Malaria causes worldwide morbidity and mortality, and while chemotherapy remains an essential means of malaria control, drug-resistant parasites necessitate the discovery of new antimalarial drugs. Peptidases are a promising class of drug targets and perform several important roles during the Plasmodium falciparum erythrocytic life cycle. Herein, we report a multidisciplinary effort combining activity-based protein profiling, biochemical, and peptidomics approaches to functionally analyze two genetically essential P. falciparum metallo-aminopeptidases (MAPs), PIA-M1 and PF-LAP. Through the synthesis of a suite of activity-based probes (ABPs) based on the general MAP inhibitor scaffold, bestatin, we generated specific ABPs for these two enzymes. Specific inhibition of PIA-M1 caused swelling of the parasite digestive vacuole and prevented proteolysis of hemoglobin (Hb)-derived oligopeptides, likely starving the parasite resulting in death. In contrast, inhibition of PF-LAP was lethal to parasites early in the life cycle, prior to the onset of Hb degradation suggesting that PF-LAP has an essential role outside of Hb digestion.


The authors declare no conflict of interest.

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Data deposition: The crystal coordinates have been deposited in the Protein Data Bank, www.pdb.org [PDB ID codes 3T8V (PIA-M1-BTA) and 3T8W (PF-LAP-PNAP)].

See Author Summary on page 13885.
of enzyme classes, including serine hydrolases (24), peptidases (25), histone deacetylases (26), and kinases (27).

(-)-Bestatin is a natural product dipeptide analog of actinomycetes that potently inhibits multiple families of MAPs including the M1 and M17 families (28–31) (Fig. 1A). Importantly, bestatin has been shown to inhibit growth of P. falciparum parasites in culture and in mouse models of malaria (32–34). In addition, a recent study has indirectly implicated aminopeptidases in hemoglobin catabolism showing that parasites treated with bestatin had decreased levels of hemazoin formation, the detoxified biomineroy byproduct of HB digestion (34); likewise, isolectine uptake was decreased in these bestatin treated parasites. However, the MAP(s) targeted by bestatin, which are responsible for these processes, were not identified.

Herein, we report on a multidisciplinary effort combining bestatin-based, small molecule ABPs with biochemical and peptidic approaches to functionally analyze two essential aminopeptidases, PfA-M1 and Pf-LAP.

**Results**

Identification of Bestatin Targets in *P. falciparum*. Because of the paucity of genetic tools for analysis of essential proteins in *P. falciparum* and a lack of highly specific inhibitors with which to probe the individual roles of MAPs, we chose to study the functions of these essential parasite MAPs through the development and application of a MAP-specific ABPP platform. ABPP utilizes tagged mechanism-based inhibitors, or activity-based probes (ABPs), to characterize families or individual active peptidases within complex proteomes. ABPs typically possess two main structural components that contribute to their target specificity: (i) a mechanism-based inhibitor scaffold to covalently or noncovalently target catalytic residues or the active site of peptidases and (ii) a reporter tag, such as a fluorophore or biotin, for the visualization, characterization of labeling events, and eventual affinity purification of target proteins. The mechanism-based inhibitor scaffold ensures that ABPs bind to the enzyme(s) in an activity-dependent manner.

We decided to use the natural product, bestatin, as the scaffold for the development of ABPs for MAPs because it is a general MAP inhibitor, kills *Plasmodium* parasites, and is synthetically tractable using both solution and solid-phase chemistry (22, 35, 36). To identify the target(s) of bestatin in *P. falciparum*, we utilized a previously published bestatin-based ABP, MH01 (Fig. 1B) (22). MH01 contains a biotin moiety to allow for monitoring of protein binding and affinity purification for target identification (22) and also utilizes a benzophenone for irreversible UV cross-linking to protein targets, as the inhibition of MAPs with bestatin is noncovalent. Asynchronous cultures of 3D7 parasites were treated with saponin to lyse the erythrocyte and parasitophorous vacuole membranes and isolated whole parasites were harvested by centrifugation. Crude parasite lysates, including both soluble and membrane proteins, were treated with 5 μM MH01, exposed to UV light, and analyzed by Western blot using streptavidin-HRP to detect biotinylated proteins. The observed labeling pattern consisted of four bands at approximately 100 kDa, 70 kDa, 55 kDa, and 25 kDa (Fig. 1C). Western blot analysis with PfA-M1 or Pf-LAP antibodies of parasite lysates labeled with MH01 showed that all four labeled species could be accounted for with these two antibodies: Three bands corresponded to different species of PfA-M1 and one to Pf-LAP (Fig. 1C). PfA-M1 is known to be proteolytically processed from a 120 kDa proform to a 115 kDa intermediate yielding the p96 and p68 forms (16), both of which contain the catalytic domains and are labeled by MH01. The labeled p25 band is likely a secondary proteolytic breakdown product. We further confirmed that MH01 bound PfA-M1 and Pf-LAP using individual parasite lines expressing YFP-tagged versions of these proteins (13). After incubation of each YFP-tagged transgenic line with MH01, immunoprecipitation using streptavidin and Western blotting for YFP revealed that both PfA-M1-YFP and Pf-LAP-YFP were targeted by MH01 (Fig. 1D and E). Likewise, the reciprocal experiment involving the immunoprecipitation of YFP and Western blotting for biotin revealed that each YFP-tagged peptidase protein was biotinylated by MH01 (Fig. 1D and E). Specificity of the interaction was confirmed by pretreatment with unlabeled bestatin, which blocked labeling. Attempted labelings using a parasite line expressing PfAPP-YFP; the other DV-localized MAP, confirmed that it was not a target of MH01 (Fig. S1B in SI Appendix). These results

![Fig. 1. Identification of PfA-M1 and Pf-LAP as the targets of the antiparasitic MAP inhibitor bestatin. (A) Structure of bestatin. (B) Structure of MH01. (C) Identification of PfA-M1 and Pf-LAP as the parasite targets of bestatin. Parasite lysates were prepared by freeze-thaw lysis and subsequently labeled with 5 μM MH01, UV crosslinked, and analyzed by Western blot for biotin. Four proteins were labeled by MH01. The same blot was stripped and reprobed sequentially with antibodies for PfA-M1 and Pf-LAP; three MH01 labeled proteins were accounted for by PfA-M1 and fourth by Pf-LAP. (D) A parasite line expressing a YFP-tagged PfA-M1 protein was incubated with MH01 followed by immunoprecipitation for biotin (MH01) and Western blot analysis for YFP, which confirmed that PfA-M1 is targeted by MH01 (first panel). The reciprocal experiment involving the immunoprecipitation of PfA-M1-YFP (after incubation of parasites with MH01) using a YFP-specific antibody confirmed PfA-M1-YFP is biotinylated and thus labeled by MH01 (second panel). (E) Likewise, the same analysis was performed using a parasite line expressing a YFP-tagged Pf-LAP protein; incubation of these parasites with MH01, followed by immunoprecipitation of biotin (MH01) and Western blot analysis for YFP confirmed that MH01 labels Pf-LAP. The reciprocal experiment involving immunoprecipitation of Pf-LAP-YFP and analysis by Western blot for biotin confirmed that Pf-LAP-YFP is biotinylated by MH01. Lastly, MH01 labeling of YFP-tagged MAPs, in both cases, is blocked by pretreatment with unbiotinylated bestatin as seen as a lack of labeling. Input lanes below each panel show Western blot analysis using anti-YFP of total parasite lysate just before immunoprecipitation.
indicate that PfA-M1 and Pf-LAP are likely the only targets of bestatin in P. falciparum parasites. In addition, they highlight the difficulty in understanding the mechanism of bestatin toxicity, which could be due to the inhibition of either PfA-M1 or Pf-LAP or both.

