Esterase mutation is a mechanism of resistance to antimalarial compounds

Eva S. Istvan
Washington University School of Medicine in St. Louis

Jeremy P. Mallari
Washington University School of Medicine in St. Louis

Victoria C. Corey
University of California San Diego School of Medicine

Neekesh V. Dharia
University of California San Diego School of Medicine

Garland R. Marshall
Washington University School of Medicine in St. Louis

See next page for additional authors

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Esterase mutation is a mechanism of resistance to antimalarial compounds

Eva S. Istvan1,2, Jeremy P. Mallari1,2, Victoria C. Corey3, Neekesh V. Dharia3,†, Garland R. Marshall4, Elizabeth A. Winzeler3 & Daniel E. Goldberg1,2

Pepstatin is a potent peptidyl inhibitor of various malarial aspartic proteases, and also has parasiticidal activity. Activity of pepstatin against cultured Plasmodium falciparum is highly variable depending on the commercial source. Here we identify a minor contaminant (pepstatin butyl ester) as the active anti-parasitic principle. We synthesize a series of derivatives and characterize an analogue (pepstatin hexyl ester) with low nanomolar activity. By selecting resistant parasite mutants, we find that a parasite esterase, PfPARE (P. falciparum Prodrug Activation and Resistance Esterase) is required for activation of esterified pepstatin. Parasites with esterase mutations are resistant to pepstatin esters and to an open source antimalarial compound, MMV011438. Recombinant PfPARE hydrolyses pepstatin esters and de-esterifies MMV011438. We conclude that (1) pepstatin is a potent but poorly bioavailable antimalarial; (2) PfPARE is a functional esterase that is capable of activating prodrugs; (3) Mutations in PfPARE constitute a mechanism of antimalarial resistance.
R esistance to antimalarial drugs is hampering efforts in disease management. The recent emergence of tolerance/resistance to the artemisinins has given rise to the spectre of failure of artemisinin combination therapies, the mainstay of control regimens. Indeed, resistance has developed to virtually all drugs used for malaria. Resistance mechanisms include efflux pump gene mutation and copy number variation (Mdr1, Crt, Mrp1), enzyme active site mutation (DHFR, DHPS, SoxP, CytC) and Na+/H+ exchanger gene mutation (ATP4) (refs 2,3). There is a pressing need for new antimalarials and for a better understanding of extent and potential resistance mechanisms. Pepstatin is a natural product isolated nearly 50 years ago from Streptomyces argenteolus and related actinomycetes. It is a well-characterized aspartic protease inhibitor and derivatives of this scaffold are in clinical use as antiretroviral therapies. Pepstatin has been shown to kill malaria parasites in culture and to cure infection in a rodent malaria model. However, the activity of pepstatin against cultured Plasmodium falciparum is highly variable depending on the commercial source.

In this study, we fractionated an active commercial preparation of microbial pepstatin and identified a small contaminant as the active principle, pepstatin butyl ester. By selecting parasite mutants resistant to pepstatin esters, we found that a parasite alpha/beta hydrolase, PIPARE (P. falciparum Prodrug Activation and Resistance Esterase), is required for activation of the esterified compound. Esterase mutation is a new mechanism of antimalarial resistance.

**Results**

**Isolation and characterization of potent pepstatin analogues.** We used LC/MS to fractionate and characterize the components of a potent pepstatin batch (Fig. 1a). Fractions were tested for antimalarial activity. The dominant peak eluted at 4.5 min and had a mass of 686.47, corresponding to pepstatin. Pure pepstatin was inactive against cultured *P. falciparum* (Fig. 1e). One minor component (~5% of the total chromatographic signal) that eluted later in the gradient was highly active. Mass spectrometric analysis indicated a m/z of 742.53 for the molecule in the active fraction. Tandem mass spectrometry showed a peptide-like fragmentation that closely resembled pepstatin; however, an extra mass of 56.06, corresponding to a butyl group, was present on the C-terminal statine residue (Fig. 1b–d). We synthesized pepstatin n-butyl ester (PBE) and determined that it is three orders of magnitude more potent than unmodified pepstatin (Fig. 1e).

