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Overexpression of Leucyl Aminopeptidase in *Plasmodium falciparum* Parasites

*TARGET FOR THE ANTIMALARIAL ACTIVITY OF BESTATIN**

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Malaria aminopeptidases are important in the generation and regulation of free amino acids that are used in protein anabolism and for maintaining osmotic stability within the infected erythrocyte. The intraerythrocytic development of malaria parasites is blocked when the activity of aminopeptidases is specifically inhibited by reagents such as bestatin. One of the major aminopeptidases of malaria parasites is a leucyl aminopeptidase of the M17 family. We reasoned that, when this enzyme was the target of bestatin inhibition, its overexpression in malaria cells would lead to a reduced sensitivity to the inhibitor. To address this supposition, transgenic *Plasmodium falciparum* parasites overexpressing the leucyl aminopeptidase were generated by transfection with a plasmid that housed the full-length gene. Transgenic parasites expressed a 65-kDa protein close to the predicted molecule size of 67.831 kDa for the introduced leucyl aminopeptidase, and immunofluorescence studies localized the protein to the cytosol, the location of the native enzyme. The product of the transgene was shown to be functionally active with cytosolic extracts of transgenic parasites exhibiting twice the leucyl aminopeptidase activity compared with wild-type parasites. *In vitro* inhibitor sensitivity assays demonstrated that the transgenic parasites were more resistant to bestatin (EC_{50} 64 μM) compared with the parent parasites (EC_{50} 25 μM). Overexpression of genes in malaria parasites would have general application in the identification and validation of targets for antimalarial drugs.

During the development and asexual reproduction of malaria parasites within erythrocytes, as much as 65–75% of erythrocyte hemoglobin is internalized and digested (1, 2). There is still some conjecture as to the reason why the parasite needs to degrade such a large proportion of the erythrocyte hemoglobin, but several theories have been proposed (3, 4). It has been known for some time that malaria parasites require amino acids derived from host hemoglobin for protein synthesis and development (4), although recent studies suggest that the fraction of digested hemoglobin used for this purpose is surprisingly small (in the region of 16%) (5). Malaria parasites acquire additional amino acids from the extracellular medium and incorporate these into their proteins (5–7) and at the same time extrude unneeded amino acids through new permeability pathways that appear in the infected host cell membranes (8). It is now believed that the excess hemoglobin digestion is necessary to reduce the colloid-osmotic pressure within the infected erythrocyte and thus prevent premature cell lysis (9). Apart from contributing to the regulation of the internal osmotic pressure, the constant influx and efflux of amino acids may also ensure that there is a sufficient supply of each amino acid, especially those that are rare in hemoglobin (isoleucine, cysteine, glutamine, methionine, and glutamate), for protein synthesis (3, 7).

Hemoglobin is endocytosed into double membrane vesicles that bud off from the cytosome of the parasite and are transported to the acidic digestive vacuole (DV)[5] (10, 11). Within this specialized organelle, hemoglobin is digested by endopeptidases at a pH of ~5.5 in a semiordered fashion, initially at the hinge region by the aspartic peptidases plasmepsins I and II and then further by the aspartyl peptidases I, II, and IV, and the histoaspartic peptidase and three cysteine peptidases, falcipain-2, -2′, and -3 (1, 3, 10, 11). Peptides generated from this digestion are processed by a metalloprotease, falcilysin, into short oligopeptides of 5–10 amino acids and then by a dipeptidyl aminopeptidase I, which reduces these to dipeptides (12). However, free amino acids are not generated within the DV, as no exoaminopeptidase activity has been detected in isolated DV (13) and *in vitro* digestion of hemoglobin by extracts of DVs does not generate free amino acids (13, 14). Accordingly, we and others have suggested that peptides/dipeptides produced within the digestive vacuole are transported outside for conversion into individual amino acids (13–16).

