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The activity and inhibition of the food vacuole plasmepsin from the rodent malaria parasite *Plasmodium chabaudi*

Tiago M. Martins [a], Ana Domingos [a,b], Colin Berry [c,*], David M. Wyatt [c]

[a] *Instituto Nacional de Engenharia, Tecnologia e Inovação, Departamento de Biotecnologia, UTPAM, Edifício F, Estrada do Paço do Lumiar, 1649-038 Lisboa, Portugal*
[b] *Instituto de Higiene e Medicina Tropical, Centro de Malária e Outras Doenças Tropicais, Universidade Nova de Lisboa, R. da Junqueira 96, 1349-008 Lisboa, Portugal*
[c] *Cardiff School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, UK*

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Abstract

The rodent malaria parasite *Plasmodium chabaudi* encodes one food vacuole plasmepsin—the aspartic proteinases important in haemoglobin degradation. A recombinant form of this enzyme was found to cleave a variety of peptide substrates and was susceptible to a selection of naturally occurring and synthetic inhibitors, displaying an inhibition profile distinct from that of aspartic proteinases from other malaria parasites. In addition, inhibitors of HIV proteinase that kill *P. chabaudi* in vivo were also inhibitors of this new plasmepsin. *P. chabaudi* is a widely used model for human malaria species and, therefore, the characterisation of this plasmepsin is an important contribution towards understanding its biology.

Keywords: Rodent malaria model; *Plasmodium chabaudi*; Aspartic proteinase; New plasmepsin

1. Introduction

Malaria remains a disease with a terrifying death toll. Despite advances in medical science, over 1 million people, mostly children, die every year as a result of infection with the human forms of this disease, a figure that is predicted to rise (Greenwood et al., 2005). Parasite resistance to currently available therapeutic agents, increased resistance of the mosquito vector to insecticides and even global warming (which is predicted to lead to increased transmission of the disease in certain areas) are all factors contributing to the increased prevalence of this disease (Greenwood et al., 2005). Although the malaria parasite responsible for most deaths (*P. falciparum*) does not appear to have a direct rodent equivalent (Qari et al., 1996), rodent forms of malaria have been used as models for the study of the human pathogenic parasites and have emerged as powerful tools for the in vivo testing of potential therapeutic agents. One species of rodent malaria, *Plasmodium chabaudi*, is of particular use as it can be kept indefinitely within the laboratory with repeated passage through infected mice. Although not a direct equivalent, *P. chabaudi* is considered a good model for *Plasmodium falciparum* due to similarities in their basic biology which include their preferential invasion of mature erythrocytes, the synchronicity of their asexual blood forms and the delayed formation of game-

* Corresponding author. Tel.: +44 29 20874508; fax: +44 29 20874486.
E-mail address: Berry@cf.ac.uk (C. Berry).

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tocytes until late on in the infection cycle (Carlton et al., 1998).

Plasmodium aspartic proteinases are known as plasmepsins (PMs) and in the human parasite *P. falciparum* at least, comprise a diverse family of enzymes of which nine are aspartic proteinases and one related histo-aspartic proteinase (HAP) (see review of plasmepsins in Coombs et al. (2001)). Four of these PMs (PMI, II, IV and HAP) from *P. falciparum* are found in the parasite's food vacuole (FV), where they are involved in the degradation of host cell haemoglobin (Banerjee et al., 2002). Previous studies have revealed that whilst *P. falciparum* has four food vacuole PMs, the other parasites that infect humans (*P. malariae*, *P. ovale* and *P. vivax*) and the rodent malarial parasites *P. berghei* and *P. chabaudi*, appear to only encode only one of these enzymes (Dame et al., 2003; Humphreys et al., 1999; Martins et al., 2003). Why *P. falciparum* encodes four of these food vacuole enzymes and the other parasites seemingly maintain just one of these proteinases is unclear. Recent studies exploring the antimalarial potential of HIV proteinase inhibitors against both *P. falciparum* and *P. chabaudi* have suggested that plasmepsins may be the in vivo targets of these compounds (Savarino et al., 2005; Skinner-Adams et al., 2005). Compared to *P. falciparum*, the FV PMs from rodent malarias are less well characterised and so these studies describing the antimalarial activity of certain HIV proteinase inhibitors have made studying FV PMs from rodent forms of malaria a priority. Partial characterisation of the FV PM from *P. berghei* has been previously reported, but full study of this enzyme was hampered by the inability to obtain appropriate quantities of this protein in an active recombinant form (Humphreys et al., 1999). This is in contrast to the studies reported herein, as we detail for the first time the recombinant production and enzymatic characterisation of the FV PM from *P. chabaudi*, including details of the interactions between this new enzyme and currently licensed HIV proteinase inhibitors. We also describe for the first time a rapid and simple assay, which allows the unequivocal distinction between the proteolytic activities of certain plasmepsins.

