch CD-Search [40] in the Conserved Domain Database (CDD). The CD-search results revealed that Ddi1 gene has a pepsin_retropepsin-like domain, with a catalytic motif, within its sequence. Interestingly, this motif is conserved in both the *Plasmodium falciparum* and *P. berghei* parasites (Fig 6).

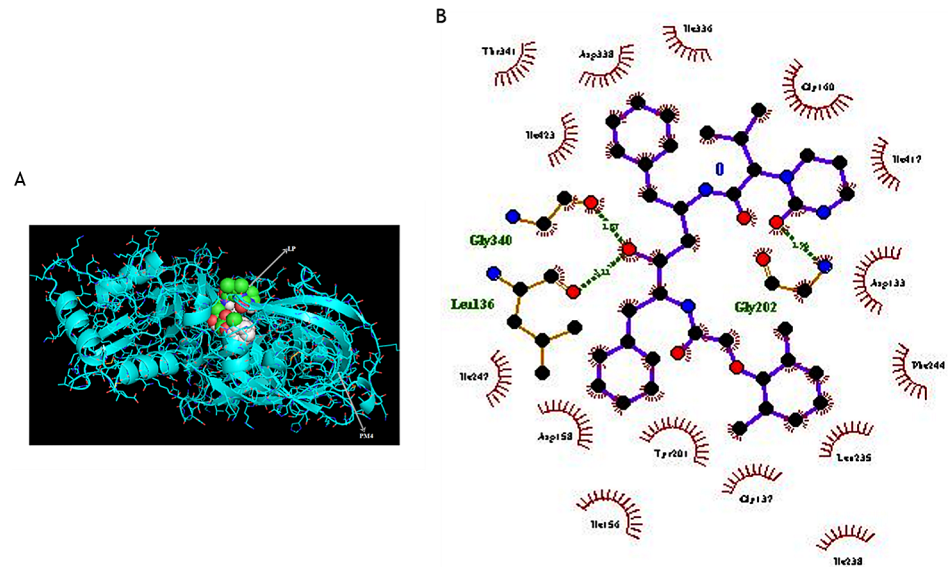


Fig 5. (A) The crystallographic structure of PM4 protein in complex with the lopinavir as visualized using PyMOL. (B) 2D LP-PM4 interaction diagram with the oxygen atoms shown in red, nitrogen atoms shown in blue while hydrogen bonds are shown as olive green dotted line. The interaction plots were generated using LigPlot+.

<https://doi.org/10.1371/journal.pone.0201556.g005>

Discussion

The emergence and fast-spreading resistance against artemisinin-based combination drugs have spurred the identification and validation of novel antimalarial targets. Proteases are proven drug targets as evidenced by the use of protease inhibitors such as LP and SQ for the treatment of HIV/AIDS and malaria parasite [21,41]. This study considered both *Plasmodium* pepsin-like proteases; PM4, PM7 or PM8 as well as a retropepsin-like protease (Ddi1) as potential targets for RPIs. We investigated the essentiality of PM4, PM7, PM8 or Ddi1 by creating KO lines and used the lines to understand the possible mechanisms of action of LP or SQ.