MAP ABP Library Design and in Vitro Analysis Against PfA-M1 and Pf-LAP. To investigate the individual functions of the two MAP targets of bestatin and to gain insight into the mechanism of how bestatin kills parasites, we synthesized several libraries of bestatin-based ABPs with the intention of generating specific ABPs for both PfA-M1 and Pf-LAP. Bestatin has two side chains that can be diversified, which are derived from the constituent α-hydroxy-β-amino acid and a natural α-amino acid (Fig. 1A). These side chains straddle the active sites of MAPs where the α-hydroxy-β-amino acid side chain (termed P1) fits into the S1 pocket of the enzyme (N-terminal to the scissile bond) and the adjacent natural amino acid side chain (P1′) interacts with the S1′ pocket (C-terminal to the scissile bond) (37, 38). In our initial library, the P1′ leucine residue in bestatin was replaced with a series of natural amino acids (except cysteine and methionine, which are prone to oxidation; norleucine was included as an isostere for natural amino acids (except cysteine and methionine, which are the P1′ chains straddle the active sites of MAPs where the α-hydroxy-β-amino acid side chain (termed P1) fits into the S1 pocket of the enzyme (N-terminal to the scissile bond) and the adjacent natural amino acid side chain (P1′) interacts with the S1′ pocket (C-terminal to the scissile bond) (37, 38). In our initial library, the P1′ leucine residue in bestatin was replaced with a series of natural amino acids (except cysteine and methionine, which are prone to oxidation; norleucine was included as an isostere for methionine) and a limited number of nonnatural amino acids (Fig. 2A). Each library member was designed to incorporate a benzophenone to enable covalent attachment of the ABP to its targets and a terminal alkyne at the C terminus, to allow for the later addition of a variety of reporter tags using the bioorthogonal copper(I)-catalyzed [3 + 2] azide/alkyne cycloaddition (“click reaction”) (39-41). This library construction strategy increases the flexibility of downstream applications as each member has a “taggable” arm; thus, they can be used to directly treat live cells (as well as cellular lysates or recombinant enzymes) for target identification and activity profiling using a reporter tag, without any necessity for resynthesis or troubleshooting of tag placement.

We initially screened a P1′ diverse library against both recombinant PfA-M1 and Pf-LAP via standard fluorescence protease activity assays. Results of this experiment are presented as a heat map based on percent inhibition of PfA-M1 and Pf-LAP for each ABP (Fig. 2A). The S1′ pocket of Pf-LAP tended to favor aromatic side chains such as Phe, Tyr, and Naphthyl. One probe, Phe-Naphthyl (PNAP), showed strong specificity for Pf-LAP over PfA-M1. In addition, several side chains favored binding toward PfA-M1 over Pf-LAP, which tended to be either small (Ser, Ala) or positively charged (Lys, Arg). The probes Phe-Ala, Phe-Lys, and Phe-Arg showed moderate specificity for PfA-M1; however, we decided not to pursue further studies with the positively charged probes because of potential issues with cell permeability. Although the Phe-Ala ABP was somewhat specific for PfA-M1, we felt it was not yet suitably specific for further biological studies; thus we investigated modifications to the P1 side chain to achieve higher specificity.

To increase specificity for PfA-M1, we synthesized a second bestatin-based library that diversified the α-hydroxy-β-amino acid side chain (P1 position) using a fixed alanine at the P1′ position. Given structural information indicating that the S1 pocket of the PfA-M1 enzyme was hydrophobic (38), the P1 library was synthesized with a variety of natural and nonnatural hydrophobic P1 side chains including: Ala, Leu, Diphenyl, Naphthyl, Biphenyl, and (Benzyl)Tyr (Fig. 2B). Each library member had a clickable alkyne C-terminal to the benzophenone. This secondary ABP library was profiled against recombinant PfA-M1 and Pf-LAP and from the initial heat map analysis, the (Benzyl)Tyr-Ala ABP (BTA) gave the highest specificity of PfA-M1 over Pf-LAP.

**Fig. 2.** Bestatin-based ABP libraries reveal distinct chemotypes produced by ABPs with increased specificity for either PfA-M1 or Pf-LAP. (A) Representative structure of the bestatin-based ABP scaffold showing the point of diversification at the P1′ position. This ABP library was screened against recombinant PfA-M1 and Pf-LAP in single fixed concentrations. Results of the assay are displayed as a heat map: Red indicates higher potency; blue indicates lower potency. The Phe-Naph ABP showed high specificity for Pf-LAP. (BES* indicates parental bestatin) (B) A library of ABPs was synthesized to identify a probe with increased specificity for PfA-M1. Representative structure of the bestatin-based ABP scaffold showing the point of diversification at the P1 position. All compounds had an Ala at the P1′ position. Results of the assay are displayed as a heat map: Red indicates higher potency; blue indicates lower potency. The (Benzyl)Tyr-Ala ABP showed high specificity for PfA-M1. (C) Synchronized parasites were treated with each compound at 1 μM and assayed for morphological changes by light microscopy of Giemsa-stained blood smears through the erythrocytic lifecycle. Scale bar, 5 μm. ABPs more specific for PfA-M1 showed swelling of the DV, while probes more specific for Pf-LAP showed an early death chemotype.
To determine the morphological effects of inhibition using the probe libraries, parasite development was monitored throughout the entire lifecycle by Giemsa staining of thin blood smears (Fig. 2C). Three chemotypes (small molecule-induced morphological changes) were observed from this analysis: (i) no overt effect, (ii) early parasite death at the ring/trophozoite transition marked by pyknotic bodies, and (iii) swelling of the DV with parasite death occurring at the trophozoite/schizont transition. Importantly, the swollen DV as observed with these bestatin-based ABPs appeared translucent in Giemsa-stained smears, which distinguishes them from the dark swollen DV containing undigested Hb seen after treating parasites with papain family cysteine protease inhibitors such as E64 that target DV falcipains 2 and 3 (42).

Correlating the in vitro results with the live cell morphological screening results revealed that ABPs that produced the swollen DV chemotype displayed a high degree of specificity for PfA-M1 while ABPs more specific for Pf-LAP produced the early death, pyknosis chemotype. Compounds that failed to inhibit both MAPs, such as Phe-Asp (P1’-Asp) showed no chemotypes and were useful as negative control compounds (Fig. 2C). The contrasting chemotypes displayed by parasites treated with the most specific compounds, BTA or PNAP, suggested that PfA-M1 and Pf-LAP have essential yet distinct roles in the parasite.

**Evaluation of BTA and PNAP Potency and Specificity.** To quantitatively assess the relative specificity of BTA and PNAP, inhibition constants against recombinant PfA-M1 and Pf-LAP were determined (Fig. 3A). All ABPs bound rapidly to PfA-M1; in contrast, binding to Pf-LAP was slow, as has been reported for bestatin (see Materials and Methods for more details). Analysis of BTA inhibition revealed that the substitution of the P1’ Leu for Ala shifted the specificity moderately toward PfA-M1. With the substitution of the P1 Phe with (Benzyl)Tyr in BTA, the affinity of the ABP was only moderately changed for PfA-M1 (Fig. 3A and Fig. S2 in SI Appendix), while the inhibition constant for Pf-LAP radically dropped nearly 100-fold, resulting in an ABP with an estimated micromolar dissociation constant, $K_d$ (an estimate was necessary due to insolubility at high micromolar concentrations). Thus the overall change in the ratio of inhibition constants of PfA-M1 over Pf-LAP from bestatin to BTA was approximately 75-fold and the absolute specificity difference for PfA-M1 over Pf-LAP was at least 15-fold, making BTA a useful biological tool to study PfA-M1 function. Likewise, the substitution of a naphthyl group for the P1’ leucine of PNAP increased the affinity of PNAP for Pf-LAP, resulting in a 170-fold change in the specificity of PNAP for Pf-LAP relative to BTA and approximately a 12-fold difference in absolute specificity, creating a relatively specific inhibitor for Pf-LAP.