We tested a series of PBE analogues (Table 1) to probe the structural basis for antimalarial activity. All pepstatin esters were significantly more active than pepstatin, with pepstatin n-hexyl ester (PHE) being the most potent (EC50 = 25 nM). Penetratin peptide-derivatized pepstatin, as well as pepstatin n-butyl amide, however, were poorly active on blood-stage parasites. N-terminally acetylated pepstatin was also inactive. Our results indicate that esterification at the C-terminus of pepstatin is critical for compound activity and that increasing the length of the ester alkyl chain increases antimalarial potency (hexyl >> butyl >> ethyl >> methyl).

**Selection of pepstatin ester-resistant parasites.** Selection of mutants resistant to compounds is a powerful way to determine antimalarial mode of action. We raised pepstatin ester-resistant parasites by treating 4 × 10^6 3D7 parasites with PBE in a single-step selection. Compound treatment initially cleared cultures of visible parasites and surviving parasites multiplied to detectable levels by 2–3 weeks. Multiple selections yielded viable parasites. Resistant parasites were recloned and tested for sensitivity to pepstatin ester. Selections from different concentrations of PBE were all similarly highly resistant to pepstatin ester. Four clones were analysed by full-genome sequencing (Table 2). Each had a non-synonymous coding mutation in the same gene, Pf3D7_0709700 (pfpare). Sequencing of the putative polymorphism-containing gene in other PBE-resistant clones selected from a different parasite strain, HB3, revealed four additional mutations (Supplementary Table 1). Seven of the eight mutations we isolated are missense and one results in an early stop.

**Confirmation of the role of PIPARE mutations in resistance.** We recombinantly modified the parasites in a clean genetic background to confirm that the mutations isolated in resistant parasites provided resistance to pepstatin esters. One of the C-terminal mutations (L357P) was introduced into parasites by single-crossover allelic exchange. Concurrently, we generated recombinants preserving the wild-type sequence. Introduced wild-type or mutant protein is expressed from the endogenous promoter and contains a C-terminal GFP, while the original genomic version is promoterless. Correct integration in clonal parasite lines was confirmed by sequencing of PCR products from integrated plasmids and by Southern blot (Supplementary Fig. 1). GFP-expressing, wild-type parasites were similarly sensitive to PHE compared to the un-transfected, parental parasites, while L357P-expressing parasites were highly resistant to the compound (Fig. 2a).

Live microscopy showed diffuse cytoplasmic GFP fluorescence (Fig. 2b). The intensity of the GFP fluorescence was weak in ring stages and increased with parasite maturity. Western blots of parasite extracts confirmed expression of intact GFP fusion proteins (Fig. 2c). Compared to the wild-type GFP-tagged parasites, the GFP band in the L357P PIPARE parasite extracts was consistently weaker (Fig. 2c).

**PIPARE has sequence similarity to alpha/beta hydrolases.** PIPARE is annotated as a lysophospholipase and is homologous to putative plant lysophospholipases. Structure prediction (Fig. 2d) supports the notion that the esterase belongs to the family of alpha/beta hydrolases, which are enzymes with very diverse specificities. Carboxylic acid esters, epoxides, lipids and phospholipids are substrates of this enzyme class. The gene is present on chromosome seven and not close to the telomeres. However, it belongs to a conserved gene family in *Plasmodium* known as PST-A, many of whose members are subtelomeric. At least three other genes predicted to encode hydrolases closely related to *pfpare* are present in *P. falciparum*: Pf3D7_1252600 (54% identical over 349 residues); Pf3D7_1401500 (55% identical over 350 residues) and Pf3D7_1038900 (53% identical over 338 residues). These three homologous hydrolases contain key catalytic residues and likely encode active enzymes.

Three putative catalytic residues in PIPARE are readily identifiable (S179, D308 and H338). S179 resides in the highly conserved pentapeptide G-x-S-x-G, also known as the ‘nucleophile elbow’. As in other hydrolases, the core of the alpha/beta fold accommodates less conserved insertions that dictate substrate specificity. Inspection of the structural model in Fig. 2d suggested that some of the resistance-conferring mutations we had obtained likely abolish enzymatic activity. The mutation M180K (clone HB3-R4) resides adjacent to the putative catalytic serine, while in clone 3D7-R4 the predicted protein is truncated and three key catalytic residues (S179, D308 and H338) are missing. Five different mutations cluster in the C-terminal region of the esterase, which is predicted to adopt an alpha-helical conformation. This part of the protein participates in homo-dimer formation in related hydrolases and may stabilize...
the enzymes\textsuperscript{14}. We observed low GFP fluorescence and decreased signal on western blots from parasite extracts containing the L357P resistance-conferring mutation. Possibly, the enzyme fold is destabilized by a proline-induced break in the C-terminal helix. Alternatively, the change of leucine to proline may slow the folding kinetics of PfPARE, as prolyl cis/trans isomerizations are frequently rate-limiting steps in protein folding\textsuperscript{15}.