2. Materials and methods

Genomic DNA from *P. chabaudi chabaudi* strain AS DNA was isolated according to Martins et al. (2003). The gene encoding a truncated form of the *P. chabaudi* food vacuole proplasmepsin (beginning at residue Ser76 of this precursor (Martins et al., 2003)) was amplified by the polymerase chain reaction (PCR) from this genomic DNA using the Expand High Fidelity PCR System (Roche, Mannheim, Germany) and forward (*GGATCC*TCCAATTATTTAACAATTCGTGG) and reverse (*GGATCC*TTAGTTTTTTGCAAGTGCGAAACCA) primers which contained *Bam*HI sites (in italics) at their 5′ends. The conditions used were: 95 °C for 5 min followed by 30 cycles of: 95 °C for 1 min; 58 °C for 1 min; 72 °C for 1 min 15 s; prior to a final elongation step of 72 °C for 10 min. The resultant 1137 bp product was ligated into the pGEM-T vector (Promega, Southampton, UK) and sequenced using an Applied Biosystems ABI 3100 capillary DNA sequencer. The DNA insert was excised with *Bam*HI, ligated into the expression vector pET3a and a clone with the cDNA in the correct orientation was selected after sequencing and termed pPcPMpET. A mutant form of the semi-proPcPM gene carrying a lysine to alanine substitution at residue 110 (numbering according to ProPcPM (Martins et al., 2003)) was generated using a site directed mutagenesis PCR method. Two complementary oligonucleotides (forward primer CATTAAAGAATCATTTGCATTATTAAAATCCGG and reverse primer CCGGATTTTAATAATGCAAATGATTCTTTAATG) containing the desired mutation (underlined) were used in a PCR reaction using *Pfu* Turbo (Stratagene) and pPcPMpET DNA as template. Following thermocycling (16 cycles of: 95 °C for 30 s; 55 °C for 30 s; 68 °C for 12 min) the parental template plasmid was digested with *Dpn*I and after isopropanol precipitation was transformed into *E. coli* DH5α cells. One clone containing the desired mutation was selected after sequence analysis and termed pPcPMpETMUT. The pET3a constructs encoding both wildtype and the mutant forms of PcPM protein were transformed into *E. coli* BL21(DE3)pLysS cells and the preparation of both enzymes proceeded as described previously for the production of *P. falciparum* semi–proplasmepsin II (Hill et al., 1994). With the exception that the urea solubilised plasmepsins were refolded by dialysis against a 200× volume of 10 mM Tris–HCl pH 8.5 at 25 °C for 16 h. After dialysis, the preparations were acidified by the addition of one-tenth volume of 1 M sodium acetate buffer pH 4.7 (the ionic strength of which had been adjusted to 1 M by the addition of NaCl), followed by immediate centrifugation at 15,000 × *g* for 10 min to pellet insoluble matter. The resulting soluble proteins in both preparations were then concentrated separately using an Amicon (Millipore, Watford, UK) stirred cell fitted with a 3 kDa cut-off membrane and were stored in 50% (v/v) glycerol at −20 °C. For N-terminal analysis, proteins were resolved by SDS-PAGE, blotted onto polyvinylidene difluoride membrane and N-terminal sequence was determined by automated Edman degradation (performed by Alta Bio-