Although in vitro data suggested that BTA and PNAP were quite specific for PfA-M1 and Pf-LAP, respectively, these data do not rule out the possibility that the ABPs have other targets in parasites. To confirm the specificity of each probe in parasites, we utilized the alkyne on each ABP to “click” on a fluorophore (BODIPY) tag, to identify target(s) of BTA and PNAP in crude P. falciparum proteomes. The fluorescent ABPs were incubated with parasite lysates, UV-crosslinked and targets analyzed via in-gel fluorescent scanning. As predicted from the in vitro kinetic assays, BTA exclusively labeled bands identical in migration on gels to those recognized by antibodies to PfA-M1 in this complex proteome, while PNAP was specific for a band that correlated in migration with Pf-LAP (Fig. 3B).

To investigate the structural basis for the specificity of BTA for PfA-M1 we solved the X-ray cocystal structure of PfA-M1 bound to the BTA probe. The cocystal structure was solved to 1.8 Å, and electron density clearly resolved the BTA probe and linker but lacked any visible density for the “clickable” alkyne C-terminal tag (Fig. 3C, Left; see Fig. S3A in SI Appendix for stereoview and Table S1 in SI Appendix for statistics). The BTA ABP bound to the essential active site zinc ion via the hydroxyl and carbonyl groups (O2/O3) and central nitrogen of the bestatin scaffold (Fig. S3A in SI Appendix). The S1 pocket showed a slight movement (approximately 1.2 Å between c-o atoms of the key S1 residue, Glu572, of the two structures) to accommodate the (Benzyl)Tyr at the P1 position. The P1’ Ala moiety did not reach far into the S1’ pocket of PfA-M1 as the remaining probe positioned itself close to the S1 pocket (Fig. 3C, Left). We also modeled BTA into the X-ray crystal structure of Pf-LAP bound to bestatin (PDB ID code 3KR4). Superposition of BTA onto the bestatin core showed that the large (Benzyl)Tyr residue at the P1’ position clashed with the narrow S1 pocket of Pf-LAP (Fig. 3C, Right).

We also solved the cocystal structure of Pf-LAP bound to PNAP (Fig. 3D, Right; see Fig. S4 in SI Appendix for hexameric structure). The 2.0-Å X-ray structure resolved the structural basis for the PNAP specificity and potency for Pf-LAP. As expected the PNAP ABP bound in a similar manner to the parent bestatin (43)
dominated by coordination of two Zn^{2+} ions of the active site (Fig. S3B in SI Appendix). The P1-Phe ring of PNAP fit neatly into the small hydrophobic S1 pocket of Pf-LAP, and the P1’ naphthyl group also formed a series of hydrophobic interactions in the S1’ cleft. The only alteration noted to accommodate the P1’ naphthyl group was the movement of Ser550 (approximately 2.9 Å between c-α atoms of the Pf-LAP-PNAP structure versus the Pf-LAP-bestatin structure). This residue is located in a loop that lines the S1’ cleft, and the movement noted in the Pf-LAP-PNAP structure effectively flips the serine residue away from the naphthyl group, dragging the loop and preventing any close contacts with the P1’ residue (Fig. S3B in SI Appendix). It was also possible to model PNAP into the X-ray crystal structure of Pf-LAP bound to bestatin (PDB ID code 3EBH). Superposition of PNAP onto the bestatin core showed that the naphthyl side chain at the P1’ position clashed with the wall of the S1’ pocket of the active site in Pf-LAP (Fig. 3D, Left).

Inhibition of PfA-M1 Kills Parasites via Disruption of Hb Digestion Whereas Inhibition of Pf-LAP Kills via a Distinct Mechanism. After confirming the specificities of BTA and PNAP, we next wanted to more fully characterize the effects of these ABPs on parasites through their life cycle. To do this, synchronized parasites were treated at the ring stage and followed by light microscopy evaluation of Giemsa-stained thin blood smears (Fig. 4A). We found that treating parasites with BTA at its IC_{50} caused a delay in the life cycle and swelling of the DV at the trophozoite stage with eventual parasite death around 60 hr posttreatment. As a comparison, parasites treated with E-64d, a cysteine protease inhibitor that blocks DV falcipains (and initial endoproteolytic cleavage of Hb) had a similar delay and swollen DV (darkly stained rather than translucent) but remained alive at the 60-hr time point. In contrast, PNAP-treated parasites were arrested at the transition to the trophozoite stage; therefore it appeared that PNAP exerted its effect on parasites significantly earlier than the time of major Hb digestion. PNAP treatment caused no prominent morphological features (other than death), thus complicating hypothesis generation as to its mechanism of action. To our knowledge the only other inhibitor that kills rings is artesunate and the other members of the artemisinin family (also shown in Fig. 4A for comparative purposes); although the mechanism of action for artesunate may be different than PNAP it is intriguing

![Fig. 4. Inhibition of PfA-M1 kills parasites via disruption of Hb digestion whereas inhibition of Pf-LAP kills via a distinct mechanism. (A) Parasites were treated with BTA (10 μM), E-64d (10 μM), PNAP (3 μM), and artesunate (10 nM) at concentrations roughly equivalent to their IC_{50} and followed by Giemsa staining and light microscopy throughout the lifecycle. Scale bar, 5 μm. (B) DV swelling was confirmed by the dose-dependent enlargement of the average DV area. Parasites expressing YFP-tagged plasmepsin II (PMII-YFP), which localizes to the DV, were treated with increasing concentrations of BTA and measuring fluorescent DV area 20 hrs later using a minimum of 10 parasites (± SEM standard deviation). (C) DV swelling was determined to be saturable and quantified by treating the PMII-YFP parasites with increasing concentrations of BTA (PNAP was also used at 250 nM) at midring stage and measuring fluorescent DV area 20 hrs later using a minimum of 10 parasites (± SEM standard deviation). (D) Treatment of parasites with BTA in media lacking exogenous amino acids, except for isoleucine, results in a more than twofold decrease in the IC_{50} of BTA, while parasites treated with PNAP or artesunate show a nonsignificant difference. Shown are representative IC_{50} plots for each compound in both I-Media (lacking exogenous amino acids except for isoleucine), and AA-Media (containing all natural amino acids). The inlay bar graphs show differences of the mean IC_{50} of three experiments carried out in triplicate (*P < 0.05, Student’s t test).]
that there could be overlap among these two structurally divergent inhibitor classes (44).

Inhibition of PfA-M1 by BTA treatment of parasites caused a novel swollen DV chemotype. To investigate this phenomenon more closely we visualized the swelling of the DV in live parasites using a parasite line expressing YFP-tagged plasmepsin II that serves as a DV marker (45). Transgenic parasites were treated with increasing concentrations of BTA and evaluated by fluorescent microscopy (Fig. 4B). From these images, we estimated the relative average DV size (measuring 10 DVs) after each treatment. Parasites treated with as little as 250 nM BTA (the \( K_i \) of BTA for PfA-M1) showed a statistically significant increase in DV size relative to untreated parasites, with saturation of this swelling at 1 \( \mu \)M (Fig. 4B and C). The observation that the degree of DV swelling is dose-dependent and saturable is consistent with the hypothesis that, within this concentration range, PfA-M1 is likely the sole target and performs a key function in the DV.

In contrast, parasites treated with PNAP at a concentration over 50 \( \mu \)M, an inhibitor of other DV peptidases: DPAP1, PfAPP and falcipain 2. No inhibition of other proteases; thus we assayed for inhibition of other proteases such as aspartic proteases: E-64d, PAP, and falcipain 2. No inhibition of any enzyme was seen at a concentration up to 30 \( \mu \)M BTA, indicating that there is likely no cross-reactivity of BTA with these enzymes in live parasites and that the DV swelling is caused solely by inhibition of PfA-M1 (Fig. S6 in SI Appendix).