Leucyl aminopeptidase-like activity has been detected in the soluble extracts of various *Plasmodium* species and functions in the release of hemoglobin-derived amino acids (13–21). The activity separates into the cytosolic fraction of the parasite, exhibits a pH optimum value of 7.2, and is inactive below pH 6.0, which is consistent with a function outside the DVs (13). *Plasmodium falciparum* aminopeptidase activity, partially purified by affinity chromatography employing the inhibitor bestatin as a ligand, cleaved synthetic peptides representative of the products of endopeptidase-degraded hemoglobin (13, 15) and exhibited a general preference for substrates containing leucine and alanine at the N terminus (amino acids that constitute 25% of the amino acid residues in human hemoglobin) (15). Aminopeptidase activity predominates in trophozoites, and the greatest *in vitro* growth inhibition by bestatin and nitrobestatin occurs when parasites are at the late ring and early trophozoite stage (13). This coincides with the period of increased metabolic activity within the infected erythrocyte, increased hemoglobin digestion, the induction of new permeability pathways, and rapid growth of the parasite (4, 8, 9).

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[5] The abbreviations used are: DV, digestive vacuole; NHMec, 7-amino-4-methyl-coumarin.

The leucyl aminopeptidase-like activity detected in soluble extracts of malaria parasites could be derived from two distinct aminopeptidases that display overlapping substrate specificities. The first is represented by a previously characterized 122-kDa zinc aminopeptidase of the M1 family, which may be membrane-bound but processed to two soluble forms of 96 and 68 kDa (21, 22). The second is the product of a gene described in the present study that encodes a 67.831-kDa leucyl aminopeptidase of the M17 family (13–15). We have overexpressed the M17 leucyl aminopeptidase gene in malaria parasites and shown that functional enzyme is expressed in the cytosol of transgenic parasites, the same compartment as the native enzyme (13, 15). More importantly, we have demonstrated that malaria parasites overexpressing M17 leucyl aminopeptidase are more resistant to killing by bestatin, an aminopeptidase inhibitor with antimalarial activity compared with wild-type parasites.

EXPERIMENTAL PROCEDURES

Parasites—*P. falciparum* clone D10 derived from *P. falciparum* isolate FC27 was obtained from The Walter and Eliza Hall Institute, Melbourne, Australia. Parasites were cultured as described previously (23).

Construction of the Transgenic Expression Plasmids and Transfection of Malaria Parasites—Pf14_0439, as annotated by PlasmoDB (plasmodb.org), is a gene located on chromosome 14 of *P. falciparum*. It consists of a single exon of 1818 bp that encodes a 605-amino-acid protein of the M17 leucyl aminopeptidase family. A PCR fragment corresponding to the predicted mRNA/RNA sequence was amplified from DNA isolated from *P. falciparum* clone D10. The forward primer for Pf14_0439 was AP14F (**AGATCT**ATGTATTTTTCTTCCTTATGT) and contained a BglII restriction site (in bold). The reverse primer was AP14R (**CTGCAG**TAGAGCGTCATTGAGTACAAA) and contained a PstI site (in bold) but not the putative stop codon of the gene. The PCR products were cloned into pGEM using a TA cloning system (Promega) and sequenced to confirm that no *Taq*-associated errors have been introduced into the DNA. The full-length fragment was digested out of the pGEM vector using the appropriate restriction enzymes and ligated into the Gateway™-compatible entry vector pHcmycB (Gateway, Invitrogen) that had previously been digested using BglII and PstI. A c-Myc tag was ligated in-frame at the 3′ end of the introduced gene sequence. This vector is a variant of the pHGB vector described by Tonkin *et al.* (24), in which the c-Myc 10-amino-acid epitope tag flanked by a 5′ PstI site and a 3′ NsiI site has been cloned into the original PstI site of pHGB. The entry vector containing the Pf14_0439 transgene was designated pHAP14cmycB. A clonase reaction was then performed using this entry vector and a Gateway™-compatible destination vector. The destination vector contained not only a Gateway™ destination cassette but also a second cassette comprising the human dihydofolate reductase synthetase gene under the control of the *P. falciparum* calmodulin promoter. This second cassette acts as a selectable marker in *P. falciparum*. This final plasmid was designated pHH1-HAP14cmycB.

