Fingerprinting the Substrate Specificity of M1 and M17 Aminopeptidases of Human Malaria, Plasmodium falciparum

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Fingerprinting the Substrate Specificity of M1 and M17 Aminopeptidases of Human Malaria, *Plasmodium falciparum*

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Abstract

**Background:** *Plasmodium falciparum*, the causative agent of human malaria, expresses two aminopeptidases, PFM1AAP and PFM17LAP, critical to generating a free amino acid pool used by the intraerythrocytic stage of the parasite for proteins synthesis, growth and development. These exopeptidases are potential targets for the development of a new class of anti-malaria drugs.

**Methodology/Principal Findings:** To define the substrate specificity of recombinant forms of these two malaria aminopeptidases we used a new library consisting of 61 fluorogenic substrates derived both from natural and unnatural amino acids. We obtained a detailed substrate fingerprint for recombinant forms of the enzymes revealing that PFM1AAP exhibits a very broad substrate tolerance, capable of efficiently hydrolyzing neutral and basic amino acids, while PFM17LAP has narrower substrate specificity and preferentially cleaves bulky, hydrophobic amino acids. The substrate library was also exploited to profile the activity of the native aminopeptidases in soluble cell lysates of *P. falciparum* malaria.

**Conclusions/Significance:** This data showed that PFM1AAP and PFM17LAP are responsible for majority of the aminopeptidase activity in these extracts. These studies provide specific substrate and mechanistic information important for understanding the function of these aminopeptidases and could be exploited in the design of new inhibitors to specifically target these for anti-malaria treatment.

Introduction

Malaria is one of the deadliest infectious diseases of humans in the world. It is endemic in tropical and subtropical regions, with about 500 million cases of malaria infections and 1.4–2.6 million deaths each year [1]. Four *Plasmodium* species commonly infect humans (*P. vivax*, *P. malariae*, *P. falciparum* and *P. ovale*) [2,3]. Among them *P. falciparum* is of special interest because it is the most lethal and responsible for most deaths, particularly in pregnant women and children under the age of five. Drugs such as chloroquine and mefloquine have played major roles in the treatment of malaria in the past. However, the spread of drug resistant parasites has meant that treatment has become increasingly reliant on artemisinin-based combination therapies (ACTs) [4,5]. Accordingly, there is a pressing need to develop new anti-malarial drugs targeting biochemical pathways critical for parasite survival and/or transmission.

Malarial parasites digest the infected host’s hemoglobin to obtain free amino acids [6]. These amino acids are used to maintain osmotic pressure within infected red blood cells, for protein synthesis during parasite development and reproduction, and to set-up a concentration gradient by which rare or absent amino acids are transported into infected red blood cell from host serum [7,8]. Two metallo-aminopeptidases M1 alanyl aminopeptidase (PFM1AAP) and M17 leucine aminopeptidase (PFM17LAP) expressed by *P. falciparum* may be responsible for the terminal steps of hemoglobin digestion [9,10,11]. It is proposed that these enzymes hydrolyze small peptides generated by the endoproteolytic digestion of hemoglobin within the parasite’s digestive...
vacuole to generate a pool of free amino acids. Prevention of 
PfM1AAP and PfM17LAP activity by aminopeptidase-specific 
inhibitors, such as bestatin, block development of malaria parasites 
{	extit{in vitro}} and {	extit{in vivo}}, suggesting that these enzymes are attractive 
targets for the development of a new class of anti-malaria drugs 
[12,13,14]. Recently, Valmourougane et al. [15] used the bestatin 
scaffold to develop several derivatives and employed these to 
explore the active sites of the two malaria enzymes. Subsequently, 
Harbut et al. [16] synthesised additional bestatin-based inhibitors 
that exhibited specificity for PfM1AAP and PfM17LAP enzymes 
and showed that these can block malaria growth in culture, thus 
indicating that both enzymes represent targets for anti-malaria 
drug design.

Sequence alignment of malaria PfM1AAP and PfM17LAP 
aminopeptidases with mammalian orthologs reveals significant 
differences in their overall primary structure and in residues that 
influence substrate binding (Figure 1). In particular, these data 
suggest that the S1 pockets of the malaria enzymes, which accepts 
the N-terminal P1 amino acids of a peptide substrate, has different 
topology in these enzyme orthologs, which could influence the 
binding and catalytic turnover of different classes of amino acids.