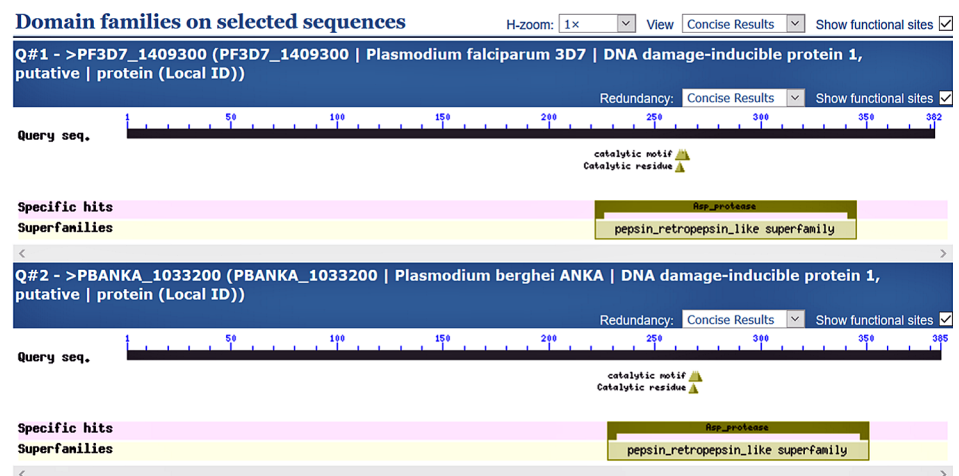


Fig 6. The Ddi1 protein has a conserved catalytic motif in its sequence. The Batch CD-Search showing the pepsin_retropepsin-like domain, with a catalytic motif conserved among the *Plasmodium* parasite species. The search involved *P. falciparum* and *P. berghei* parasites and both show the conserved motif between amino acid positions 250 and 280.

<https://doi.org/10.1371/journal.pone.0201556.g006>

We generated KO *Plasmodium berghei* parasites deficient in the PM4, PM7 or PM8 genes but the deletion of the Ddi1 gene seemed toxic to the growth of the asexual blood stage parasites. The domain search within the Ddi1 protein confirmed the presence of a conserved catalytic motif among all the *Plasmodium* parasites species. The Ddi1 protein belongs to the A2 family of proteases, a retropepsin-like protease and is an active and functional aspartyl protease in *Leishmania major* parasites [42]. The protease is involved in a novel, ubiquitin-dependent proteolytic, cell cycle control and reduction of extracellular protein secretion in *Saccharomyces cerevisiae* [43]. Recently, studies have shown that an ortholog of yeast Ddi1 in *L. major* and human can complement protein secretion phenotype in Ddi1-deficient *S. cerevisiae* cells and that RPIs forestall the resultant complementation phenotype [44]. The retropepsin-like family of proteases includes retroviral aspartyl proteases, retrotransposons and DNA-damage-inducible proteins in eukaryotic cells. From this study, the high binding affinities of the Ddi1 to the LP coupled with its essential role in the growth of the asexual blood parasite suggest that chemical compounds that inhibit the Ddi1 may form next generation of novel drugs.

To study the role of other aspartyl proteases in mediating drug action, we used the generated KO lines (PM4_KO, PM7_KO, and PM8_KO) to investigate the effect of gene deletion on the parasite growth rate phenotypes. Although a recent study on the functional profile of *Plasmodium* genome deleted the genes in *P. berghei*, the effect of the gene deletion on drug susceptibility was not measured [45]. In agreement with this study, we have demonstrated that, whereas the PM7 and PM8 genes are dispensable during the asexual stages of parasite development, they exhibit an indirect interaction with the growth of the asexual parasite stage. The PM8 controls egress of the sporozoite from the oocysts and regulates the gliding motility of the parasite for the invasion of the salivary gland in the mosquito vectors [46]. The loss of PM7 and PM8 genes increased the growth rate of asexual blood stage parasites suggesting that the deletion confer a growth advantage to the KO parasite. At D4 PI, the parasitemia for the PM7_KO and PM8_KO parasites was 2.5-fold higher than that of the WT parasites. On further scrutiny, we observed that, despite the PM4 being a dispensable gene, its deletion significantly reduces parasite asexual stage growth rates. The parasitemia for PM4_KO parasites was 2-fold lower than that of the WT parasites, suggesting a substantial contribution to the fitness of the parasite within the red blood cells. Whereas *P. falciparum* expresses four digestive vacuoles (DV) PMs; (PM 1, 2, histo-aspartic protease; HAP and PM4), there is only one identified DV PM in *P. berghei*, PM4 [22]. Therefore, successful deletion of PM4 in *P. berghei* suggests that the parasite could be relying on other enzyme pathways for haemoglobin degradation that sustains the supply of the amino acids for the intraerythrocytic parasite growth. The findings of PM4 deletion in *P. berghei* are consistent with the findings from [18], who documented that a quadruple KO of all the DV PMs (PM1, PM2, HAP, and PM4) in *P. falciparum* does not entirely impair parasite growth. However, the effect of the quadruple gene KO on drug susceptibility was not determined. Recent studies on parasite proteases identified PM9 and PM10 as a protein for the invasion of the erythrocytes, with PM10 also controlling egress of the parasite from the red blood cells [21]. Indeed compounds that inhibit PM10 action also result in complete blockage of parasite egress from the red blood cells [21]. The PM9 and PM10, though essential for the asexual blood stage growth in all the malaria species including the rodent parasite *P. berghei* [45], are not localised within the DV thus not involved in the digestion of hemoglobin.