Ring-stage parasites were subjected to electroporation in the presence of 150 μg of plasmid as described previously (25). Parasites resistant to WR99210 were obtained 15–22 days later.

Northern Blotting—Northern blotting was carried out essentially as described previously using total RNA extracts from trophozoite stage parasites (26). To distinguish between transgene and endogenous gene expression, the blot was probed with a DNA fragment containing the c-Myc epitope tag and the 3′ untranslated region (UTR) of the transgene labeled with [α-^{32}P]dATP by random priming (DECAprime II, Ambion).

Immunoblotting—Proteins of saponin-lysed parasite extracts were resolved by reducing 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane using a semidry electroblotter (Trans-Blot, Bio-Rad) (26). The membranes were probed with a mouse monoclonal antibody to c-Myc (1:2500 dilution; Sigma) followed by a horseradish peroxidase-labeled anti-mouse IgG antibody (1:5000 dilution, Chemicon International Inc.). The membrane was stripped and reprobed with an anti-glyceraldehyde-3-phosphate dehydrogenase mouse monoclonal antibody (1:2000 dilution) to demonstrate equal loading and transfer of malaria proteins (26).

Immunofluorescence Analysis of Parasites—Acetone-fixed thin blood smears of the transgenic parasite and wild-type D10 were probed with a mouse monoclonal antibody to c-Myc (1:2000) and bound antibody visualized with goat anti-mouse IgG-Cy2 (10 μg/ml) (26). The parasite nuclei were visualized with Hoechst dye (0.5 μg/ml). The slides were viewed with a fluorescence microscope (×1000 magnification).

Assays for Leucine Aminopeptidase Activity—Aminopeptidase activity was measured by monitoring the release of the leaving group 7-amino-4-methyl-coumarin (NHMec) from the fluorogenic peptide substrates H-leucine-NHMec and H-alanine-NHMec (13, 15). Samples of soluble parasite extracts were added to the substrate (10 μM) in phosphate-buffered saline in a final volume of 200 μl in wells of a 96-microtitre plate. The reaction was monitored at 37 °C over a 30-min period in a spectrofluorimeter with excitation at 370 nm and emission at 440 nm. Protein assays were performed using the BCA protein acid kit employing bovine serum albumin as a standard.

Parasite Growth and Sensitivity to Inhibitors—The *in vitro* sensitivities of the wild-type and transfected parasite populations to the aminopeptidase inhibitor bestatin {(-)-*N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-butyryl]-L-leucine} (Sigma) were determined using the [^{3}H]hypoxanthine incorporation assay (27). Serial dilutions of inhibitor were prepared in culture medium (0.05–50 μM) and added with [^{3}H]hypoxanthine (0.5 μCi/well) to asynchronous cultures at a 0.5% parasitaemia and 2% hematocrit. After a 48-h incubation, the amount of [^{3}H]hypoxanthine incorporated into the parasites was measured, and the concentrations of inhibitor required to prevent incorporation by 50% (EC_{50}) were determined by linear interpolation of inhibition curves (28). Each assay was performed in triplicate on three separate occasions, and data were pooled and are presented as mean ± S.E.

RESULTS

Transfection of Plasmid pHH1-HAP14cmycB Generates an Aminopeptidase Transgenic Parasite—*P. falciparum* clone D10 ring-stage parasites were transfected with the Pf14_0439 transgene and selected on WR99210. Parasites resistant to WR99210 were observed 15–22 days later. DNA was extracted when transgenic cultures reached 1% parasitaemia and the presence of the plasmid determined by PCR amplification using vector-specific primers located in the heat shock protein 86 5′ UTR and the *Plasmodium berghei* dihydrofolate reductase 3′ UTR (*i.e.* flanking the inserted aminopeptidase gene) (24). Gel analysis of the PCR-generated DNA revealed a band of 2 kb for pHH1-HAP14cmycB (Fig. 1*A*), the expected size for the M17 aminopeptidase gene plus additional vector sequence. The transgenic parasite cultures were maintained on WR99210 selection thereafter to sustain the episomally carried plasmids. The growth rate and intraerythrocytic development of the transgenic parasites were not distinguishable from wild-type parasites (data not shown).