Because disruption in the endocytosis or subsequent catabolic breakdown of Hb is thought to be lethal to parasites (9), we hypothesized that PfA-M1 inhibition by BTA leads to starvation of the parasite via blockage of proteolysis of Hb peptides. To test this idea, we assayed whether parasites forced to rely only on Hb catabolism are more sensitive to BTA than parasites cultured with exogenous amino acids, by assaying the potency of BTA on parasites cultured in media lacking all amino acids except isoleucine (the only amino acid not present in Hb). Indeed, parasites were sensitized by approximately 2.4-fold to inhibition by BTA in media with only isoleucine (Fig. 4D). In contrast, parasites treated with PNAP or the antimalarial arsunate, which kills ring stage parasites prior to initiation of large-scale Hb degradation and thus acts as a negative control, showed statistically insignificant differences in sensitization to either compound in the isoleucine media. This evidence suggests a role for PfA-M1 in the Hb digestion pathway and also provides further evidence that the primary role of Pf-LAP is not within the Hb digestion pathway.

Initial proteolytic events are thought to be carried out by the redundant endopeptidases falcipains 2/3, plasmepsins I, II, IV, and HAP (46). Hb-derived oligopeptides are then broken down by exopeptidases. Considering that PfA-M1 is an aminopeptidase, its likely role in the DV would occur after initial Hb proteolysis by the endopeptidases. To confirm this, we treated synchronous cultures of parasites during the trophozoite stage, in which the majority of Hb degradation takes place. Fig. S7 in SI Appendix shows that parasites treated with E-64d leads to an accumulation of full-length Hb and causes the swelling of the DV with undigested Hb (42). Conversely, parasites treated with BTA showed no inhibition of proteolysis of full-length Hb but still caused the DV to swell. Treatment of parasites with PNAP was similar to DMSO.

### Inhibition of PfA-M1 Blocks Proteolysis of Specific Hb-Derived Oligopeptides

To obtain direct evidence for the role of PfA-M1 in the proteolysis of Hb-derived oligopeptides, we endeavored to find peptide substrates for this enzyme. We used a mass spectrometry-based peptidomics approach to assay the relative abundance of peptides (<10 kDa) in parasites either untreated or treated with the specific PfA-M1 inhibitor BTA. To do this, trophozoite stage parasites were treated with BTA or DMSO for 24 hr. Whole parasite extracts were prepared and peptides were enriched by an acid extraction followed by a filtration through a 10 kDa filter column. Resulting peptide extracts were analyzed by nano LC-MS/MS. Mass spectrometry analysis of the peptide peaks against both Hb \( \alpha \) and \( \beta \) sequences revealed that the great majority of these oligopeptides showed no difference between the treated and un-

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**Fig. 5.** Inhibition of PfA-M1 blocks proteolysis of specific Hb oligopeptides. (A) Global peptide profiling of treated parasites identifies a subset of peptides that accumulate in BTA-treated parasites relative to DMSO. Peptides were extracted from either DMSO or BTA-treated parasites and peptides analyzed by LC-MS/MS. Peptides identified are displayed according to elution time, intensity, and M/Z. Area of the peptide peak corresponds to relative abundance. (B) LC-MS/MS sequencing of the peptides reveals accumulated peptides are derived from Hb. Ratio of peptide abundance was calculated by determining the area of peptide intensity of BTA-treated vs untreated. (C) A synthesized version of an abundantly accumulated peptide, identified in the prior LC-MS/MS analysis, was efficiently proteolyzed at the N-terminal valine by PfA-M1, while DPAP1, the other likely essential DV aminopeptidase, failed to catalyze any proteolysis. Shown are HPLC traces displaying the synthetic peptide without enzyme (top trace), with PfA-M1 (middle trace), or with DPAP1 (lower trace). Insets show peptide sequences corresponding to HPLC peaks.

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treated samples; however, several oligopeptides, from both the α and β chains of Hb, appeared to accumulate after treatment of parasites with BTA (Fig. 5 A and B).

Further sequence analysis of the accumulated Hb-derived oligopeptides revealed that the majority were likely poor substrates for DPAP1, the other essential aminopeptidase with broad substrate specificity found in the DV (47). We thus hypothesized that PFA-M1 was necessary for proteolysis of these oligopeptides. To test this idea, a highly enriched peptide that was identified from the peptidomics study of BTA-treated samples was resynthesized and shown to be resistant to cleavage by DPAP1, yet efficiently cleaved by PFA-M1 (Fig. 5C). This data provides direct evidence of a role for the genetically essential enzyme, PFA-M1, in the digestion of small Hb-derived oligopeptides in *P. falciparum*.

**Discussion**

Peptidases likely have many essential functions in *P. falciparum*, yet the biological roles of the majority of putative proteases encoded by the parasite genome remain to be characterized. One reason for this rests in the difficulty in genetically manipulating essential parasite genes. To circumvent this deficit in genetic tools, a small molecule approach may be used to perturb and thus investigate essential protein functions in the parasite. Several issues arise from the use of small molecule probes, including (i) target identification, (ii) specificity, and (iii) permeability in live cells. To address some of these issues, we generated a library of MAP-specific, “clickable” ABPs. Replacement of a bulky reporter tag with an alkyne group resulted in smaller, more versatile ABPs that is thought to be exchanged for isoleucine via an antiport (i.e., it is hard to completely rule out the possibility of a minor role for Pf-LAP in the major period of Hb degradation suggests that the essential role for Pf-LAP is complicated by the fact that its inhibition did not yield any overt morphological change in the parasite other than death.

From our peptidomics experiments we showed that Hb oligopeptides were substrates for PFA-M1 proteolysis. One issue with this analysis is that small peptides were not found using our mass spectrometry method, which was limited to the identification of peptides greater than four amino acids in length. It is likely that the concerted action of DV endo- and exoproteases also produced smaller tri- and dipeptides, which are substrates for PFA-M1 in the DV. In support of this idea, we also attempted to identify small peptide species using a Single Quad LC/MS (which allows for profiling peptides between 200 and 400 kDa) that accumulated in BTA-treated parasites (Fig. S8 in *SI Appendix*). These low molecular weight species all matched to predicted dipeptide molecular weights, and more than half were the molecular weight of dipeptides found in Hb. However, this method precluded the definitive identification of these molecules as peptides (as opposed to metabolites) and their origin (i.e., Hb). However, we believe these data are suggestive that several dipeptides, in addition to oligopeptides, are likely important substrates for the PFA-M1 enzyme.

Our data using the PNAP ABP for Pf-LAP indicated that this enzyme has an important role quite early in the intraerythrocytic life cycle rather than during the major period of Hb digestion. Formulating a testable hypothesis about a specific role for PNAP is complicated by the fact that its inhibition did not yield any overt morphological change in the parasite other than death. However, we suspect PNAP may have an essential housekeeping function in the cytosolic turnover of dipeptides (49) and perhaps acts in concert with the parasite proteasome, as has been shown for other neutral cytosolic leucine aminopeptidases pathways (50). Like PNAP, lethal amounts of proteasome inhibitors exert their effect in the ring-trophozoite transition and parasites do not progress into the later trophozoite stage (51). Our data does not completely rule out the possibility of a minor role for PNAP in the Hb degradation pathway via proteolysis of Hb-derived dipeptides that have been transported from the DV into the cytoplasm. However, the fact that PNAP-treated parasites die prior to the major period of Hb degradation suggests that the essential role for Pf-LAP is not within the Hb digestion pathway.