Malaria parasite haemoglobin degradation is a vital process during intra-erythrocytic parasite development. The plasmepsins, aminopeptidases, metalloprotease, falcilysin, and falcipains are found in the DV and are involved in degrading the haemoglobin. The presence of multiple enzyme pathways with overlapping specificity and function is strategic to increase parasite fitness [47,48]. As earlier alluded, the deletion of PM4 confers significant growth

disadvantage, and the KO parasite is less susceptible to LP and SQ. We thus argue that since the PM4 participates in the haemoglobin degradation [24,27], then the protein may be a target for both the LP and SQ. However, the presence of alternative enzymes for haemoglobin degradation provides the asexual blood stage parasites with an advantage albeit with cost in the growth rate.

Our drug profile results show that the activity of all the tested drugs against PM7_KO and PM8_KO parasites was nearly equivalent to the WT parasites. The activity of LP/RT and SQ/RT ranged between 15% and 21% against both the mutant parasites and WT parasites. These findings augment the fact that the asexual parasites do not express the PM7 and PM8 genes within the erythrocytes [24,48]. Our study focused on the asexual blood stage parasites; both the LP and SQ are predicted to act on the parasite within the erythrocytes, thus although the LP and SQ may bind PM7 and PM8, the inhibition effect may not be observed in the asexual parasites within the erythrocytes. Indeed, studies involving PM8 have shown that it plays an essential role in the sporogonic stages of malaria parasite development [46]. On the contrary, compared to the WT parasites, the LP/RT or SQ/RT activity against PM4_KO lines was 3-fold lower, demonstrating a potential role in the LP and SQ action. The PM4 is the only DV specific PM found in all *Plasmodium* species suggesting an essential role for the parasite in the growth of the asexual blood stage [41].

The modeling and molecular docking studies revealed that LP or SQ have high binding energies towards the *Plasmodium* aspartyl proteases. In fact, the drugs' binding affinity towards PM4, PM7, PM8 or Ddi1 were higher than the affinity towards the HIV-1 aspartyl protease. However, because parasites contain many proteases with functional redundancy, unlike retroviruses, the *in vivo* activity of LP and SQ against the parasites is not proportional to the *in silico* binding affinities. The high binding affinity of LP or SQ towards PM4 correlates with the reduced sensitivity of PM4_KO parasites to the drugs. However, because of the redundant enzyme system involved in haemoglobin digestion for parasite asexual stage development, inhibition of PM4 does not entirely deprive of parasite survival. The high binding affinity shown by LP or SQ towards PM7 or PM8 (-8.4 kcal/mol to -9.4 kcal/mol) does not correlate with the *in vivo* drug suppression of asexual stage parasites probably because the enzymes are not essential and thus possibly not expressed during the erythrocytic asexual stages of parasite growth. Future work tailored towards understanding the functions of Ddi1 in *Plasmodium* parasites and its candidature as a target for drugs may inform the development of future drugs against the malaria parasite.