The Aminopeptidase Transgene Is Transcribed and Translated—Northern blot analysis showed that the pHH1-HAP14cmycB-transfected malaria parasite cultures transcribed a single species of mRNA with an apparent size of 2.3 kb (Fig. 1*B*) when hybridized using a DNA fragment corresponding to the c-Myc tag and the 3′ UTR of the transgene. When saponin-lysed parasite-infected erythrocytes of the same cultures were probed by immu-

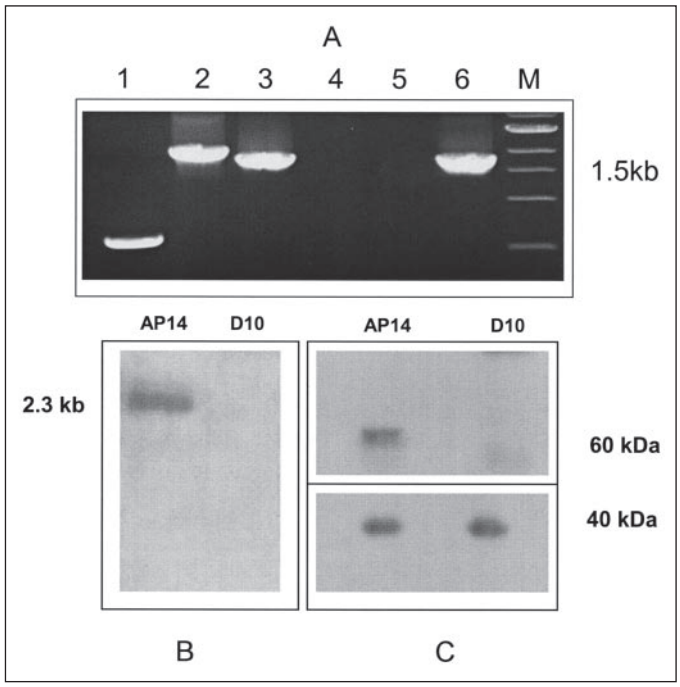

FIGURE 1. **Transfection of *P. falciparum* parasites with gene encoding M17 leucyl aminopeptidase.** *A*, PCR amplification of DNA extracted from the transgenic parasite Pf14_0439 clone (*lanes 1–3*) and wild-type parasite clone D10 (*lanes 4–6*). *Lanes 1* and *4*, PCRs using primers human dihydrofolate reductase (hDHFR) forward and reverse, which amplify the human dihydrofolate reductase gene from the vector pHH1-HAP14cmycB; *lanes 2* and *5*, PCR with primers, which are vector-specific primers that flank the inserted transgene; *lanes 3* and *6*, PCR using primers AP14F and AP14R, which amplify both the endogenous and transgene copies of Pf14_0439. *B*, Northern blot using 20 μg of RNA fractionated on a 1% Tris acetate-EDTA gel and probed with a DNA fragment containing the c-Myc epitope tag and the 3′ UTR of the transgene labeled with [α-^{32}P]dATP. AP14 refers to the transgenic parasite clone, whereas D10 is the wild-type parental clone. *C*, Western blot of saponin-lysed parasite extracts from the transgenic parasite clone (*AP14*) and the wild-type parasite line D10 probed sequentially with antibodies to c-Myc (*upper panel*) and glyceraldehyde-3-phosphatedehydrogenase (*lower panel*).