Our collective data suggest that these two MAPs are both potential antiparasitic drug targets. In fact, PNAP is, to our knowledge, the most potent parasite MAP inhibitor with an IC$_{50}$ in the 200 nM range, which gives us hope that these types of inhibitors could be further developed into more drug-like therapeutics. In addition, *P. falciparum* MAPs share little homology with their human counterparts; less than 35% in the case of the M1 family proteases. It is therefore reasonable to suggest that potent, specific inhibitors of *P. falciparum* MAPs can be designed over human MAPs. In addition, information gleaned from our preliminary SAR and crystallography efforts may provide a jumping off point for future medicinal chemistry efforts against both enzymes. Our data here suggest that combination therapy involving endopeptidase inhibitors, such as those for falcipains, and PFA-M1-specific inhibitors might provide an opportunity for a synergistic drug combination (52). Ultimately, this strategy may represent a good way to reduce the chance of parasite resistance.

**Materials and Methods**

**General Methods.** See *SI Appendix* for additional chemical and experimental protocols. A summary of the methods is given below.

**Parasite Culture and IC$_{50}$ Determination for Bestatin-Based ABPs.** Briefly, 3D7 parasites were cultured in RPMI 1640 (Invitrogen) supplemented with Albu- max II (Invitrogen). For synchronization, schizont stage parasites were magnet purified using a SuperMACS™ II Cell Separation Unit (Miltenyi Biotech). For IC$_{50}$ determinations, synchronized parasites were plated at 1% parasitemia and 6% hematocrit in 96-well plates at a total volume of 50 μL. Serial dilutions of 2x concentration of the respective compound were added to the wells to bring the total volume up to 100 μL and 0.5% parasitemia and 3% hematocrit. Compounds were assayed for a 72 h period, after which...
2X Vybrant DyeCycle Green DNA (Invitrogen) in PBS was added for a final concentration of 10 μM and incubated at 37 °C for 30 min. DNA content, as an indicator of parasitemia, was analyzed on an Accuri C6 Flow Cytometer with C-Sampler. IC₅₀ curves were generated using GraphPad Prism (GraphPad Software).

Labeling of Parasite MAPs with Activity-Based Probes. For parasite labeling, mixed stage parasites were harvested and released from erythrocytes with 1% saponin followed by centrifugation at 1,500 × g for 5 min and 3 washes in cold PBS. Parasite lysates were prepared by freeze-thaw in 50 mM Tris-HCl pH 7.0, 50 mM NaCl, 10 μM ZnCl₂, and protease inhibitor cocktail (EDTA-free) (Roche) and extracts were clarified by centrifugation at 1,100 × g for 10 min at 4 °C. Labeling was performed with indicated concentrations of the ABP for 1 hr at 37 °C followed by UV crosslinking (365 nm) for 1 hr on ice. Competition of labeling was carried out by preincubating lysates for 1 hr at 37 °C. For immunoprecipitation, lysates were passed through 7 K MWCO desalting columns (Pierce) after UV crosslinking then incubated overnight with streptavidin Ultralink Resin (Pierce). Proteins were visualized by standard Western blotting and VECTASTAIN ABC kit (Vector Labs) or rabbit anti-GBP (ab6556, Abcam). For fluorescent probes, labeled proteins were visualized in-gel using a Typhoon flatbed scanner (GE Healthcare).

Synthesis of Bestatin-Based ABP Libraries. A detailed description of the synthesis and characterization of these compounds may be found in SI Appendix.

Recombinant Proteins. Details of the expression in Escherichia coli and purification of recombinant Pf-M1 (residues 192 to 1,085) will be described separately (53). Pf-LAP lacking the N-terminal Asn-rich region (residues 79-260) was expressed with a C-terminal hexahistidine tag in Escherichia coli and purified as previously described (43). The estimated molecular mass of the purified species from size exclusion chromatography (343 kDa) was in good agreement with the predicted mass for the hexameric enzyme (357 kDa). The purification of recombinant DPA1 has been published (47).

X-ray Crystallography. Pf-M1 and Pf-LAP enzymes were purified and crystallized as previously described (38). Crystals of the Pf-M1-BTA complex were obtained by cryocrystallization of BTA with Pf-M1 in mother liquor containing 20% PEG 3350, 100 mM HEPES pH 7.0, 10 mM MgCl₂, and protease inhibitor cocktail (EDTA-free) (Roche). The estimated molecular mass of the purified species from size exclusion chromatography (343 kDa) was in good agreement with the predicted mass for the hexameric enzyme (357 kDa). The purification of recombinant DPA1 has been published (47).

Determination of Kᵢ and Kₒ Values. Kᵢ values for inhibition of Pf-M1 were determined by Dixon plots and a detailed protocol for determining Pf-LAP Kₒ values may be found in SI Appendix.

Mass Spectrometry-Based Peptide Profiling. Briefly, parasites were treated with 2 μM BTA or DMSO at the midring stage. Parasites were treated for 24 hr at which point they were harvested by saponin treatment, centrifuged, and stored at −80 °C in the presence of protease inhibitors. To isolate peptides, parasite samples were boiled in water for 10 min and then centrifuged for 10 min at 18,000 × g. The supernatant was saved, and the pellet was re-suspended in 0.25% acetic acid and disrupted by freeze-thaw and microcentrifuged. All fractions were combined and centrifuged at 20,000 × g for 20 min. The supernatant was passed through a 10 kDa molecular weight cutoff filter (Millipore).

The retention times and m/z values of the peptides identified were used to map corresponding peptide peaks in the chromatograms generated from in-source fragmentation with C-Sampler. IC₅₀ values may be found in SI Appendix. Mass spectrometry analysis of common peptides was performed in order to verify the accuracy of the tandem MS analysis. Multiple peptides were manually aligned and then for semiquantitative assessment of the abundances of individual peptides, the total peak areas were determined using the Bioworks algorithm PepQuan (the Area/Height Calculation) with parameters set to area, mass tolerance of 1.5, minimum threshold of 5,000, five smoothing points, and including all proteins. The alignment was based on retention times, m/z values, and patterns of peaks in close proximity.

ACKNOWLEDGMENTS. We thank the Australian Synchrotron for beamtime and Tom Caradoc-Davis in particular for technical assistance. We thank John Dalton, PhD, McGill University, for the Pf-LAP antisera. Authors acknowledge the support by Penn Genome Frontiers Institute (D.C.G.), Ritter Foundation (D.C.G.), 10°A1-076342-01 (D.B.), 10°A1-077638 (M.K.), NHST32A007532 (M.B.H.), and 1R56-A1-081770-01A2 (D.C.G.). S.M. is an Australian Research Council (ARC) Future Fellow, J.C.W. is an ARC Federation Fellow and a National Health and Medical Research Council (NHMRC) Principal Research Fellow. We thank the NHMRC and the ARC for funding support.
A bestatin-based chemical biology strategy reveals distinct roles for malarial M1- and M17-family aminopeptidases

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S1: Full blots from Figure 1E and attempted labeling of PfAPP by MH01. (a) Complete blots of the IP/Westerns from Figure 1E show that PfA-M1-YFP is processed and Pf-LAP-YFP migrates as a single band. (b) A parasite line expressing a YFP-tagged PfAPP protein was incubated with MH01 followed by immunoprecipitation for biotin (MH01) and western blot analysis for YFP, which confirmed is not targeted by MH01. The reciprocal experiment involving the immunoprecipitation of PfAPP-YFP (after incubation of parasites with MH01) using a YFP-specific antibody revealed no biotinylation of the protein (second panel).