Differences between the N-terminal amino acid preferences of 
malaria and mammalian enzymes could be exploited in the design 
of inhibitors that could kill malaria parasites without inhibition of 
their mammalian homologs.

In the present paper, we have examined and compared the 
detailed substrate specificities of functionally-active recombinant 
forms of PfM1AAP and PfM17LAP. To obtain substrate 
fingerprints for each enzyme we employed our recently developed 
substrate library consisting of natural and unnatural amino acids 
attached to an ACC fluorophore [17]. The information gained 
from this library provided extensive activity profiling of functional 
recombinant forms of PfM1AAP and PfM17LAP. We also profiled 
the general aminopeptidase activity in soluble cell lysates derived 
from the 3D7 clone of P. falciparum malaria. Our data show major 
differences in the substrate specificity between the two malaria 
enzymes that are related to the structure/shape of their active site. 
Significant difference was observed between these and the human 
aminopeptidase homologs for which we previously determined a 
substrate specificity profile [17]. Furthermore, we show that 
PfM1AAP and PfM17LAP represent the major aminopeptidase 
activity in soluble malaria extracts. This information will be

\[\text{Figure 1. Multiple sequence alignments of PfM1AAP and PfM17LAP with mammalian orthologs, H: human Homo sapiens, P: pig, Sus} \]
\[\text{scrofa, R: rat, Rattus norvegicus and X: Plasmodium falciparum. Dashes represent gaps that optimize sequence adjustment. Small or} \]
\[\text{hydrophobic amino acids are colored in magenta, acidic are red, basic are blue and amino acids with an amine or hydroxyl group are green. Conserved amino acids} \]
\[\text{are highlighted in gray. Amino acids from active site residues are presented on a black background and those participating in metal binding are} \]
\[\text{outlined. doi:10.1371/journal.pone.0031938.g001}\]
important for the future characterization of the malaria aminopeptidases and elucidation of their function in malaria, and in the design of specific inhibitory compounds for anti-malarial treatment.

**Methods**

**Materials**

All chemicals and solvents were obtained from commercial suppliers and used without further purification, unless otherwise stated. Analytical high performance liquid chromatography (HPLC) analysis used a Beckman-Coulter System Gold 125 solvent delivery module equipped with a Beckman-Coulter System Gold 166 Detector system by using a Varian Microsorb-MV C18 (250×4.8 mm) column. Preparative HPLC analysis used a Beckman-Coulter System Gold 125 P solvent delivery module equipped with a Beckman-Coulter System Gold 168 Detector system with a Kromasil 100-10 C18 (20 mm ID) column (Richard Scientific). Solvent composition system A (water/0.1%TFA) and system B (acetonitrile/water 80%/20% with 0.1% of TFA). LC-MS data were recorded with the aid of the Burnham Medicinal Chemistry facility using Shimadzu LCMS-2010EV system. The solid phase substrate library was synthesized using a semiautomated FlexChem Peptide Synthesis System (Model 202). Enzymatic kinetic studies were performed using Spectra MAX Gemini EM fluorimeter (Molecular Devices) operating in the kinetic mode in 96-well plates.

**Preparation of malaria cell extracts and recombinant PfM1AAP and PfM17LAP**

The intra-erythrocytic stages of 3D7 P. falciparum parasites were cultured in RPMI containing 10% human serum [18]. Parasites were lysed from erythrocytes using 0.03% saponin [19] and extracted by three rounds of freeze-thaw in phosphate-buffered saline, pH 7.3, prepared as described previously [20]. The production of recombinant malaria aminopeptidases PfM1AAP and PfM17LAP in Escherichia coli and their isolation by Ni-chelate affinity chromatography has been described elsewhere [21,22].

**Substrate library screening**

To first compare the substrate specificity of the two malarial aminopeptidases (PfM1AAP and PfM17LAP) an initial screening of 19 natural amino acids was performed. PfM17LAP was assayed in 50 mM Tris-HCl, pH 8.0, and was activated with Co2+ ions at a final concentration of 1.0 mM. PfM1AAP was assayed in 50 mM Tris-HCl, pH 7.5, without additional metal ions. These two buffers were made at 25°C and assays were carried out at 37°C. Before adding to the substrate, all enzymes were preincubated at 37°C for 30 minutes (the enzyme concentration in these additional screens was between 0.06–0.10 μM). These screens were carried out using substrate concentrations of 50 μM and 2.5 μM for PfM1AAP and PfM17LAP, respectively.