Acknowledgments

We thank the team from the *PlasmoGEM* project at the Wellcome Trust Sanger Institute for providing the highly efficient gene modification resources used in this study. We also are grateful to Florence Ng'ong'a and Rosaline Macharia who assisted in the execution of the *in silico* work.

Author Contributions

Conceptualization: Noah Machuki Onchieku, Daniel Kiboi, Gabriel Magoma.

Data curation: Noah Machuki Onchieku, Reagan Mogire.

Formal analysis: Noah Machuki Onchieku, Reagan Mogire, Daniel Kiboi, Gabriel Magoma.

Funding acquisition: Noah Machuki Onchieku, Peter Mwitari, Daniel Kiboi, Gabriel Magoma.

Investigation: Noah Machuki Onchieku, Loise Ndong'u, Francis Kimani, Daniel Kiboi, Gabriel Magoma.

Methodology: Noah Machuki Onchieku, Reagan Mogire, Loise Ndong'u, Daniel Kiboi, Gabriel Magoma.

Project administration: Noah Machuki Onchieku, Peter Mwitari, Francis Kimani, Damaris Matoke-Muhia, Daniel Kiboi, Gabriel Magoma.

Resources: Noah Machuki Onchieku, Peter Mwitari, Francis Kimani, Damaris Matoke-Muhia, Daniel Kiboi, Gabriel Magoma.

Software: Noah Machuki Onchieku, Reagan Mogire, Daniel Kiboi, Gabriel Magoma.

Supervision: Peter Mwitari, Daniel Kiboi, Gabriel Magoma.

Validation: Noah Machuki Onchieku, Daniel Kiboi, Gabriel Magoma.

Visualization: Noah Machuki Onchieku, Loise Ndong'u.

Writing – original draft: Noah Machuki Onchieku.

Writing – review & editing: Noah Machuki Onchieku, Reagan Mogire, Peter Mwitari, Damaris Matoke-Muhia, Daniel Kiboi, Gabriel Magoma.