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PcProplasmepsin      ~K E S F K L L K S G L L K↓R↓E H L V K~
PbProplasmepsin      ~K E S F K L L K S G L L K K E H L T K~
PfProplasmepsin-I    ~K E S L K F F K T G L T Q K P H L G N~
PfProplasmepsin-II   ~K E S V N F↓L N S G L T K T N Y L G S~
PfProplasmepsin-IV   ~K E S F K F↓F↓K S G Y A Q K G Y L G S~
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Fig. 1. Comparison of the Propart sequences of PcPM, PbPM and PfPMI, II and IV. Underlined is the residue that was substituted within the PcPM sequence (Lys → Ala) to generate a form of PcPM zymogen that autoactivated to produce a pseudo-mature form of PcPM. ↓ represents autoprocessing sites in the proplasmepsin sequences.

science, Birmingham, UK). Recombinant *P. falciparum* plasmepsin II was prepared according to Hill et al. (1994) and *P. falciparum* plasmepsin IV was produced according to Wyatt and Berry (2002). The hydrolysis of chromogenic peptide substrates at pH 4.7 was monitored spectrophotometrically as described previously (Moon et al., 1997; Tyas et al., 1999). The derived kinetic constants K_m and k_{cat} always had estimated errors of <15%. The concentration of active enzyme used in each assay was determined by active site titration with isovaleryl pepstatin as described previously (Jupp et al., 1990). K_i values for inhibitor interactions with recombinant plasmepsins were determined at pH 4.7 as described previously (Jupp et al., 1990) using the chromogenic substrate Lys-Pro-Ile-Glu-Phe*Nph-Arg-Leu (where * is the scissile peptide bond and Nph is *para*-nitrophenylalanine). Stock milk solution for milk clotting assays was prepared by dissolving 3 g dried, skimmed milk powder in 25 ml of water. An aliquot (1 ml) of this was added to a volume (49 ml) of 180 mM sodium acetate pH 5.3, 8.5 mM $CaCl_2$ and the solution allowed to stand for 4 h at 25 °C. The milk clotting activity of recombinant protein preparations was determined by monitoring the increase in absorbance at 500 nm, of a 200 μl portion of milk solution following the addition of a small quantity of enzyme.

3. Results

The gene encoding an FV proplasmepsin (proPcPM) from *P. chabaudi* has previously been reported (Martins et al., 2003). A fragment encoding a semi-proform of this enzyme, which lacked the first 75 amino acids of the predicted propart sequence was amplified from *P. chabaudi* genomic DNA by the polymerase chain reaction, cloned and its nucleotide sequence was confirmed. The DNA encoding this truncated enzyme was expressed in *E. coli* according to Hill et al. (1994) and the resultant recombinant semi-proplasmepsin protein was recovered from the insoluble portion, where upon it was solubilised and refolded by dialysis.

Recombinant forms of semi-proplasmepsin II and IV when incubated at acidic pH values undergo a process of autoactivation, liberating a pseudo-mature form of the aspartic proteinase (Fig. 1). The wild type semi-proPcPM however, resolutely failed to undergo this conversion process even when incubated for extended time periods at pH 4.7 (greater than 72 h at 37 °C—data not shown). Therefore, a clone encoding a semi-proform of proPcPM that was predicted to autoactivate, was generated by site directed mutagenesis. This clone encoded a lysine to alanine substitution at residue 110 (proPcPM numbering) and was used to produce recombinant precursor proplasmepsin in an identical fashion to the wildtype zymogen. When incubated at acid pH values, this mutant semi-pro form of PcPM underwent an auto-conversion process to yield a pseudo-mature length aspartic proteinase in a reaction that could be inhibited by the general aspartic proteinase inhibitor isovaleryl pepstatin (not shown). N-terminal sequencing showed this "pseudo-mature" form of PcPM to be composed of two populations beginning K-R-E-H-L-V∼ and R-E-H-L-V-K∼ (Fig. 1). These N-termini corresponded to the "pseudo-mature" forms of PcPM beginning at residues 118 and 119 of the proPcPM sequence (Martins et al., 2003) (Fig. 1). This protein preparation was then used in all subsequent kinetic experiments.