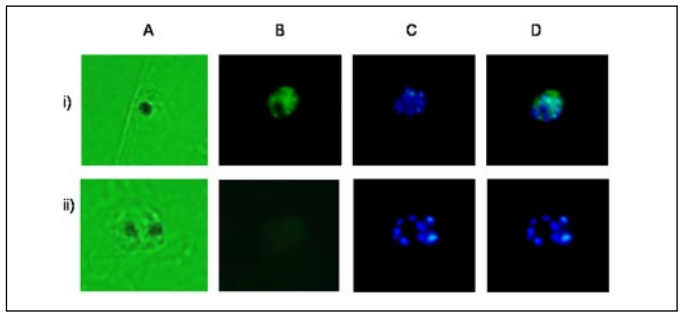

FIGURE 2. **Immunofluorescence analysis of the transgenic parasites.** A mouse monoclonal antibody to c-Myc was used to probe Pf14_0439 transgenic (series *i*) and D10 wild-type parasites (series *ii*). *A* shows parasites under bright field illumination. *B* shows specific *green* staining of the transgenic parasite (*i*), which is absent in the wild-type parasite clone (*ii*). *C*, *i* and *ii* show parasite nuclei visualized with 4′,6-diamidino-2-phenylindole (Hoechst dye). *D* shows a merge of *panels B* and *C*.

noblotting with anti-c-Myc tag monoclonal antibody, a single protein with an apparent molecular mass of 65 kDa (slightly smaller than the theoretical molecular mass of 67.831 kDa) was detected (Fig. 1*C*).

Immunolocalization of Transgene Products—To determine the cellular location of the protein product of the leucyl aminopeptidase transgene, an immunofluorescence assay was performed using anti-c-Myc monoclonal antibody (Fig. 2). A diffuse staining characteristic of localization to the parasite cytoplasm was observed. Staining with the antiserum was observed only in parasitized red blood cells as confirmed by nuclear staining with Hoechst dye (Fig. 2), and no staining was observed in the wild-type D10 parasites (Fig. 2).

The Pf14_0439 Transgene Expresses a Functional Protein—Soluble extracts of malaria parasites were prepared and assayed for leucyl aminopeptidase activity using the fluorogenic substrates H-leucine-NHMec and H-alanine-NHMec. The activity of these extracts against H-leucine-NHMec was higher than against H-alanine-NHMec, which would be expected of a M17 cytosolic leucyl aminopeptidase (29). Malaria parasites transfected with the Pf14_0439 transgene exhibited approximately twice the specific aminopeptidase activity (relative fluorescence units/10^6 infected cells) against both substrates compared with wild-type parasites (Fig. 3). Activity in all extracts was completely inhibited by 10 μM bestatin (data not shown).

The Transgenic P. falciparum Clone Has Altered Sensitivity to Aminopeptidase Inhibitors—Parasites transfected with pHH1-HAP14cmycB and maintained on 50 nM WR99210 were more resistant to bestatin than either D10 parent parasites or parasites transfected with the pHH1 vector without the AP14cmyc cassette and maintained under a similar selection regime; the combined data of three separate tests showed that the parasites exhibited an EC_{50} of 64, 25, and 20 μM, respectively (Fig. 4).

DISCUSSION

Aminopeptidases are exopeptidases that catalyze the sequential removal of amino acids from the N termini of peptides and play a major role in regulating the balance between catabolism and anabolism in all living cells. In malaria parasites, leucyl aminopeptidase-like enzymes are believed to function in the terminal stages of hemoglobin digestion to generate free amino acids that are then used for parasite protein synthesis (13–15). Because malaria parasites also use amino acids taken from their external environment for protein synthesis, it appears that the aminopeptidases may also be important in regulating the internal free amino acid pool for additional purposes, including maintenance of the osmotic stability of the cell (9).