References

1. Breman JG, Mills A, Snow RW, Mulligan J, Lengeler C, Mendis K, et al. Malaria Conquering. Disease Control Priorities in Developing Countries. 2nd ed. New York: Oxford University Press; 2006. p. 413–32.
2. WHO. World Malaria Report 2017 [Internet]. 2017 [cited 2017 Jan 4]. Available from: <http://www.who.int/malaria/publications/world-malaria-report-2017/en/>
3. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, et al. Spread of Artemisinin Resistance in *Plasmodium falciparum* Malaria. 2014; 371(5):411–23.
4. Noedl H, Se Y, Schaefer K, Smith BL, Socheat D, Fukuda MM. Evidence of Artemisinin-resistant malaria in Western Cambodia. N Engl J Med. 2008; 359(24):2619–20. <https://doi.org/10.1056/NEJMc0805011> PMID: 19064625
5. Phyto AP, Nkhoma S, Stepniwska K, Ashley EA, Nair S, McGready R, et al. Emergence of artemisinin-resistant malaria on the western border of Thailand: A longitudinal study. Lancet. 2012; 379(9830):1960–6. [https://doi.org/10.1016/S0140-6736\(12\)60484-X](https://doi.org/10.1016/S0140-6736(12)60484-X) PMID: 22484134
6. Talisuna AO, Karema C, Ogutu B, Juma E, Logedi J, Nyandigisi A, et al. Mitigating the threat of artemisinin resistance in Africa: improvement of drug-resistance surveillance and response systems. Lancet Infect Dis. 2012; 12(11):888–96. [https://doi.org/10.1016/S1473-3099\(12\)70241-4](https://doi.org/10.1016/S1473-3099(12)70241-4) PMID: 23099083
7. WHO. Malaria in HIV/AIDS patients [Internet]. 2017 [cited 2017 Jan 4]. Available from: http://www.who.int/malaria/areas/high_risk_groups/hiv_aids_patients/en/
8. Andrews KT, Fairlie DP, Madala PK, Ray J, Wyatt DM, Hilton PM, et al. Potencies of Human Immunodeficiency Virus Protease Inhibitors *In Vitro* against *Plasmodium falciparum* and *In Vivo* against Murine Malaria. Antimicrob Agents Chemother. 2006; 50(2):639–48. <https://doi.org/10.1128/AAC.50.2.639-648.2006> PMID: 16436721
9. Hobbs C V, Voza T, Coppi A, Kirmse B, Marsh K, Borkowsky W, et al. HIV Protease Inhibitors Inhibit the Development of Preerythrocytic-Stage *Plasmodium* Parasites. J Infect Dis. 2009; 199:134–41. <https://doi.org/10.1086/594369> PMID: 19032102
10. Hobbs C V, Neal J, Conteh S, Donnelly L, Chen J, Marsh K, et al. HIV Treatments Reduce Malaria Liver Stage Burden in a Non-Human Primate Model of Malaria Infection at Clinically Relevant Concentrations *In Vivo*. PLoS One. 2014; 9(7):3–9.
11. Lek-Uthai U, Suwanarusk R, Ruengweerayut R, Nosten F, Gardiner DL, Boonma P, et al. Stronger Activity of Human Immunodeficiency Virus Type 1 Protease Inhibitors against Clinical Isolates of *Plasmodium vivax* than against Those of *P. falciparum*. Antimicrob Agents Chemother. 2008; 52(7):2435–41. <https://doi.org/10.1128/AAC.00169-08> PMID: 18443130
12. Nsanzabana C, Rosenthal PJ. *In Vitro* Activity of Antiretroviral Drugs against *Plasmodium falciparum*. Antimicrob Agents Chemother. 2011; 55(11):5073–7. <https://doi.org/10.1128/AAC.05130-11> PMID: 21876053