PfPARE is an active esterase. To test whether PfPARE activity is important for parasite viability or resistance to pepstatin esters, we generated a parasite clone in which we mutated a predicted key catalytic residue (S179T). The S179T mutation resulted in PHE resistance (Fig. 2a). Active site mutant parasites were also resistant to other pepstatin esters (Supplementary Table 2). Growth rates were unaffected by either L357P or S179T mutations, indicating that catalytic activity is dispensable for asexual malaria parasites (Supplementary Fig. 2). We assessed whether PfPARE had esterase activity by generating wild-type and active site mutant proteins in E. coli. We initially attempted to express the same active site mutant that we had used in the parasite (S179T). However, S179T expressed at low levels, was unstable and could not be purified. We did succeed in expressing another active site mutant (S179G), and this protein was used in subsequent studies. Affinity purified proteins were incubated with the generic esterase substrate p-nitrophenyl butyrate (PNPB), and free p-nitrophenol (PNP) generated by hydrolysis was observed.

**Figure 1** | Potent pepstatin preparations contain pepstatin esters. (a) Separation of older, potent pepstatin by C18 reverse phase chromatography. The prominent peak eluting at 4.5 min has a mass of 686.47 and does not kill malaria cultures at 40 μM. The minor peak eluting at 6.4 min has a mass of 742.53 and is highly potent. (b, c) MS/MS fragmentation of the 686.47 mass peak (b) and the 742.53 mass peak (c). Mass-to-charge ratios (m/z) of selected fragments are indicated in black numbers. The blue letters and red numbers match fragments of the structure key in (d). (d) Structure and MS fragmentation key of pepstatin or pepstatin ester. Blue lines and letters mark N-terminal fragments present in the free acid or ester of pepstatin. Red lines and numbers show C-terminal fragments which have an additional 56 amu in the pepstatin ester. (Note that there is loss of a proton on pepstatin upon the addition of the C\textsubscript{4}H\textsubscript{9} ester). (e) Concentration responses of blood-stage 3D7 parasites (all in 0.1% DMSO) with pepstatin (black) or two syntheses of pepstatin n-butyl ester (PBE) (red, blue) measured using a flow cytometry-based assay. PBE inhibition data were fit to a sigmoidal growth inhibition curve. Error bars show s.d. ± indicates standard errors calculated for the biological triplicates of the experiment shown. N = 4.
could hydrolyse pepstatin esters. Affinity-purified wild-type or mutation (Fig. 3c,d). We postulated that PfPARE can act directly compound processing, but not uptake, was affected by the compound, indicating that changes in the parasite determine efficacy of pepstatin esters.

PfPARE is active on another antimalarial ester scaffold. We were curious whether PfPARE could act on ester-containing antimalarials other than pepstatin esters and performed concentration-response experiments with three open source antimalarial compounds. MMV011438, a benzodiazepine, showed differential sensitivity in wild-type and mutant (L357P or S179T) esterase-expressing parasites described above (Fig. 4a), indicating that the recombinant enzyme on PME suggests that esterase activity in the parasite determines efficacy of pepstatin esters.