A final screening of the 61-membered library of the natural and unnatural amino acids was then performed: for PfM1AAP screens the library concentration was 2.5 μM and for PfM17LAP screens 300 nM (total enzyme concentrations in these assays were 0.03–0.10 μM). The fluorescence signal was monitored continuously and the wavelength values were following, excitation at 355 nm and emission at 460 nm. The total time of each assay was between 15–45 minutes. From each single experiment only the linear portion of progress curve was used to calculate final substrate specificity represented by RFU/s (production of Relative Fluorescence Unit per second) value. Experiments using the entire 61-membered library were repeated three times and for natural amino acids sublibrary were repeated twice. The average value with standard deviations for each substrate were compared with the best cleaved substrate (100% of specificity) and all data are presented on plot where x axis represents given fluorogenic substrate and y axis represents the specificity signified as percent participation in velocity of the most specific substrate.

**Determination of kinetic parameters for best cleaved substrates (Km, kcat, kcat/Km)**

The kinetic parameters of the best substrates were determined using the above assay conditions. However, before adding to the substrate, all enzymes were preincubated at 37°C for 30 minutes. The ACC concentration was calculated by total digestion assay for each enzyme separately. In each measurement 6 independent substrates with known concentration were chosen and the average value was calculated. To measure Km value eight different concentrations of given substrates and constant enzyme concentration were used. Reaction volume was at 100 μl and enzyme concentrations were 0.0315 μM and 0.300 μM for PfM1AAP and PfM17LAP, respectively. To measure kcat/Km value six different concentrations of given substrates and constant enzyme concentration were used. All experimental conditions were as above. The fluorescence signal was monitored continuously and the wavelength value was the following, excitation at 355 nm and emission at 460 nm. The total time of each assay was between 15–30 minutes. All experiments were repeated at least three times and the average value with standard deviation was calculated. Concentration of DMSO in each experiment was less than 2% (v/v).

**Results**

**Design of the substrate library**

To determine substrate specificity of the enzyme-substrate complex in the S1 pocket of malaria aminopeptidases, we utilized a substrate-profiling approach in which a fluorogenic substrate library containing 61 amino acids was synthesized and used to profile three mammalian orthologs of the M1 aminopeptidase N [17]. This library was designed to screen substrate preferences for 19 natural amino acids (to avoid oxidation artifacts we omitted cysteine) and 42 unnatural amino acids representing a broad spectrum of side chain substitutions (Figure S1). Most of the compounds in the library contain an unblocked ε-amino group to satisfy the primary specificity of aminopeptidases.

Additionally, we also synthesized several substrates with diverse functionalities (for example, a secondary amine derivatives, an ε-hydroxy group, or an amine group in other than the ε position) linked to a fluorophore leaving group (in the P1’ position) to determine how this would influence substrate recognition by malaria aminopeptidases. It was anticipated that this approach would provide additional information that could be used to identify good substrates, design of inhibitors, as well as comparison of different aminopeptidases. In our present investigation of substrate specificity, we used functionally-active recombinant forms of the two P. falciparum aminopeptidases - PfM1AAP and PfM17LAP (Figure 1) [9,12]. As the fluorophore leaving group we employed 7-amino-4-carbamoylmethylcoumarin (ACC) because of its convenience in solid phase synthesis [23].

**Recombinant enzyme substrate-specificity results**

An initial library screen for each malaria aminopeptidase was performed to establish optimal screening conditions. For each enzyme, the best cleaved substrates were chosen and their kinetic parameters (kcat, kcat/Km) measured. After measurement of Km we performed a second screen in which the concentration of...
each substrate was maintained well below the lowest $K_m$ value. This procedure ensures that substrate cleavage (measured as a fluorescence signal) is proportional only to $k_{cat}/K_m$ and is not correlated with individual values of $K_m$ or $k_{cat}$. An equal concentration of the given substrates in each well was obtained by placing calculated amounts of substrate in the well and then mixing with enzyme to a total volume of 100 µL. Final substrate concentrations for the enzymes were as follows: 10 µM for PfM1AAP (lowest $K_m = 60.8$ µM) and 0.3 µM for PfM17LAP (lowest $K_m = 0.35$ µM). It is important to note that the most challenging library screening was with the PfM17LAP. To obtain satisfactory fluorescence signals and avoid depletion of substrate at high enzyme concentration we performed the screen at 0.3 µM, only slightly below the $K_m$ value of the best-cleaved substrate – Igl.