13. Parikh S, Gut J, Istvan E, Goldberg DE, Havlir D V, Rosenthal PJ. Antimalarial Activity of Human Immunodeficiency Virus Type 1 Protease Inhibitors. *Antimicrob Agents Chemother*. 2005; 49(7):2983–5. <https://doi.org/10.1128/AAC.49.7.2983-2985.2005> PMID: 15980379
14. Skinner-adams TS, Mccarthy JS, Gardiner DL, Hilton PM, Andrews KT. Antiretrovirals as Antimalarial Agents. *J Infect Dis*. 2004; 190(1):1998–2000.
15. Achan J, Kakuru A, Ikilezi G, Ruel T, Clark TD, Nsanzabana C, et al. Antiretroviral Agents and Prevention of Malaria in HIV-Infected Ugandan Children. *N Engl J Med*. 2012; 367(22):2110–8. <https://doi.org/10.1056/NEJMoa1200501> PMID: 23190222
16. Patick AK, Potts KE. Protease Inhibitors as Antiviral Agents. *Clin Microbiol Rev*. 1998; 11(4):614–27. PMID: 9767059
17. Abbenante G, Fairlie D, Abbenante G, Fairlie DP. Protease Inhibitors in the Clinic. *Med Chem (Los Angeles)*. 2005; 1(1):71–104.
18. Bonilla A, Bonilla TD, Yowell CA, Fujioka H, Dame JB. Critical roles for the digestive vacuole plasmepsins of *Plasmodium falciparum* in vacuolar function. *Mol Microbiol*. 2007; 65(1):64–75. <https://doi.org/10.1111/j.1365-2958.2007.05768.x> PMID: 17581121
19. Goldberg DE. Hemoglobin Degradation. In: Compans R. W., Cooper M. D., Honjo T., Koprowski H., Melchers F., Oldstone M. B. A., Olsnes S., Potter M., Vogt P. K., Wagner H., David J. Sullivan M.D., Sanjeev KD. *Malaria: Drugs, Disease and Post-genomic Biology*. SpringerLink; 2005. p. 275–91.
20. Li F, Bounkeua V, Pettersen K, Vinetz JM. *Plasmodium falciparum* ookinete expression of plasmepsin VII and plasmepsin X. *Malar J*. 2016; 15(111):1–10.
21. Nasamu AS, Glushakova S, Russo I, Vaupel B, Oksman A, Kim AS, et al. Plasmepsins IX and X are essential and druggable mediators of malaria parasite egress and invasion. *Science*. 2017; 358(6362):518–22. <https://doi.org/10.1126/science.aan1478> PMID: 29074774
22. Dame JB, Yowell C, Omara-Opyene L, Carlton JM, Cooper RA, Li T. Plasmepsin 4, the food vacuole aspartic proteinase found in all *Plasmodium* spp. infecting man. *Mol Biochem Parasitol*. 2003; 130(1):1–12. PMID: 14550891
23. Liu P, Robbins AH, Marzahn MR, Mcclung SH. Enzymatic Characterization of Recombinant Food Vacuole Plasmepsin 4 from the Rodent Malaria Parasite *Plasmodium berghei*. *PLoS One*. 2015; 10(10):1–28.
24. Banerjee R, Liu J, Beatty W, Pelosof L, Klemba M, Goldberg DE. Four plasmepsins are active in the *Plasmodium falciparum* food vacuole, including a protease with an active-site histidine. *Proc Natl Acad Sci U S A*. 2002; 99(2):990–5. <https://doi.org/10.1073/pnas.022630099> PMID: 11782538
25. Hodder AN, Sleebs BE, Czabotar PE, Gazdik M, Xu Y, Neill MTO, et al. Structural basis for plasmepsin V inhibition that blocks export of malaria proteins to human erythrocytes. *Nat Struct Mol Biol*. 2015; 22(8):1–9.
26. Sleebs BE, Lopaticki S, Marapana DS, Neill MTO, Rajasekaran P, Whitehead LW, et al. Inhibition of Plasmepsin V Activity Demonstrates Its Essential Role in Protein Export, PfEMP1 Display, and Survival of Malaria Parasites. *PLoS Biol*. 2014; 12(7).
27. Rosenthal PJ. Proteases of malarial parasites: new targets for chemotherapy. *Emerg Infect Dis*. 1998; 4(1):49–57. <https://doi.org/10.3201/eid0401.980107> PMID: 9452398
28. PlasmoDB. PBANKA_1033200 DNA damage-inducible protein 1, putative [Internet]. 2016. Available from: http://plasmodb.org/plasmo/app/record/gene/PBANKA_1033200
29. Franke-fayard B, Trueman H, Ramesar J, Mendoza J, Keur M Van Der, Linden R Van Der, et al. A *Plasmodium berghei* reference line that constitutively expresses GFP at a high level throughout the complete life cycle. *Mol Biochem Parasitol*. 2004; 137(1):23–33. <https://doi.org/10.1016/j.molbiopara.2004.04.007> PMID: 15279948
30. Janse CJ, Ramesar J, Waters AP. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite *Plasmodium berghei*. *Nat Methods*. 2006; 1(1):346–56.
31. Fidock DA, Rosenthal PJ, Croft SL, Brun R, Nwaka S. Antimalarial drug discovery: efficacy models for compound screening. A Protocol for Antimalarial Efficacy Testing in vitro. *Nat Rev Drug Discov*. 2004; 3(6):509–20. <https://doi.org/10.1038/nrd1416> PMID: 15173840
32. Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, et al. SWISS-MODEL: Modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res*. 2014; 42(W1):252–8.
33. Delano WL. The PyMOL Molecular Graphics System. DeLano Sci [Internet]. 2002; Available from: <http://www.pymol.org>
34. Laskowski RA, Macarthur MW, Moss DS, Thornton J. PROCHECK: A program to check the stereochemical quality of protein structures. *J Appl Crystallogr Appl Crystallogr*. 1993; 26(2):283–91.