Table 1 | Structures and in vitro activities of pepstatin and analogues.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Potency* (µM)</th>
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<tr>
<td>Pepstatin (P)</td>
<td>Iva-Val-Val-Sta-Ala-Sta-OH</td>
<td>none at 40</td>
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<tr>
<td>Acetyl pepstatin</td>
<td>Ac-Val-Val-Sta-Ala-Sta-OH</td>
<td>none at 20</td>
</tr>
<tr>
<td>Pepstatin-Penetratin</td>
<td>Pepstatin-RQIKWFQNRMKWKK-OH</td>
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<tr>
<td>Pepstatin n-butyl amide</td>
<td>Pepstatin-CO-NH-(CH2)3CH3</td>
<td>none at 20</td>
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<td>Pepstatin methyl ester (PME)</td>
<td>Pepstatin-COO-CH3</td>
<td>5.12 ± 0.15</td>
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<tr>
<td>Pepstatin ethyl ester (PEE)</td>
<td>Pepstatin-COO-CH2-CH3</td>
<td>0.730 ± 0.012</td>
</tr>
<tr>
<td>Pepstatin 3-methyl-1-butyl ester (PMBE)</td>
<td>Pepstatin-COO-(CH2)3-CH(CH2)2</td>
<td>0.071 ± 0.010</td>
</tr>
<tr>
<td>Pepstatin n-butyl ester (PBE) prep 1</td>
<td>Pepstatin-COO-(CH2)5-CH3</td>
<td>0.083 ± 0.006</td>
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<tr>
<td>Pepstatin n-butyl ester (PBE) prep 2</td>
<td>Pepstatin-COO-(CH2)5-CH3</td>
<td>0.092 ± 0.006</td>
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<tr>
<td>Pepstatin n-hexyl ester (PHE) prep 1</td>
<td>Pepstatin-COO-(CH2)5-CH3</td>
<td>0.019 ± 0.003</td>
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<tr>
<td>Pepstatin n-hexyl ester (PHE) prep 2</td>
<td>Pepstatin-COO-(CH2)5-CH3</td>
<td>0.026 ± 0.003</td>
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</tbody>
</table>

*Potency—where appropriate, potency is presented as EC50. For inactive compounds, inhibition at highest concentration is presented.

Table 2 | Whole genome sequencing of PBE and MMV011438 resistant parasite clones.

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<tr>
<th>Resistant clone</th>
<th>Compound used for selection</th>
<th>Chr Position</th>
<th>Gene name</th>
<th>Gene description</th>
<th>Effect</th>
<th>Impact</th>
<th>Amino acid</th>
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<td>1066792</td>
<td></td>
<td>Intergenic</td>
<td>Ribosomal RNA</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>5</td>
<td>1290269</td>
<td>PF3D7_0531600</td>
<td>18S ribosomal RNA</td>
<td>Non synonymous coding</td>
<td>Mis-sense</td>
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<td></td>
<td></td>
<td>7</td>
<td>435126</td>
<td>PF3D7_0709700</td>
<td>Putative esterase</td>
<td>Synonymous coding</td>
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<td>Sec24 subunit b</td>
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<td>7</td>
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<td>Stop gained</td>
<td>Non-sense</td>
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<tr>
<td>3D7-R3</td>
<td>PBE</td>
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<td>1066792</td>
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<tr>
<td>3D7-R4</td>
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<td>6</td>
<td>566727</td>
<td>PF3D7_0613800</td>
<td>Transcription factor (AP2)</td>
<td>Non synonymous coding</td>
<td>Missense</td>
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<tr>
<td>Dd2-2G1</td>
<td>MMV011438</td>
<td>5</td>
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<td></td>
<td>Intergenic</td>
<td>Ribosomal RNA</td>
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<td></td>
<td>7</td>
<td>435160</td>
<td>PF3D7_0709700</td>
<td>Putative esterase</td>
<td>Non-synonymous coding</td>
<td>Missense</td>
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Whole genome sequencing from gDNA of compound resistant clones reveals SNPs in PF3D7_0709700. PCR sequencing of PF3D7_0709700 in additional MMV011438 resistant clones indicated that they either had the same mutation as clone 2G1 or a missense (T to G) at bp 435138, resulting in 353S. Filter parameters used to analyse data are in Supplementary Table 3.

Photometrically. Wild-type PfPARE was catalytically active, while the S179G mutant protein activity was near background levels (Fig. 2e).

Esterified pepstatin is taken up by cells and processed. We performed in-cell metabolism experiments to determine the fate of pepstatin and its ester analogues in parasitized RBCs. Pepstatin or pepstatin esters were incubated with mature ring-stage parasite cultures for 5–6 h. Samples from HPLC-fractionated extracts were analysed by mass spectrometry. Cellular uptake of pepstatin esters were incubated with mature ring-stage parasite extracts (Fig. 3a). In contrast, cellular accumulation of exogenously added free pepstatin was undetectable. These data suggest that the ionizable carboxylic acid moiety of pepstatin decreases cellular penetration, resulting in poor parasiticidal activity. Replacing the carboxylic acid with an ester results in a neutral molecule, likely resulting in the greatly improved activity. Replacing the carboxylic acid with an ester results in a neutral molecule, likely resulting in the greatly improved activity. Replacing the carboxylic acid with an ester results in a neutral molecule, likely resulting in the greatly improved activity.
PfPARE is an active esterase with moderate substrate specificity that hydrolyses ester-containing prodrugs.