To gain a better insight into substrate specificity of the enzymes toward natural amino acids, we performed an additional screen at higher substrate concentration. This did not affect the observed data because the $K_m$ values recorded for these substrates were also higher, which guaranteed a proportional correlation between fluorescence signal and $k_{cat}/K_m$ at 50 µM for PfM1AAP (lowest $K_m = 138.2$ µM) and 2.5 µM for PfM17LAP (lowest $K_m = 3.44$ µM).

**PfM1AAP aminopeptidase**

The natural amino acids preferred by PfM1AAP are leucine and methionine (Figure 2). Alanine and arginine are also readily cleaved by this enzyme, but with a slightly lower affinity. Other amino acids susceptible to hydrolysis by PfM1AAP aminopeptidase include Lys, Phe, Tyr, Trp, Gln, Ser and Gly. Negligible activity was observed for Glu, Asp, Pro, Ile, Thr, Val, His and Asn.

Analysis of the whole library revealed that PfM1AAP exhibits very broad substrate specificity with this aminopeptidase capable of cleaving a range of substituents particularly the bulky, hydrophobic amino acids (Figure 3). The most preferred substrates were hCha, hPhe and Nle, all of which were cleaved more efficiently than the best natural amino acid, methionine. A second series of unnatural amino acids were also hydrolyzed by PfM1AAP at about 50% of the activity seen for methionine. These were hArg, Cha, Nva, 4-Cl-Phe, 2-Nal, Igl, hLeu or styryl-Ala.

**PfM17LAP aminopeptidase**

PfM17LAP exhibited strikingly narrow substrate specificity toward natural amino acids, particularly in comparison to PfM1AAP (Figure 2). The most readily accepted substrates are the hydrophobic amino acids Leu and Trp. Other amino acids

![Figure 2](image-url)
susceptible to hydrolysis, albeit at a very much lower level included Phe, Met, Thr and Tyr. Cleavage of Ile and His were slightly above background.

Analysis of the complete substrate library revealed a highly restricted specificity of PfM17LAP for hydrophobic substrates (Figure 3). The amino acid derivatives most efficiently cleaved by PfM17LAP were hPhe and hCha. These two substrates are cleaved approximately three and five times better than the best-cleaved natural amino acids Trp and Leu, respectively. Other amino acid derivatives that are cleaved by PfM17LAP include Igl and Nle, and less so Nva and hLeu.

To study the distinct substrate differences between PfM1AAP and PfM17LAP in more detail we determined their kinetic parameters ($K_m$, $k_{cat}$, $k_{cat}/K_m$) against a panel of selected natural and unnatural substrates (Table 1). These studies showed that the substrates Arg, Ala, HArg, 2-Nal, 3-NO2-Phe and styryl-Ala were exclusively cleaved by PfM1AAP. By contrast, we did not observe cleavage of any substrate by PfM17LAP that was not cleaved by PfM1AAP. For those substrates that both enzymes cleave, the efficiency or turnover rate ($k_{cat}/K_m$) was always far higher with PfM1AAP in comparison to PfM17LAP, even for those substrates most preferred by PfM17LAP (e.g. Leu, hLeu, Phe, hPhe, hCha). However, the substrate binding affinities, as assessed by $K_m$, for PfM17LAP were between one or two orders of magnitude lower as compared to PfM1AAP. These data indicate that the two enzymes function in milieu of different substrate concentration; PfM1AAP works more effectively at relatively high substrate concentration while PfM17LAP functions more efficiently at much lower substrate concentrations.

**Malaria cell lysate substrate specificity results**

To understand the nature of the aminopeptidase activity expressed by malaria parasites we screened our substrate library with a soluble malaria cell extract derived from the 3D7 clone of *P. falciparum*. We employed the substrate library at an arbitrary final concentration of 5 μM. This concentration was determined in a preliminary screening test to be sufficient to obtain a good and linear fluorescence signal (data not shown). No activity was observed against fluorogenic peptides substrates when lysates of uninfected erythrocytes were prepared in a similar manner to the parasite-infected erythrocytes as reported previously [9].