35. Wiederstein M, Sippl MJ. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Res.* 2007; 35(2):407–10.
36. Bowie JU, Lüthy R, Eisenberg D. A Method to Identify Protein Sequences That Fold into a Known Three-Dimensional Structure. *Science* (80-). 1991; 253(5016):164–70.
37. Williams AJ, Pence HE. ChemSpider: An Online Chemical Information Resource. *J Chem Educ.* 2010; 87(11).
38. Forli S, Huey R, Pique ME, Sanner M, Goodsell DS, Arthur J. Computational protein-ligand docking and virtual drug screening with the AutoDock suite. *Nat Protoc.* 2016; 11(5):905–19. <https://doi.org/10.1038/nprot.2016.051> PMID: 27077332
39. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. *J Comput Chem.* 2010; 31(2):455–61. <https://doi.org/10.1002/jcc.21334> PMID: 19499576
40. Marchler-bauer A, Bo Y, Han L, He J, Lanczycki CJ, Lu S, et al. CDD / SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res.* 2017; 45(D):200–3.
41. Moura PA, Dame JB, Fidock DA. Role of *Plasmodium falciparum* Digestive Vacuole Plasmepsins in the Specificity and Antimalarial Mode of Action of Cysteine and. *Antimicrob Agents Chemother.* 2009; 53(12):4968–78. <https://doi.org/10.1128/AAC.00882-09> PMID: 19752273
42. Perteguer MJ, Gómez-puertas P, Cañavate C, Dagger F, Gárate T, Valdivieso E. Ddi1-like protein from *Leishmania major* is an active aspartyl proteinase. *Cell Stress Chaperones.* 2013; 18:171–81. <https://doi.org/10.1007/s12192-012-0368-9> PMID: 22933181
43. Gabriely G, Kama R, Gelin-licht R, Gerst JE. Different Domains of the UBL-UBA Ubiquitin Receptor, Ddi1 / Vsm1, Are Involved in Its Multiple Cellular Roles. *Mol Biol Cell.* 2008; 19:3625–37. <https://doi.org/10.1091/mbc.E07-05-0462> PMID: 18562697
44. White RE, Powell DJ, Berry C. HIV proteinase inhibitors target the Ddi1-like protein of *Leishmania* parasites. *FASEB J.* 2016; 25(5):1729–36.
45. Bushell E, Gomes AR, Sanderson T, Wengelnik K, Rayner JC, Billker O, et al. Functional Profiling of a *Plasmodium* Genome Reveals an Abundance of Essential Genes Article Functional Profiling of a *Plasmodium* Genome Reveals an Abundance of Essential Genes. *Cell.* 2017; 170(2):260–272.e8. <https://doi.org/10.1016/j.cell.2017.06.030> PMID: 28708996
46. Mastan BS, Kumar S, Dey S, Arun K, Mishra S. *Plasmodium berghei* plasmepsin VIII is essential for sporozoite gliding motility. *Int J Parasitol.* 2017; 47(5):7–13.
47. Liu J, Istvan ES, Gluzman IY, Gross J, Goldberg DE. *Plasmodium falciparum* ensures its amino acid supply with multiple acquisition pathways and redundant proteolytic enzyme systems. *Proc Natl Acad Sci U S A.* 2006; 103(23):8840–5. <https://doi.org/10.1073/pnas.0601876103> PMID: 16731623
48. Omara-Opyene AL, Moura PA, Sulsona CR, Bonilla JA, Yowell CA, Fujioka H, et al. Genetic disruption of the *Plasmodium falciparum* digestive vacuole plasmepsins demonstrates their functional redundancy. *J Biol Chem.* 2004; 279(52):54088–96. <https://doi.org/10.1074/jbc.M409605200> PMID: 15491999