Two leucyl aminopeptidase-like enzymes are expressed by malaria parasites, a 122-kDa membrane alanine aminopeptidase of the M1 family and a 67.8-kDa leucyl aminopeptidase of the M17 family (21, 22). Aminopeptidases of the M1 family are generally considered to be membrane-bound (30), but Florent and co-workers (22) have shown by soluble extraction and immunofluorescence studies that 96- and 68-kDa forms, presumably processed from the parent molecule, are found in the cytosol of malaria cells. The M17 family of aminopeptidase, on the other hand, are found in the cytosol of all cells and do not undergo processing events (29). Both classes of enzyme exhibit optimal activity at neutral pH and have similar preferences for amino acids, although as their names imply, they show a preference for either alanine or leucine. These overlapping biochemical features have led to confusion as to the true nature of the leucine-aminopeptidase activity measured in many studies that used soluble extracts of malaria parasites as their enzyme source (13–20) and to the identity of the target of aminopeptidase inhibitors that have antimalarial activity. Recent studies (31, 32) using microarrays have provided expression profiles for the M1 and M17 aminopeptidase throughout the intraerythrocytic stages of malaria parasites and demonstrate that the both enzymes are most highly expressed in early trophozoites, which is consistent with earlier biochemical studies (13, 15). However, the expression level of the M17 leucyl aminopeptidase is ~18-fold higher than the M1 aminopeptidase. The corollary of these findings is that the M17 leucyl aminopeptidase is the predominant source of leucyl aminopeptidase-like activity in malaria parasites and thus is most likely the subject of the previous biochemical and inhibitor studies.

In the present study, we have shown that the M17 leucyl aminopeptidase can be overexpressed in malaria cells by transfecting ring-stage