The ability of the mature form of PcPM to cleave a variety of peptide substrates was examined (Table 1). PcPM consistently displayed significantly higher specificity constants (k_{cat}/K_m) for the cleavage of these substrates compared to the *P. falciparum* FV aspartic proteinases, indicating the greater ability of PcPM to cleave these substrates. The higher specificity constants were generally as a result of increased substrate turnover (values for k_{cat}), compared to the FV aspartic proteinases from *P. falciparum*, as the affinities (K_m values) for the interaction of substrates with PcPM were broadly similar to those obtained for the *P. falciparum* enzymes.

The capacity of selected FV plasmepsins from *P. falciparum* and PcPM to clot milk was analysed. Milk

Table 1
Kinetic parameters for the hydrolysis of chromogenic peptide substrates

Peptide sequence	PcPM			PfPMI			PfPMII			PfPMIV		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
KEFAF*ZALK	30	25	830	45[a]	3[a]	70[a]	40[a]	17[a]	430[a]	35[b]	13[b]	370[b]
KEFNF*ZALK	30	190	6300	20[a]	2[a]	100[c]	25[c]	7[c]	280[c]	20[b]	20[b]	1000[b]
KERVF*ZALK	45	80	1800	20[a]	0.3[a]	20[a]	30[a]	7[a]	230[a]	11[b]	6[b]	140[b]
KPIEF*ZRL	20	190	9500	–	<0.01[a]	–	20[a]	1[a]	50[a]	36[b]	5[b]	140[b]

Values were measured at 37 °C, pH 4.7, ionic strength = 0.1 M. In the peptide sequences, asterisk (*) represents the cleavage point.
[a] Values for recombinant plasmepsins I and II are taken from Tyas (1997).
[b] Values for recombinant plasmepsins I and II are taken from Wyatt and Berry (2002).
[c] Values for recombinant plasmepsins I and II are taken from Tyas et al. (1999).

clotting assays performed with a solution of skimmed milk in sodium acetate buffer (pH 5.3) readily distinguished between the activities of PfPMII and PfPMIV, as it emerged that PfPMII was incapable of clotting milk, whereas PfPMIV rapidly clotted milk (assessed by an increase of absorbance at 500 nm). PcPM also rapidly clotted milk under the assay conditions, in a pepstatin inhibitable fashion.

The interaction of PcPM with inhibitors of aspartic proteinases was also analysed. Firstly, the effects on the activity of PcPM of three HIV proteinase inhibitors that are currently licensed for the treatment of HIV were investigated. Of these three inhibitors, a preparation containing lopinavir and ritonavir in a 1:4.6 molar ratio (sold as Kaletra®, Abbott Laboratories) was the most potent inhibitor of PcPM and displayed a K_i-value of 30 nM. Ritonavir alone (Norvir®, Abbott Laboratories) was a less potent inhibitor of PcPM (K_i-value of 190 nm) and another HIV proteinase inhibitor saquinavir (Fortovase®, Roche) inhibited PcPM with a K_i-value of 80 nm (Table 2).

As is typical of aspartic proteinases, PcPM was potently inhibited by a series of pepstatin inhibitors (Table 2). Isovaleryl pepstatin was the most effective inhibitor of this series, offering more potent inhibition than the second best of these inhibitors, acetyl pepstatin. The third inhibitor in this series, lactoyl pepstatin was a poorer inhibitor (K_i-value of 300 nm) of PcPM than of PfPMII (K_i-value of 100 nm) and of PfPMIV (K_i-value of 25 nm). PcPM was also assayed with a range of chemically synthesised inhibitors that have previously been used to distinguish partially between the activities of certain *P. falciparum* FV plasmepsins (Wyatt and Berry, 2002). This same set of inhibitors can also be used to display the differences between PcPM and FV plasmepins from *P. falciparum* as most strikingly, the inhibitor Ro40-4388, a potent inhibitor of PfPMI and PfPMIV offered no inhibition towards PcPM even at 4 μM. The inhibitors Ro42-1118 and Ro40-5576 offered comparable potency towards PcPM compared to PfPMI, II and IV.