S2: Morphological and biochemical profiling of bestatin and PheLeu. (a) In vitro kinetic evaluation of the PheLeu ABP. (b) Activity-based profiling using a fluorescent version of MH01 reveals no change in probe specificity relative to the biotinylated MH01.
S3 : Stereo diagrams of inhibitors bound to active site of PfA-M1 and Pf-LAP. (a) 1.8 Å PfA-M1-BTA and (b) 2.0 Å Pf-LAP-PNAP. Inhibitors BTA and PNAP are colored in green, carbon atoms of PfA-M1 and Pf-LAP residues are colored grey. Zinc is shown as black spheres. Hydrogen and metallo-bonds are indicated (dashed black lines). Electron density shown is a composite omit map contoured to 1.0 \( \sigma \) and calculated using CNS. For clarity only electron density of each ligand, zinc ions and Pf-LAP carbonate ion (b) is shown.
**S4** : Cartoon diagrams of the biologically functional Pf-LAP hexamer colored by chain: A (green); B (cyan); C (wheat); D (yellow); E (salmon); F (grey). (a) The cartoon diagram of PNAP bound to active sites of biological unit of Pf-LAP. PNAP is colored in magenta. Zinc ions are shown as black spheres. (b) Cartoon diagram of Pf-LAP-PNAP where chains D-F are excluded to show PNAP binding to active sites of chains A-C that line the interior cavity of the hexamer.

**S5** : Bestatin and PheLeu probe treatments do not cause DV swelling. (a) Parasites expressing YFP-tagged plasmepsin II, which localizes to the DV, were treated with bestatin or PheLeu probe (each 10 µM) and imaged by fluorescence microscopy. (b) Quntitation of DV size of the treated PMII-YFP parasites indicates a lack of DV swelling upon treatment with either compound. (c) A concentration-response curve showing the effect of bestatin-treatment on *P. falciparum* culture in 1-Media versus media containing all amino acids.
**S6 : DV-localised endoproteases are not inhibited by BTA.** Activity assays for falciparin 2/2’, DPAP1 and PfAPP show that no inhibition occurs at 30 µM BTA.

**S7 : Inhibition of PfA-M1 causes DV swelling but does not prevent proteolytic cleavage of full length Hb.** Parasites treated with BTA (1 mM) and PNAP (0.25 mM) are capable of initiating Hb degradation, as shown by an absence of full length Hb subunits (17 kDa) in both untreated and BTA-treated parasites, in contrast to parasites treated with E64-d, which disrupts the initial endoproteolytic cleavage of Hb.
S8: Small dipeptide species accumulate in BTA-treated parasites. (a) The LC trace identifies a peak that increases in the lysates of BTA-treated parasites. (b) The MS profile identifies the species with a molecular weight of 282 Da, which may corresponds to a Thr-Tyr dipeptide from Hb. Another MS trace identifies a putative Hb-derived His-Lys dipeptide, with a molecular weight of 283. (d) A table of the putative dipeptides species identified in BTA-treated parasites.
## Supplementary Table 1 | Data Collection and refinement statistics

<table>
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<th>Data collection</th>
<th>rP/A-M1_BTA</th>
<th>Pf-LAP-PNAP</th>
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<td>$P2_12_12_1$</td>
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<td>a=173.8, b=177.1, c=231.2, b=90.0°</td>
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<tr>
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</tr>
<tr>
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<tr>
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<td>$R_{	ext{pim}}$ (%)</td>
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### Structure refinement

#### Non hydrogen atoms

<table>
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<th></th>
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<th>Pf-LAP-PNAP</th>
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<td>Mean water molecule</td>
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### Agreement between intensities of repeated measurements of the same reflections and can be defined as: $\sum (I_{h,i} - <I_h>) / \sum I_{h,i}$, where $I_{h,i}$ are individual values and $<I_h>$ is the mean value of the intensity of reflection $h$. **Values in parentheses refer to the highest resolution shell.**
Supplementary Methods

Determination of $K_i$ and $K_i^*$ values

Bestatin has been shown to be a slow-binding inhibitor of leucine aminopeptidases, including Pf-LAP, with the slow step involving a conformational change of the initially-formed low affinity enzyme-inhibitor complex (EI) to form a tight complex (EI*; Scheme X)\(^1\)-\(^3\). $K_i$ is the dissociation constant for the initial enzyme-inhibitor complex whereas $K_i^*$ is the overall inhibition constant and is defined as $[E][I]/([EI]+[EI^*])$ \(^4\). $K_i^*$ values were determined for the di-Zn form of Pf-LAP\(^3\) in 50 mM Tris-HCl pH 8.0 containing 50 mM ZnCl\(_2\), 250 mM Leu-AMC, 0.1% Triton X-100, 180 ng/mL Pf-LAP and inhibitor at 25 °C. Changes in fluorescence upon mixing of substrate and inhibitor with enzyme were monitored in 96 well plates using a Victor\(^3\) microplate fluorometer. Progress curves were followed for 160 minutes and fit to the equation for slow-binding inhibition $[P] = v_o t + (v_o - v_s)(1 - e^{(-k_{obs} t)}/k_{obs}$, where $v_o$ is the initial rate, $v_s$ is the steady-state rate and $k_{obs}$ is an apparent first-order rate constant for the formation of the high affinity enzyme-inhibitor complex, EI*\(^4\). At the inhibitor concentrations necessary to produce well-defined progress curves (at least two half-times), $v_s$ was typically $< 5\%$ of the uninhibited velocity making steady-state approaches to determining $K_i^*$ (i.e. Dixon plot) unfeasible. Instead $K_i^*$ was determined from plots of $k_{obs}$ vs. $[I]$. In the case of bestatin, the data defined a hyperbolic curve. $k_6$ was determined from the relationship $k_6 = v_o/v_o^*k_{obs}$ and was added to the data set ($k_{obs} = k_6$ when $[I] = 0$) to better define the hyperbolic curve. Data were fit by non-linear regression to the equation $k_{obs} = k_6 + k_5[I]/(K_{i}^{app} + [I])$, where $K_{i}^{app} = K_i(1 +$
Under our assay conditions the $K_m$ for Leu-AMC was 1.1 mM. $K_i^*$ was calculated from the relationship $K_i^* = k_6K_i/(k_5 + k_6)$ where $K_i$ and $(k_5 + k_6)$ were determined from curve fits and $k_6$ was determined as described above. For bestatin probe, Phe-Ala probe and BTA probe, plots of $k_{obvs}$ vs. $[I]$ were linear, a situation that can arise if $K_i$ is much greater than the inhibitor concentrations used in the assays. In these cases $k_{obvs}$ vs. $[I]$ plots were fit by linear regression yielding a slope of $k_6/K_{i*app}$, determination of $k_6$ as described above enabled calculation of $K_{i*app}$ and thus $K_i^*$. With BTA probe, the combination of low affinity for Pf-LAP and insolubility at high micromolar concentrations restricted the range of $k_{obvs}$ values that could be determined compared to those for bestatin and Phe-Ala probes. However, sufficient data were available to allow an estimate for the $K_i^*$ value to be made.

Scheme 1:

\[ \begin{align*}
E + I & \overset{k_4}{\underset{k_5}{\rightleftharpoons}} EI \\
& \overset{k_6}{\underset{k_4}{\rightleftharpoons}} EI^* \\
\end{align*} \]

**X-ray Crystallography**

Diffraction images were processed using MOSFLM and SCALA from the CCP4 suite. 5% of each dataset was flagged for calculation of $R_{Free}$ with neither a sigma nor a low-resolution cut-off applied to the data. Subsequent crystallographic and structural analysis was performed using the CCP4i interface to the CCP4 suite, unless stated otherwise. The inhibitor complex was initially solved and refined against the unbound PfA-M1 and Pf-LAP
structure (protein atoms only) as described previously\textsuperscript{11} and clearly showed unbiased features in the active site for both structures. Superposition of BTA into the Pf-LAP active site was performed using the X-ray crystal structure of Pf-LAP-bestatin (3KR4.pdb) where the bestatin scaffold was used to superpose BTA. Superposition of PNAP into the PfA-M1 active site was performed using the X-ray crystal structure of PfA-M1-bestatin (3EBH.pdb) where the bestatin scaffold was used to superpose PNAP. Pymol\textsuperscript{12} was used to produce structural representations (http://www.pymol.org). Hydrogen bonds (excluding water-mediated bonds) were calculated using Lig_contact and CONTACT\textsuperscript{8}. CCP4MG was used to produce electrostatic diagrams\textsuperscript{10}. Lys and Arg residues were assigned a single positive charge, and Asp and Glu residues were assigned a single negative charge; all other residues were considered neutral. The calculation was done assuming a uniform dielectric constant of 80 for the solvent and 2 for the protein interior. The ionic strength was set to zero. The color of the surface represents the electrostatic potential at the protein surface, going from blue (potential of $+10 \, kT/e$) to red (potential of $-10 \, kT/e$), where T is temperature, e is the charge of an electron, and k is the Boltzmann constant. The probe radius used was 1.4 Å. The coordinates and structure factors will be available from the Protein Data Bank (XXX.pdb and XXX.pdb). Raw data and images will be available from TARDIS\textsuperscript{13} (www.tardis.edu.au).