**Discussion**

We have clarified the differences in potency of different pepstatin preparations reported previously and found that antimalarial activity is due not to pepstatin, but to low levels of a highly potent pepstatin ester. It is unclear whether active microbial pepstatin preparations contain pepstatin ester because it is produced by Actinomycetes or because pepstatin was modified during compound isolation. We found that esterification is required for parasite uptake of pepstatin, and we have synthesized a series of pepstatin esters, identified structural features important for activity, and developed a potent inhibitor with low nanomolar EC\(_{50}\) against cultured *P. falciparum*. Clearly, pepstatin esters are not good lead compounds for drug discovery as resistance arose quickly. Nevertheless, we have gained valuable information: (1) pepstatin likely inhibits the activity of an essential aspartic protease; and (2) the free carboxylic acid moiety is crucial for pepstatin’s potency. These findings may help in the development of new antimalarials that do not require esterification for cell uptake.

PfPARE is expressed during the asexual blood stages and resides in the parasite cytoplasm where it may encounter drugs or substrates. Its function is dispensable in blood-stage parasites. The physiological substrate of PfPARE is unknown. Parasites can hydrolyse a range of other ester-containing antimalarial compounds and that mutations in PfPARE can provide resistance to new potential drugs. It is possible that PfPARE is not essential because other hydrolases are capable of compensating for its activity from the pathogen box and 400 compounds from the malaria box reveals 26 esters\(^{17}\). Consideration should be given to
the possibility that some are prodrugs subject to rapid resistance development. Testing against parasites with an inactivated esterase gene provides a facile screen to identify such compounds. A limitation of our study is that other non-essential esterases may be active on different compounds. Furthermore, we did not gain full understanding of the substrate specificity of PfPARE.

Resistance to prodrug activation has precedence in the antimicrobial literature. Acyclovir is an anti-herpesvirus nucleoside analogue that is activated by a viral kinase. Since this antiviral drug is a prodrug, inactivation or deletion leads to acyclovir resistance18. Isoniazid is a prodrug for thymidine kinase is not essential to the virus, inactivation or side analogue that is activated by a viral kinase. Since this antimicrobial drug is a prodrug, inactivation or deletion leads to isoniazid resistance19. Metronidazole is a drug that needs to be reduced by the bacterial NADPH-dependent enzyme RDXA for activity against Helicobacter20. Microaerophilic protists such as Entamoeba histolytica have similar genes, but resistance to metronidazole is achieved by different mechanisms21. Esterase inactivation, then, is the first example of a parasitic mechanism to avoid killing by failure to activate a prodrug. Prodrug synthesis is an attractive way to move compounds into cells, but in Plasmodium, resistance is potentially a major liability.

Methods

Fractration of potent pepstatin. Fractionation of microbial pepstatin (Sigma Aldrich, a gift from Hagai Ginsburg, Jerusalem, Israel) was performed on a reverse-phase C18 column using a 0–50% gradient of acetonitrile/H2O with 0.1% formic acid. Fractions were analysed by MS/MS on a hybrid FTMS consisting of a Thermo LTQ-FT and a Thermo Orbitrap.

Synthesis of pepstatin analogues. Acetyl pepstatin, pepstatin methyl ester and pepstatin-penetratin were purchased from Calbiochem. Ethanol, 1-butanol, 3-methyl-1-butanol and 1-hexanol were purchased from Sigma-Aldrich. Esters were synthesized according to the esterification method of Wilcox22. Briefly, pepstatin (synthetic, Calbiochem) (10 mg, 15 μmol) was dissolved in the appropriate alcohol (8 g, 80 mmol) and 1 N HCl was added to a concentration of 0.1 N (1 mmol). Reactions were stirred at room temperature for 7–10 days and evaporated under argon. Crude products were dissolved in dimethylsulfoxide and purified by C18 chromatography. Inhibitor concentrations were determined by preparing a standard curve of absorption (218 nm) for pepstatin and calculating the areas under the curve for the pepstatin derivatives. Structures were confirmed by mass spectrometry (measured masses of Na-adducts of PEE = 738.3; PBE = 763.4; PMBE = 790.0; PHE = 793.6). MALDI-TOF spectra of PEE, PBE, PMBE and pepstatin n-butyl amide are in Supplementary Fig. 3. 1HNMR and 13CNMR assignments for PHE are in the Supplementary Note 1. Pepstatin n-butyl amide was synthesized by dissolving 13.5 mol pepstatin, 27 μmol HBTU and 27 μmol HOBr in a solution of dichloromethane (1 ml) and dimethylformamide (0.5 ml). Three equivalents of n-butylamine was added in DIEA and the reaction was stirred at room temperature for 18 h. The product was purified on HPLC and characterized by MALDI-TOF (mass 765.2, corresponding to the Na-adduct of the amide) (Supplementary Fig. 3).