4. Discussion

P. chabaudi, like *P. berghei* and the human parasites *P. vivax*, *P. malariae* and *P. ovale* encodes 7 plasmepsins, only one of which is found the parasite's food vacuole, whereas *P. falciparum* encodes 10 plasmepsins of which four are found in the food vacuole (Coombs et al., 2001; Banerjee et al., 2002; Martins et al., 2003; Dame et al., 2003). Studies on rodent parasite plasmepsins may, therefore, provide useful insights into the characteristics of non-falciparum human malarial aspartic proteinases.

Table 2
Inhibition of PcPM

Inhibitor	PcPM, K_i (nM)	PfPMI, K_i (nM)	PfPMII, K_i (nM)	PfPMIV, K_i (nM)
Kaletra®	30	nd	nd	nd
Saquinavir	190	nd	nd	nd
Ritonavir	80	nd	nd	nd
Ac-Val-Val-Sta-Ala-Sta	2	nd	0.6	0.2[a]
Iva-Val-Val-Sta-Ala-Sta	0.1	0.7[b]	<0.1	0.1[a]
Lac-Val-Sta-Ala-Sta	300	nd	100	25[a]
Ro40-4388	NI	4[b]	700[b]	10[a]
Ro40-5576	40	6[b]	250[b]	15[a]
Ro42-1118	55	300[c]	4000[b]	50[a]

Values were measured at pH 4.7 as described in the legend of Table 1. nd: not determined, NI: no inhibition at 4 μM. Ac-Val-Val-Sta-Ala-Sta: acetyl pepstatin, Iva-Val-Val-Sta-Ala-Sta: isovaleryl pepstatin, Lac-Val-Sta-Ala-Sta: lactoyl pepstatin.

[a] Values for recombinant plasmepsins I, II and IV with synthetic inhibitors are derived from Wyatt and Berry (2002).

[b] Values for recombinant plasmepsins I, II and IV with synthetic inhibitors are derived from Tyas et al. (1999).

[c] Values for recombinant plasmepsins I, II and IV with synthetic inhibitors are derived from Tyas (1997).

In *P. falciparum* the FV PM zymogens are processed by a proteolytic enzyme, suggested to be an atypical calpain-like enzyme, which generates mature, fully active proteinases (Banerjee et al., 2003). A recombinant form of the PcPM zymogen, in a similar fashion to its counterpart from *P. berghei* failed to autoactivate in vitro to generate the mature forms of these enzymes (Humphreys et al., 1999), perhaps implying the existence of such a processing enzyme within the rodent parasites *P. chabaudi* and *P. berghei*. A pseudo-mature form of PcPM could be generated by autoprocessing after a lysine to alanine substitution was introduced within the propart of PcPM (Fig. 1). This produced a form of semi-proplasmepsin that rapidly converted to an enzyme of mature size (as judged by migration on SDS-PAGE) upon incubation at acidic pH values. This "mature-sized" protein preparation was found to contain two N-terminal sequences, corresponding to proteins that appeared to have undergone an additional processing event, in addition to that at the engineered activation site (Fig. 1). Thus it is likely that the autoprocessing of this mutant semi-proPcPM has occurred by a two step process, initially by cleavage after residue 109 within the sequence ∼K-L-L*A-S∼ (where * represents the engineered cleavage junction), then potentially completed by secondary cleavage events after residues 118 and 119, respectively (ProPcPM numbering) within the sequence ∼L-L-K*R*E-H-L∼ (where * represents the cleavage junction). A likely explanation for the failure of wild-type PcPM to autoactivate is that the predicted activation sequence places a Lys residue in the S1′ subsite of PcPM (subsite numbering according to Schechter and Berger (1967)) (Fig. 1), where it is seemingly not well tolerated. The mutation of this residue to Ala, removes this unfavourable interaction and, therefore, the propeptide is rapidly liberated from the zymogen upon incubation at acidic pH values.