**Fluorescence imaging**

Trophozoite state parasites were treated for 12 hr with the compounds and
concentrations as shown in the figure. Images were obtained using a Leica DMI6000 B microscope and Leica LAS AF software. Parasite and DV sizes were quantified using ImageJ.

**Mass spectrometry-based peptide profiling**

No reduction and alkylation was performed. The resulting peptide extracts were desalted and concentrated on ZipTip (Millipore) according to manufacturer’s instruction and then analyzed by nanoLC-MS/MS using an LCQ Deca XP Plus ion trap mass spectrometer equipped with a nanoelectrospray ion source (Thermo Fisher Scientific Inc., San Jose, CA) coupled to an Ultimate Nano liquid chromatography system (Dionex Corporation, Sunnyvale, CA). Fully automated analyte sampling, chromatography, and production and acquisition of MS and MS-MS data were performed essentially as described earlier\(^\text{14}\) with slight modifications. A Famos 48-well plate autosampler (Dionex) was used to sample the peptide extract in batch mode with a user-defined program to withdraw 1 µl from each sample. The aqueous buffer (A) (5% acetonitrile in water containing 0.1% formic acid) and the organic buffer (B) (80% acetonitrile in water containing 0.1% formic acid) were delivered by an Ultimate (Dionex) micropump as follows: 5% B in the first 6 min, 5 to 50% B from 6 to 96 min, 50 to 95% B from 96 to 97 min, 95% B from 97 to 101 min, 95 to 5% B from 101 to 102 min, and 5% B from 102 to 122 min. The peptide mixture was fractioned on a Dionex PepMap C\(_{18}\) column (75-µm [inner diameter] by 150 mm) with a 3-µm particle size and a 100-Å-pore diameter. Mass spectrometry scans as well as HPLC solvent gradients
were controlled via the Xcalibur 2.0.7 SP1 software (Thermo Fisher Scientific Inc.). The eluted peptides were directly introduced into the MS equipment via nanoESI source. The LCQ DecaXP was operated in a data-dependent mode where the machine measured intensity of all the ions in the mass range from 200 to 800 (mass-to-charge ratios) and isolated the three most intense ion peaks for collision-induced dissociation using a normalized collision energy level of 35%. Dynamic exclusion was enabled at repeat count 2, repeat duration 0.5 min, exclusion list size 25, exclusion duration 3 min and exclusion mass width ±1.5 m/z. Data analysis was accomplished with SEQUEST (BioWorks 3.3.1 SP1, Thermo Fisher Scientific Inc.) using both hemoglobin a and b sequences. Following dta generation parameters were used: peptide mass range, 200–800 Da; minimal total ion intensity threshold, 50,000; minimal number of fragment ions, 15; precursor mass tolerance, 1.4 Da; group scan, 0; group count, 1. “No enzyme” was selected for database search. Mass tolerance for precursor and fragment ions was 2 and 1.0 Da, respectively. No chemical or posttranslational modifications were considered.

**Peptide Synthesis**

The peptide VDPENF was synthesized on an Argonaut synthesizer using standard Fmoc solid phase peptide synthesis on Rink-amide resin. Peptides were purchased from Bachem (Switzerland). Rink-amide resin (0.69 mmol/g) was purchased from Novabiochem (San Diego, CA). All other chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA). Peptide
assembly was performed using HBTU (2-(1H—benzotriazole-1-yl)-1,1,3,3-
tetramethyluronium hexafluorophosphate) activation (5 and 10 equivalents respectively) of amino acids (5 eq) in DIEA (N,N’-diisopropylethylamine) and NMP (N-methylpyrrolidone). The Fmoc protecting group was removed with 20% piperidine in DMF, for 5 min. After deprotection and again after amino acid coupling, the reaction vessel was rinsed 3 times with NMP followed by 3 rinses with DMF. The N-terminus of the peptide was not capped. The peptide was cleaved from the resin using 93% TFA (trifluoroacetic acid), 2% TIPS (triisopropylsilane), and 5% water. The peptide was precipitated twice in 40 mL cold diethyl ether and dried overnight. To purify, the peptide was dissolved in 95% water, 5% acetonitrile and 0.1% formic acid (Solvent A) and run on a semi-preparative C18 column on an Agilent HPLC (15% to 35%, Solvent B) (Agilent Technologies). Pure fractions were confirmed by ES-API (calculated m/z 719.31, found m/z 719.2).
Synthesis procedures:

I) General method for solid-phase peptide synthesis of bestatin based ABPs (P1’ library): Standard solid-phase peptide synthesis was performed on Rink amide resin, using HBTU/HOBt/DIEA in an equimolar ratio in DMF for 30 min at RT. Coupling of the \(\alpha\)-hydroxy-\(\beta\)-amino acid required HATU for 1 hr. Each amino acid was double coupled. Fmoc protecting groups were removed with 20% piperidine/DMF (30 min). The deprotection of the Aloc group was conducted under a positive flow of argon. The resin was solvated with dichloromethane for 5 min. The solvent was drained, and PhSiH\(_3\) (24 eq.) in CH\(_2\)Cl\(_2\) was added to the resin followed by Pd(PPh\(_3\))\(_4\) (0.25 eq.) in CH\(_2\)Cl\(_2\). After agitating the resin for 1 h by bubbling with argon, the solution was drained, and the resin was washed with CH\(_2\)Cl\(_2\) (3x). To cleave products from resin, a solution of 95%TFA:2.5%TIS:2.5%H\(_2\)O was added to the resin. After standing for 2 h, the cleavage mixture was collected, and the resin was washed with fresh cleavage solution (Scheme 1). The combined fractions were evaporated to dryness and the product was purified by reverse phase-HPLC. Fractions containing product were pooled and lyophilized. Reverse phase HPLC was conducted on a C18 column using an Agilent 1200 HPLC. Purifications were performed at room temperature and compounds were eluted with a concentration gradient 0-70% of acetonitrile (0.1% Formic acid). LC/MS data were acquired using LC/MSD SL system (Agilent). HRMS was recorded at the UCRiverside mass spectrometry
facility. Solid-phase peptide chemistry was conducted in polypropylene cartridges, with 2-way Nylon stopcocks (Biotage, VA). The cartridges were connected to a 20 port vacuum manifold (Biotage, VA) that was used to drain excess solvent and reagents from the cartridge. The scheme for the synthesis of ABPs of bestatin may be depicted as follows.

**Scheme 1:** Synthesis of P1' library of bestatin ABPs

Reagents and conditions: (a) i-20% Piperidine/DMF; ii-Fmoc-Lys(Alloc)OH, HBTU, HOBr, DIEA; iii-20% Piperidine/DMF; iv-Fmoc-BpaOH, HBTU, HOBr, DIEA; v-20% Piperidine/DMF; vi-Fmoc-PPGoch (9 atoms), HBTU, HOBr, DIEA; vii-20% Piperidine/DMF; viii-Fmoc-ROH, HBTU, HOBr, DIEA; ix-20% Piperidine/DMF; x-N-Boc-(2S,3R)-3-amino-2-hydroxy-4-phenyl butyric acid, HATU, DIEA; b) i-Pdc(PPh3)4, PhSH3, DCM; ii-hexynoic acid, HBTU, HOBr, DIEA; iii-95%/TFA:2.5%/TIS:2.5%H2O.