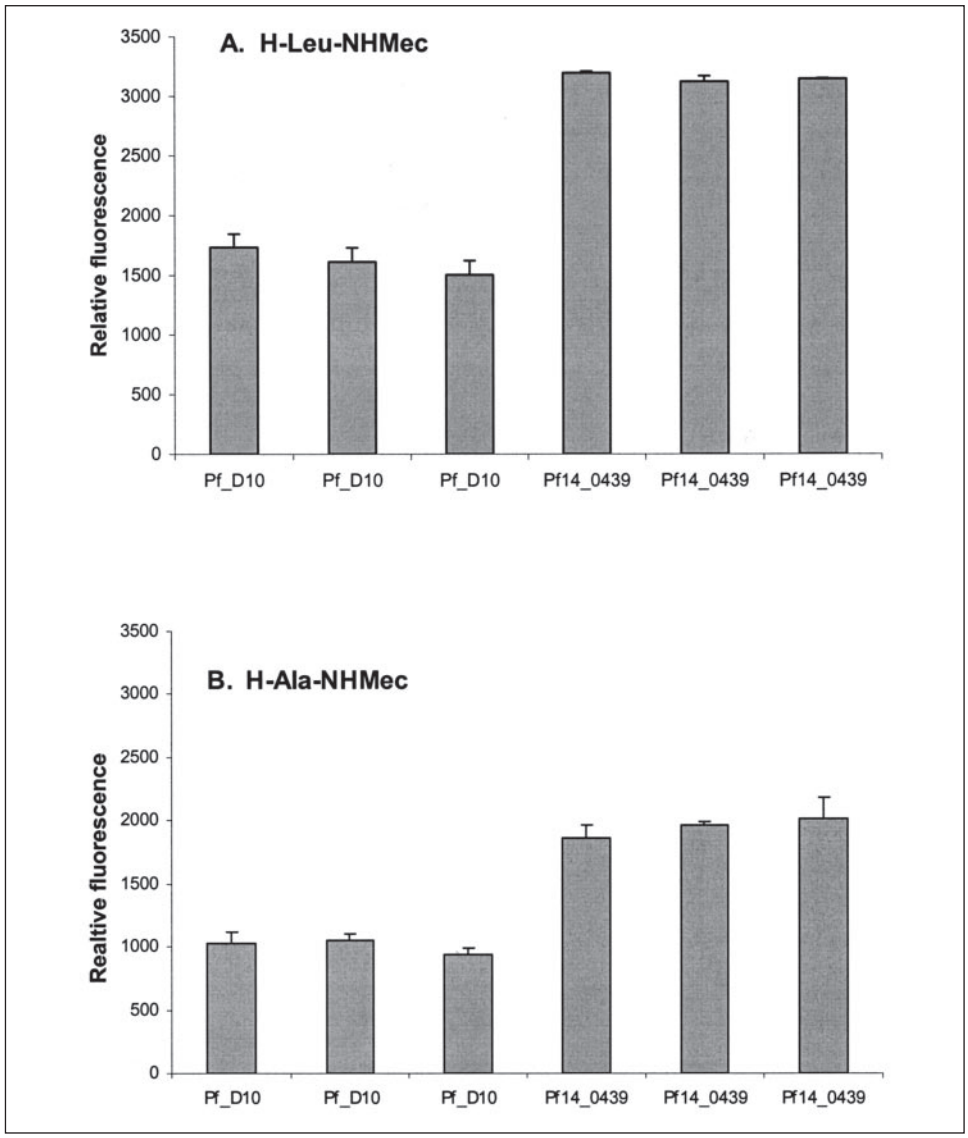

FIGURE 3. **Aminopeptidase activity in soluble extracts of wild-type and transgenic *P. falciparum* (*Pf*).** Enzyme activity in soluble extracts of three separate preparations of wild-type (Pf_D10) and transgenic (Pf14_0439) parasites was measured using the fluorogenic peptide substrates H-Leu-NHMec (*A*) and H-Ala-NHMec (*B*) and expressed as relative fluorescence units/10^6 malaria cells

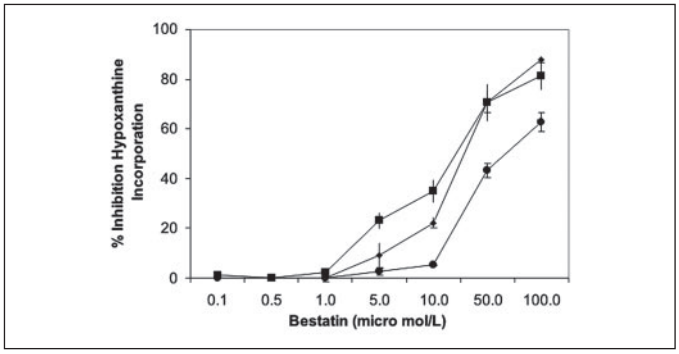

FIGURE 4. **Effect of bestatin on transgenic and wild-type *P. falciparum*.** *In vitro* sensitivity of Pf14_0439 transgenic parasites (—●—), D10 wild-type parasites (—◆—), and D10 parasites transfected with the pHH1 vector alone (—■—) to the aminopeptidase inhibitor bestatin determined by [^{3}H]hypoxanthine incorporation. Each assay was performed in triplicate for each drug concentration and on three separate occasions; therefore, the data shown represents the mean ± S.E. of nine values.

parasites with the transgene housed in the pHH1-HAP14cmycB vector prepared in our laboratory. The transgene was transcribed and expressed as a single 65-kDa protein. Immunofluorescence studies showed that it was transported correctly to the cytosol, the site of the native enzyme (13). Most importantly, enzymatic assays with specific peptide substrates showed that the transfected malaria cells expressed twice as much aminopeptidase activity as wild-type cells, which we can presume is because of the proper folding and activation of the enzyme synthesized from the transfected gene. The introduction of the transgene did not alter the *in vitro* growth rate or viability of the malaria cells. Accordingly, although we suggested that leucyl aminopepitdases are important in the regulation of the free amino acid pool in malaria cells, the increased enzyme activity within the cells did not upset this function sufficiently to affect malaria growth. It is probable that cells that overexpressed leucyl aminopeptidase to a lethal level were lost during the selection of transgene-carrying parasites using WR99210.