PcPM cleaved a variety of peptide substrates with differing kinetics to those observed for the cleavage of the same substrate series by PfPMI, II and IV (Tyas, 1997; Tyas et al., 1999; Wyatt and Berry, 2002). In general, PcPM cleaved these substrates with much higher specificity constants than the *P. falciparum* enzymes. This may be a reflection of the existence of a solitary FV plasmepsin in *P. chabaudi* with a broader substrate specificity, as opposed to the more discriminating activities of the *P. falciparum* FV aspartic proteinases. In addition, the proteolytic activities of PcPM and PfPMIV can be readily distinguished from the activity of PfPMII by the use of a simple milk clotting assay. In this assay, PcPM and PfPMIV rapidly clotted milk in a pepstatin inhibitable fashion, whereas PfPMII was incapable of initiating clotting, despite lengthy incubation times. To our knowledge, this is the first simple and cost effective assay that has been described that allows the distinction between the activities of PfPMII and PfPMIV and, therefore, may have application in the analysis of plasmepsin containing FV extracts from *P. falciparum*. The ability of PcPM to clot milk may be an additional indicator of the potential broader substrate specificity of this plasmepsin.

The effectiveness of a series of statine containing inhibitors to inactivate PcPM was investigated. Like all typical non-retroviral aspartic proteinases, PcPM was potently inhibited by iso-valeryl pepstatin and also by acetyl pepstatin. Interestingly, the shorter lactoyl pepstatin, which has a more hydrophobic lactoyl moiety at the P3 position (substituent numbering according to Schechter and Berger (1967)) compared to the valine

that is present in this position in the other pepstatins tested was a poorer inhibitor of PcPM than of PfPMII and PfPMIV, being approximately 3-fold worse against PfPMII and over 10-fold less effective against PfPMIV. The other series of inhibitors tested against PcPM were of a chemically synthesised nature, of these compounds, Ro40-5576 and Ro42-118 inhibited PcPM with potencies similar to those previously reported with PfPMIV (Wyatt and Berry, 2002), although these inhibitors were significantly more potent against PfPMI (approximately 6-fold) than they are against PcPM. These same two inhibitors were significantly more effective against PcPM than against PfPMII, displaying respective K_i values approximately 10 and 20 times lower. However, of these inhibitors, the most striking difference between PcPM and the PfPMs is with Ro40-4388. This compound is a potent inhibitor of PfPMI (K_i-value of 4 nM) and is a good inhibitor of PfPMIV (K_i-value of 10 nM) and whilst it is less effective against PfPMII, exhibiting a K_i-value of 700 nm (Tyas et al., 1999; Wyatt and Berry, 2002). This compound however, did not offer any significant inhibition against PcPM at a concentration of 4 μM. It is clear, therefore, that despite the similarities that exist between PcPM and the equivalent enzymes in *P. falciparum*, PcPM has distinct active site characteristics that perhaps demonstrate its unique role within the parasite's FV.

Previous studies have indicated that HIV proteinase inhibitors have anti-malarial activity in assays performed on cultured *P. falciparum* parasites (Parikh et al., 2005; Skinner-Adams et al., 2004) and more significantly, reduce parasite load in *P. chabaudi* infected mice (Savarino et al., 2005; Skinner-Adams et al., 2005). It has been hypothesised that HIV inhibitors may reduce parasite numbers in *P. chabaudi* infected mice by inhibiting parasite plasmepsins (Skinner-Adams et al., 2005). Here we have demonstrated that three commonly prescribed HIV drugs are capable of inhibiting the food vacuole plasmepsin from *P. chabaudi* at concentrations readily achievable under normal dose conditions. Therefore, it is possible that this enzyme may in fact be a potential target of these drugs within the in vivo murine model.

Here we have described the proteolytic activity of PcPM and shown that its features are typical of a malarial FV aspartic proteinase. The presence of one predicted FV plasmepsin in most *Plasmodia*, compared to the four enzymes present in *P. falciparum* has previously raised the question of whether redundancy exists within the *P. falciparum* FV and recent studies have shown that no single FV plasmepsin is a requirement for the parasite (Omara-Opyene et al., 2004; Liu et al., 2005). Our studies have shown that PcPM has distinct characteristics that mean that this single plasmepsin may potentially fulfil the role of the four equivalent enzymes of *P. falciparum* and, therefore, remains an interesting aspartic proteinase for study since such single FV plasmepsins may represent better drug targets than the more functionally redundant enzymes of *P. falciparum*.

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