Our data demonstrate that several substrates are efficiently cleaved by aminopeptidases in the cell lysate (Figure 4). Interestingly, the substrate profile closely represents a combination of activity of both PfM1AAP and PfM17LAP aminopeptidases. The most readily cleaved substrates (e.g. Arg, Ala, Leu, Met, hCha) show a close overlap with those cleaved by either recombinant enzymes. To validate this observation we performed a library screen in which the soluble cell lysate was preincubated for 30 minutes with 50 μM hPhe-PO$_3$H$_2$, which we have previously shown is a potent inhibitor of both PfM1AAP and PfM17LAP [14,24]. No activity was observed toward any substrate in the...
Table 1. Kinetic parameters for selected substrates.

<table>
<thead>
<tr>
<th></th>
<th>P/M1AAP</th>
<th>P/M17LAP</th>
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<tbody>
<tr>
<td></td>
<td>$K_{m}$ (μM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>Ala</td>
<td>240.6 ± 10.9</td>
<td>1.054 ± 0.154</td>
</tr>
<tr>
<td>Arg</td>
<td>214.3 ± 18.5</td>
<td>0.876 ± 0.105</td>
</tr>
<tr>
<td>hArg</td>
<td>124.3 ± 15.6</td>
<td>1.144 ± 0.100</td>
</tr>
<tr>
<td>Leu</td>
<td>140.9 ± 12.1</td>
<td>0.868 ± 0.111</td>
</tr>
<tr>
<td>hLeu</td>
<td>210.8 ± 26.6</td>
<td>1.859 ± 0.300</td>
</tr>
<tr>
<td>Phe</td>
<td>218.0 ± 6.2</td>
<td>0.422 ± 0.028</td>
</tr>
<tr>
<td>hPhe</td>
<td>60.8 ± 3.1</td>
<td>0.978 ± 0.003</td>
</tr>
<tr>
<td>Met</td>
<td>138.2 ± 7.9</td>
<td>0.887 ± 0.077</td>
</tr>
<tr>
<td>Trp</td>
<td>144.4 ± 18.7</td>
<td>0.444 ± 0.052</td>
</tr>
<tr>
<td>Cha</td>
<td>269.8 ± 30.8</td>
<td>1.358 ± 0.140</td>
</tr>
<tr>
<td>hCha</td>
<td>96.3 ± 16.2</td>
<td>1.592 ± 0.230</td>
</tr>
<tr>
<td>2-Nal</td>
<td>316.6 ± 24.9</td>
<td>0.884 ± 0.018</td>
</tr>
<tr>
<td>3-NO$_2$-Phe</td>
<td>131.7 ± 19.9</td>
<td>0.141 ± 0.026</td>
</tr>
<tr>
<td>Nva</td>
<td>267.1 ± 7.7</td>
<td>2.786 ± 0.228</td>
</tr>
<tr>
<td>allyl-Gly</td>
<td>266.7 ± 25.2</td>
<td>1.520 ± 0.301</td>
</tr>
<tr>
<td>IgI</td>
<td>84.7 ± 6.1</td>
<td>0.492 ± 0.032</td>
</tr>
<tr>
<td>styryl-Ala</td>
<td>192.7 ± 10.5</td>
<td>0.937 ± 0.154</td>
</tr>
<tr>
<td>Nle</td>
<td>110.1 ± 13.6</td>
<td>1.399 ± 0.119</td>
</tr>
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Comparison of the kinetic parameters ($K_{m}$, $k_{cat}$, $k_{cat}/K_{m}$) of the selected substrates for PfM1AAP and PfM17LAP. The results are presented as mean values with standard deviation.

doi:10.1371/journal.pone.0031938.t001

Discussion

Malaria is currently considered one of the most deadly infectious global diseases of humans, killing approximately 1 million people in sub-Saharan Africa alone each year. New approaches to overcome the spread of malaria parasites that have become resistant to currently available drugs are necessary, particularly the identification of novel drugs targeting metabolic pathways. The aminopeptidases PfM1AAP and PfM17LAP are critical to the growth and development of malaria parasites within the erythrocyte as knockout of either aminopeptidase gene is lethal to the parasite [11,12], and therefore they are both currently considered as promising targets for medicinal intervention [12]. The two enzymes are suggested to participate in the final step of hemoglobin digestion, the main source of nutrient for the parasite, resulting in the production of single amino acids, which are