Bestatin is a dipeptide originally isolated from filtrates of *Streptomyces olivoretticuli* and has immunopotentiating and antitumor activity (33, 34). We have reported that bestatin, which inhibits aminopeptidase members of both the M1 and M17 families (27, 30), was a potent inhibitor of the *in vitro* growth of malaria parasites (13, 16). Exposure of synchronized cultures of *P. falciparum* cultures to bestatin and its derivative nitrobestatin indicated that late rings/trophozoites are the most susceptible intraerythrocytic stage. This correlates with the time at which hemoglobin is being degraded and used as a source of nutrient

and also with the increased expression of both the M1 and M17 aminopeptidases (13, 21, 31, 32). Here we show that *P. falciparum* parasites overexpressing the M17 leucyl aminopepidase are >2-fold less susceptible to killing by bestatin (EC_{50} 64 μM) compared with wild-type parasites (EC_{50} 25 μM). The natural interpretation of these results is that higher amounts of bestatin, which binds aminopeptidases in an equimolar ratio, are required to reduce the intracellular activity of leucyl aminopeptidase to lethal levels in malaria cells that express more of this enzyme. By extension, our studies show that the M17 leucyl aminopeptidase is a target of bestatin in malaria cells.

During this study, malaria parasites were also transfected with the gene encoding the M1 membrane alanyl aminopeptidase, but these transgenic parasites did not exhibit an increase in aminopeptidase activity or a difference in susceptibility to bestatin compared wild-type malaria cells (data not shown). Although immunoblotting demonstrated that a protein of the expected molecular size (~120 kDa) was expressed by the transgenic parasites, this was not processed to the soluble smaller molecular size forms of 96 and 68 kDa that were detected in the native parasites by Florent and co-workers (22). The translated M1 aminopeptidase may not have been correctly folded and/or post-translationally modified to produce a functionally active enzyme, and therefore we cannot make a conclusion on its potential as a target for bestatin-mediated killing of malaria. In addition, we cannot exclude the possibility that overexpression of the M17 leucyl aminopeptidase prevents killing of malaria parasites by providing more enzyme to compete for bestatin binding and preventing it from inhibiting the M1 aminopeptidase.

Cysteinyl (falcipain 1–3) and aspartyl (plasmepsins I–IV) endopeptidases are involved in the degradation of hemoglobin within the digestive vacuole (1–4). Specific or broad range inhibitors of these endopeptidases block hemoglobin degradation and have potent effects on malaria growth, and therefore both enzyme classes are intensely studied as targets for new antimalarial drugs (3, 10, 35). Because cysteinyl and aspartyl inhibitors exhibit synergistic activity when used together, combination therapies are also possible (36). Gene disruption methods have been used to explore the function of these enzymes and their role in hemoglobin digestion. Disruption of the gene encoding falcipain 2 did not result in complete ablation of cysteine endopeptidase activity but did impair hemoglobin digestion and parasite growth (37), whereas disruption of falcipain 1 had no effect on either (38). Disruption of the genes encoding falcipain 3 (37) and dipeptidyl aminopeptidase (12) is lethal to malaria parasites, and hence these peptidases are considered critical to survival within the erythrocyte.

As an alternative to employing gene disruption methods for studying the importance of peptidases in malaria, we exploited the transfection plasmids available in our laboratory to overexpress the M17 leucyl aminopeptidase in malaria parasites. Tonkin *et al.* (24) described the design of transfection vectors for producing proteins fused with the reporter protein green fluorescent protein and exploited these to localize malaria proteins to specific intracellular compartments, such as the plastid and mitochondrion. In this study, these vectors were further modified by replacing the green fluorescent protein reporter with the significantly smaller c-Myc epitope tag allowing production of functional molecules that could be traced with anti-c-Myc antibodies. Using this approach, we also identified the M17 aminopeptidase as a target for the antimalarial activity of bestatin and therefore suggest that this could be a general approach for the characterization and validation of malaria drug targets.

Cysteinyl or aspartyl inhibitors and bestatin show little synergy against malaria parasites *in vitro*, most likely because the aminopeptidase functions in separate cell compartments from the endopeptidases (39). However, a combination of drugs targeted at multiple exoaminopeptidases involved in amino acid regulation in the parasite cytosol may offer a more reasonable proposition for combination therapies; the targets would not only include the M17 leucyl aminopeptidase and M1 membrane alanine aminopeptidases but also two uncharacterized enzymes expressed by the intraerythrocytic stages of malaria, namely prolyl aminopeptidase and aspartyl aminopeptidases (plasmodb.org).

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