Parasite culture. 3D7, mycoplasma free, was a gift from P.K. Rathod (University of Washington). The identity of the strain was confirmed by whole-genome sequencing. HB3 was obtained from the MR4 repository at ATCC (Cat. No. MRA-155). Parasites were cultured in human red blood cells (de-identified donors from the St Louis Children’s Hospital blood bank) at 2% hematocrit in RPMI 1640 with 0.25% (w/v) Albumax23,24. Resistance selections were performed with 4 x 10^5 3D7 or HB3 parasites in a single-step with either 280 nM or 1.2 μM PBE. Resistant parasites were cloned by limiting dilution and two clones for each mutant were analysed. Concentration-response experiments were done in biological triplicates with synchronous, young ring-stage cultures (1–1.2% starting parasitemia) and at least two times. Parasitemias (percentage of total erythrocytes infected with parasites) were measured approximately 70–80 h post compound addition by nucleic acid staining of iRBCs with 0.8 μg ml^-1 acidine orange in PBS.

Figure 3 | In cell metabolism of pepstatin esters. (a) Detection of pepstatin (P) or pepstatin esters (E) by MALDI-TOF. Fractions of 3D7 lysates after incubation with pepstatin (P) or analogues were spotted onto a MALDI plate and scored for the presence or absence of compounds with appropriate mass. This MS assay is not quantitative. (b–d) MALDI-TOF spectra of HPLC fractions (C18) from whole cell extracts of 3D7 (b) or S1797 mutant parasite (c,d) infected RBCs, incubated with PBE. The dominant peak of 707.0 (b) corresponds to the Na-adduct of pepstatin. The dominant peak of 766.1 (c) corresponds to the Na adduct of PBE. Pepstatin was not detected in any fractions prepared from S1797 mutant parasite extracts (d). (e) Activity of recombinant PfPARE on pepstatin esters and MDTIP compounds16. Wild-type or mutant proteins were incubated with compounds and amounts of de-esterified products were determined by LC/MS (triplicate reactions). Results for incubations with wild-type enzyme are in black, with S1797G mutant enzyme is in red. *Pepstatin from incubations with PM could be detected by MALDI-TOF, but not significantly by LC/MS. Error bars are s.d.
Figure 4 | PIPARE hydrolyzes and activates an ester-containing prodrug. Concentration-responses against 3D7 asexual parasites (right) and structures (left) of compounds from the MDTIP consortium. EC_{50} values were measured with a flow cytometry-based assay. Parasite strains are labelled black (3D7), blue (ppare wild-type allelic exchange), green (ppare L357P allelic exchange) and orange (ppare S179T allelic exchange). Inhibition data were fit to a sigmoidal concentration-response curve. (a) MMV01438, (b) MMV021735, (c) MMV030666. In a, F-test; P < 0.0001 for S179T or L357P compared to Wt. Error bars show s.d. ± indicates standard errors calculated for the biological triplicates of the experiment shown. N = 2.

Fluorescence microscopy and western blotting. Fluorescence microscopy was performed on live, GFP-expressing parasites using a Zeiss Axioskope. Nucleic acid was detected by staining with DAPI. For western blotting, 3 × 10^7 parasites were released from iRBCs with 0.035% saponin and lysed in Ripa buffer. For each sample, 9 × 10^6 parasites were loaded onto a 12% TGX gel (BioRad). Proteins were transferred onto PVDF using wet transfer with 20% methanol. Blots were blocked (1:1,000) (a gift from Dr Audrey R. Odom, Washington University). Secondary antibodies were goat-α-mouse (800) and donkey-α-rabbit (680) IR-Dyes (1:20,000) from Licor.