Figure 4. Total aminopeptidase activity explored in the 3D7 clone of P. falciparum in the absence [A]. Aminopeptidase activity in soluble malaria parasite extracts was monitored using an fmax multi-well fluorescence plate reader (Molecular Devices) at excitation wavelength of 355 nm and an emission wavelength of 460 nm. The x-axis represents the abbreviated amino acid names (for full name and structure see Figure S1). The y-axis represents the average relative activity expressed as a percent of the best amino acid. Note, the addition of the aminopeptidase-specific inhibitor hPhe-PO$_3$H$_2$ (50 μM) to any of the above experiments completely abrogated cleavage of every substrate, thus confirming that the observed signal originates only from these enzymes.

doi:10.1371/journal.pone.0031938.g004
subsequently used for production of parasite proteins as they grow and develop with the host erythrocyte. As much as 70% of the erythrocyte hemoglobin is degraded suggesting that an efficient catabolic process is required [6]. However, the aminopeptidases may also function in the regular catabolic turnover of malaria proteins or biogenesis of intracellular organelles as the parasite undergoes recognized stage-specific developments [9,10,24].

Previous studies in the search for phosphonate or phosphinite compounds that inhibit both PfM1AAP and PfM17LAP resulted in the selection of several compounds that significantly reduced development of malaria parasites both in erythrocyte cell culture and in the murine P. c. chabaudi model of malaria [14,25]. However, since these compounds block the activity of both enzymes it remains to be determined whether killing is due to inhibition of one or both enzymes. Harbut et al. [16] recently used a bestatin scaffold to develop inhibitors that showed a 12–15 fold specificity for either PfM1AAP or PfM17LAP and demonstrated that these killed malaria parasites in vitro. The PfM1AAP-specific inhibitors caused swelling of the malaria digestive vacuole and disrupted proteolysis of haemoglobin-derived peptides while the PfM17LAP-specific inhibitors killed malaria parasite prior to the onset of haemoglobin digestion. These support the idea that the two enzymes play distinct roles in malaria parasites and that both can be targeted for anti-malaria drug development [12].

Recently, the high-resolution X-ray crystal structures of both PfM1AAP and PfM17LAP were determined and revealed large differences within the S1 pockets of their active sites [19,20]. Both molecules revealed extensively buried active sites centered around the essential active site cation(s). However the nature and size of the S1 pocket varied dramatically. The PfM1AAP S1 pocket is long and contains acidic residues deep in the pocket, thus forming an excellent platform for docking amino acids of basic character. Notably, a polar glutamic acid (Glu572) residue is located at the base of the pocket where it would be available to form an ionic interaction with the side chains of long and basic side chains. Comparison of bestatin-bound and unbound PfM1AAP structures also revealed flexibility of polar residues deep within the S1 pocket, thus possibly providing further adaptability to the shape of the S1 pocket. Valmourougue et al. [15] showed using bestatin-based inhibitors that the S1 pocket, residues 570–575, is flexible and can move to accommodate large side chains. Our library-screening results confirm that PfM1AAP aminopeptidase can cleave a large variety of amino acids with small or bulky amino acids side chains. One of the best cleaved are compounds with Arg and hArg, thus confirming that a mechanistic level the crystal structure data analysis and predictions. In contrast, the PfM17LAP S1 pocket that interacts with the substrate P1 residue is a small, narrow and substantially hydrophobic. Structural analysis suggested that only hydrophobic amino acids could be tolerated in this binding pocket. In the bestatin-bound structure, the P1 Phe-like moiety was tightly packed into the S1 pocket, forming stacking interactions with the hydrophobic pocket. Analysis of substrate library data for PfM17LAP confirms predictions from its crystal structure by showing that this enzyme efficiently cleaves amino acids with bulky and hydrophobic side chains, while the presence of any hydrophilic group leads to reduced binding. The size and hydrophobic nature of this narrow pocket explains the inability ofthis enzyme to cleave peptides/proteins after polar residues. Analysis of the PfM17LAP structure reveals no suitable polar hydrogen bonding partners at the base of the S1 pocket that could interact with a charged P1 side chain.

Substrates capable of differentiating between the two malaria aminopeptidases are Ala, Arg and hArg, a property that can be applied in the future for the specific monitoring activity of PfM1AAP in cell lysates as well as for design of specific inhibitors for this enzyme. On the other hand, both PfM1AAP and PfM17LAP preferentially recognize and cleave two unnatural amino acids – hPhe and hCha. Phosphonate derivatives of these substrates were reported previously as very good inhibitors of recombinant PfM17LAP and in malaria cell culture experiments, thus confirming that substrate specificity data can yield useful information for design of aminopeptidases inhibitors [14,25].