Expression of recombinant PIPARE. A codon-optimized version (most abundant codon for E. coli) of the PFS3D7_0709700 gene was purchased from IDT (Supplementary Note 2). The gene product lacked an amino terminal low complexity region that is predicted to be disordered (amino acids 1–22). After cloning into the expression vector pET15b using the T7 promoter, the protein contains a T7 RNA polymerase promoter, N-terminal His-tag and amino acids generated from the vector sequence, the MW of the recombinant enzyme is 42,700. The S179G mutation was introduced using QuikChange Mutagenesis (Agilent) with the primer (5′-CTTGCGTTCCTATTAGGGTGGATAGCGTTTTCG-3′) and reverse (5′-CCTTATATTTTCGATATGACGG-3′). Transfections were performed in duplicate using 100 μg of supercoiled DNA with 3D7 ring-stage parasites. WR99210 (5 mM) was added to mature ring-stage parasites one cycle after transfection and used to select resistant parasites. Transfectants were cloned by limited dilution. To confirm correct integration, we used the AlkPhos Direct Kit for Southern blots as described (Fisher Scientific). PCR products for the Southern blots were generated with the following primer pairs (Supplementary Fig. S1A: 5′-GTATAAACATATGATACGTTAC-3′ and 5′-ACATTGATACGTTAC-3′; Supplementary Fig. SIC: 5′-CTATGATACGTTAC-3′). Parasite strains are labelled black (3D7), blue (ppare wild-type allelic exchange), green (ppare L357P allelic exchange) and orange (ppare S179T allelic exchange). Inhibition data were fit to a sigmoidal concentration-response curve. (a) MMV01438, (b) MMV021735, (c) MMV030666. In a, F-test; P < 0.0001 for S179T or L357P compared to Wt. Error bars show s.d. ± indicates standard errors calculated for the biological triplicates of the experiment shown. N = 2.
In-cell metabolism of pepstatin esters. A volume of 150–240 ml of mid-ring parasite cultures at 10–20% parasitemia was incubated with 2 μm compound for 5–6 h. In experiments where comparisons between parasite cultures were important (that is, wild-type and mutant esterase parasite clones), culture amounts and % parasitemia were matched. Compounds not taken up by parasites were removed by permeabilizing infected RBCs with 0.053% saponin and parasites pellets were washed three times with cold PBS. After freeze-thawing, pellets were resuspended in cold 20 mM NaPO4, pH 7.2 (350 μl) of ml of infected culture, slowly mixed at 4°C for 3 h and extracted with 3 volumes of cold acetonitrile. Centrifugation supernatants (15 min, 2,100g) of the extractions were loaded onto a reverse-phase C18 column equilibrated in H2O with 1.0% formic acid and eluted with a linear gradient (solvent B = 100% acetonitrile with 0.1% formic acid). Fractions were collected, mixed 1:1 with 1-cyano-4-hydrocinnamic acid, spotted onto a sample plate and analysed on a Voyager 4254 MALDI-TOF.

Full genome sequencing. For sequencing, DNA libraries for each gDNA sample were prepped with the Nextera XT kit (Cat. No. FC-131-1024, Illumina), using the dual index protocol. Libraries were clustered using the RapidRun mode on the Illumina HiSeq 2500, and 100 base pairs were sequenced on either fragment end for each sample. Paired-end reads were aligned to the P. falciparum 3D7 reference genome (PlasmoDB v. 26.0), as described previously with the exception that mutations were called using GATK’s HaplotyperCaller instead of UnifiedGenotyper. Mutations, including single nucleotide polymorphisms and insertion/deletions (INDELs), were filtered based on general recommendations from GATK (Supplementary Table 3). Additional filters were applied post HaplotypeCaller, which included removing positions where read coverage was <5 in the parent and any position where all samples had a heterozygous ratio >0.2 (reference/total reads).

Data availability. Sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive database with accession code SRP077277. Coordinates of the PIPARE model generated by the Roberts Structure Prediction Server are available on modelarchiver.org with DOI (10.5452/ma-aqpe). All other relevant data are available from the authors on request.

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Author contributions
E.S.I. and D.E.G. wrote the manuscript. E.S.I. and J.P.M. performed experiments. G.R.M. synthesized pepstatin amide. V.C.C., N.V.D., and E.A.W. performed sequencing and analysed sequence data.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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