Our previous studies using a restricted number of natural amino acid derivatives of fluorogenic substrates indicated that the two aminopeptidases exhibit distinct but overlapping substrate specificities [9,19]. The availability of our library of 61 individual fluorogenic substrates in the form of natural and unnatural amino acids allowed us to perform comparative screens with the aminopeptidases, PfM1AAP and PfM17LAP. This has given us a more detailed understanding of the biochemistry of each PfM1AAP and PfM17LAP, which could enable the future design of specific substrates and inhibitors of each enzyme. The enzyme kinetic parameters presented in Table 1 show that the broad-acting PfM1AAP cleaves all substrates that are also cleaved by the more restrictive PfM17LAP. Moreover, PfM1AAP cleaves these substrates with a far greater efficiency, with k_{cat}/K_{M} values in the region of 40–100 fold higher. Additionally, the K_{M} values obtained for PfM17LAP are between one or two orders of magnitude lower than those of PfM1AAP. This further supports the suggestion that both enzymes may not function together in the same catabolic pathway and/or in the same cellular compartment. It is most probable that PfM1AAP functions in a cellular environment where its substrates are in high concentration. Immunolocatino studies [11,21,26,27] suggest that this could possibly be within or adjacent to the parasites digestive vacuole where the initial endo- and exo-proteolytic cleavages of host hemoglobin would generate high concentrations of peptide substrates. In contrast, PfM17LAP, which was localized to the cytoplasm of the malaria cell [9,12,21], could function where it substrates, peptides derived from hemoglobin or other proteins, are in lower concentration. Because of its strict specificity for leucine, we have previously suggested that a prime function of PfM17LAP could be in generating high intracellular concentrations of leucine that can be exchanged via specific channels for extracellular isoleucine [28], an essential amino acid not found in human hemoglobin [9,12].

One of the objectives of this study was to characterize the aminopeptidase activity of aminopeptidases in malaria extracts and compare this to the recombinant PfM1AAP and PfM17LAP. Our data clearly show that PfM1AAP and PfM17LAP are primarily responsible for the aminopeptidase activity in the soluble lysates of the 3D7 clone of P. falciparum. There are four types of methionine aminopeptidases (MetAP) expressed in malaria cells [29] and we expected that this activity would be particularly enhanced in the substrate profile of soluble cell lysates compared to the recombinant PfM1AAP and PfM17LAP. Interestingly, this was not the case and it is most probable that MetAP activities are present at a low level in the soluble lysates, although other possibilities include that these enzymes are membrane bound. Our data strongly suggests that both PfM1AAP and PfM17LAP are the predominant exo-aminopeptidases in the soluble lysates of the malaria parasites.

In conclusion, we have used a new library of fluorogenic substrates designed from natural and unnatural amino acids to define the distinct substrate specificity and kinetic parameters of two malaria aminopeptidases PfM1AAP and PfM17LAP, potential targets for new anti-malarials. Aminopeptidase fingerprint of PfM1AAP overlaps very well with previously published data for three mammalian (human, rat and pig) orthologs of this enzyme.

Fingerprinting the Malaria Aminopeptidases
suggested that no dramatic evolutionary changes occurred to this enzyme in term of substrate recognition preferences. This suggests that designing inhibitors that block the activity of the malaria enzyme without inhibiting the host enzyme will present a major challenge. However, our results show individual features of each enzyme with regard to substrate specificity and suggest that compounds that inhibit each enzyme specifically or together could be synthesized for combination therapies. This suggestion is supported by the recent results of Valmourougane et al. [15] and Harbut et al. [16] who designed PM1AAP- and PM17LaAP-specific inhibitors using the basic bestatin scaffold, although these did not show enhanced killing of parasite over bestatin itself. We have also shown that our library could be employed for activity profiling of cell extracts from different strains of malaria. Finally, we can analyze our formation for basis for future selection of specific substrates for this group of proteases as well as for the design of inhibitors, which could further help to answer questions about their relative importance in malaria development.

Supporting Information
Figure S1 Structures of natural and unnatural amino acids fluorogenic substrates used in the library. (DOC)

Author Contributions
Conceived and designed the experiments: MP JPD GSS MD. Performed the experiments: MP SM TSSA KRT JT MD. Analyzed the data: MP DLG JCW JPD GSS MD. Contributed reagents/materials/analysis tools: MP MD JPD G. Wrote the paper: MP JPD GSS MD.

References