**PheGlyBes (1):** colourless solid; 15% yield after HPLC purification; ESI-MS: 870.3 [M+H]+; HRMS: found [M+H]+ 870.4404. C48H59N7O10+ requires 870.4402.

**PheAspBes (2):** colourless solid; 18% yield after HPLC purification; ESI-MS: 928.3 [M+H]+; HRMS: found [M+H]+ 928.4448. C48H62N7O12+ requires 928.4456.
**PheHisBes (3):** colourless solid; 16% yield after HPLC purification; ESI-MS: 950.3 [M+H]^+; HRMS: found [M+H]^+ 950.4763. C_{50}H_{64}N_{9}O_{10}^+ requires 950.4776.

**PheNleBes (4):** colourless solid; 16% yield after HPLC purification; ESI-MS: 926.3 [M+H]^+; HRMS: found [M+H]^+ 926.5032. C_{50}H_{68}N_{7}O_{10}^+ requires 926.5028.

**PheGlnBes (5):** colourless solid; 16% yield after HPLC purification; ESI-MS: 941.3 [M+H]^+; HRMS: found [M+H]^+ 941.4780. C_{49}H_{65}N_{8}O_{11}^+ requires 941.4773.

**PhelleBes (6):** colourless solid; 15% yield after HPLC purification; ESI-MS: 926.3 [M+H]^+; HRMS: found [M+H]^+ 926.5036. C_{50}H_{68}N_{7}O_{10}^+ requires 926.5028.
**PhePheBes (7):** colourless solid; 18% yield after HPLC purification; ESI-MS: 960.3 [M+H]^+; HRMS: found [M+H]^+ 960.4863. C_{53}H_{66}N_{7}O_{10}^+ requires 960.4871.

![PhePheBes structure](image)

**PheLeuBes (8):** colourless solid; 18% yield after HPLC purification; ESI-MS: 926.3 [M+H]^+; HRMS: found [M+H]^+ 926.5004. C_{50}H_{68}N_{7}O_{10}^+ requires 926.5028.

![PheLeuBes structure](image)

**PheGluBes (9):** colourless solid; 16% yield after HPLC purification; ESI-MS: 942.3 [M+H]^+; HRMS: found [M+H]^+ 942.4617. C_{49}H_{64}N_{7}O_{12}^+ requires 942.4613.

![PheGluBes structure](image)

**PheAsnBes (10):** colourless solid; 18% yield after HPLC purification; ESI-MS: 927.2 [M+H]^+; HRMS: found [M+H]^+ 927.4619. C_{48}H_{63}N_{8}O_{11}^+ requires 927.4616.

![PheAsnBes structure](image)

**PheThrBes (11):** colourless solid; 20% yield after HPLC purification; ESI-MS: 914.3 [M+H]^+; HRMS: found [M+H]^+ 914.4661. C_{48}H_{64}N_{7}O_{11}^+ requires 914.4664.
**PheProBes (12):** colourless solid; 19% yield after HPLC purification; ESI-MS:
910.3 [M+H]^+; HRMS: found [M+H]^+ 910.4693. \( \text{C}_{49}\text{H}_{64}\text{N}_{7}\text{O}_{10}^+ \) requires 910.4715.

**PheValBes (13):** colourless solid; 18% yield after HPLC purification; ESI-MS:
912.3 [M+H]^+; HRMS: found [M+H]^+ 912.4884. \( \text{C}_{49}\text{H}_{66}\text{N}_{7}\text{O}_{10}^+ \) requires 912.4871.

**PheSerBes (14):** colourless solid; 15% yield after HPLC purification; ESI-MS:
900.2 [M+H]^+; HRMS: found [M+H]^+ 900.4508. \( \text{C}_{47}\text{H}_{62}\text{N}_{7}\text{O}_{11}^+ \) requires 900.4507.

**PheTrpBes (15):** colourless solid; 16% yield after HPLC purification; ESI-MS:
999.3 [M+H]^+; HRMS: found [M+H]^+ 999.4979. \( \text{C}_{55}\text{H}_{67}\text{N}_{8}\text{O}_{10}^+ \) requires 999.4980.
PheTyrBes (16): colourless solid; 19% yield after HPLC purification; ESI-MS: 976.3 [M+H]^+; HRMS: found [M+H]^+ 976.4822. C_{53}H_{66}N_{11}O_{11}^+ requires 976.4820.

PheAlaBes (17): colourless solid; 18% yield after HPLC purification; ESI-MS: 884.3 [M+H]^+; HRMS: found [M+H]^+ 884.4511. C_{47}H_{62}N_{7}O_{10}^+ requires 884.4558.

PheArgBes (18): colourless solid; 17% yield after HPLC purification; ESI-MS: 969.3 [M+H]^+; HRMS: found [M+H]^+ 969.5196. C_{50}H_{69}N_{10}O_{10}^+ requires 969.5198.

PheLysBes (19): colourless solid; 17% yield after HPLC purification; ESI-MS: 941.3 [M+H]^+; HRMS: found [M+H]^+ 941.5158. C_{50}H_{69}N_{8}O_{10}^+ requires 941.5137.
**PheLys(Z)Bes (20):** colourless solid 18% yield after HPLC purification; ESI: 1075.3 [M+H]^+; HRMS: found [M+H]^+ 1075.522. C_{58}H_{75}N_8O_{12}^+ requires 1075.5499.

**PheBipBes (21):** colourless solid 16% yield after HPLC purification; ESI: 1036.3 [M+H]^+; HRMS: found [M+H]^+ 1036.5198. C_{59}H_{70}N_7O_{10}^+ requires 1036.5179.

**PheTyr(Bzl)Bes (22):** colourless solid 17% yield after HPLC purification; ESI: 1066.3 [M+H]^+; HRMS: found [M+H]^+ 1066.5291. C_{60}H_{72}N_7O_{11}^+ requires 1066.5284.

**PheNaphBes (23):** colourless solid 15% yield after HPLC purification; ESI: 1010.2 [M+H]^+; HRMS: found [M+H]^+ 1010.5042. C_{57}H_{68}N_7O_{10}^+ requires 1010.5022.
II) Synthesis of P1 library ABPs with Ala at the P1' site:

The synthesis of the P1 library required a strategy for the synthesis of \(\alpha\)-hydroxy-\(\beta\)-amino acids. Details of the synthesis of \(\alpha\)-hydroxy-\(\beta\)-amino acids and its purification will be described elsewhere (Velmourougane, G and Greenbaum, D. C. et al., manuscript accepted in J. Med. Chem.). The scheme for the synthesis of \(\alpha\)-hydroxy-\(\beta\)-amino acids is as follows (Scheme 2).

**Scheme 2: Synthesis of \(\alpha\)-hydroxy-\(\beta\)-amino acids**

![Scheme 2](image)

The \(\alpha\)-hydroxy-\(\beta\)-amino acid 30a-f were converted to the P1 library ABP’s as per the scheme depicted below. The yields of the products ranged from 12 to 15% (Scheme 3).

**Scheme 3: Synthesis of P1 library of Bestatin ABPs.**
AlaAlaBes (31): colourless solid; 15% yield after HPLC purification; ESI-MS: 808.2 [M+H]+; HRMS: found [M+H]+ 808.4253. C41H58N7O10+ requires 808.4245.

LeuAlaBes (32): colourless solid; 12% yield after HPLC purification; ESI-MS: 850.3 [M+H]+; HRMS: found [M+H]+ 850.4734. C44H64N7O10+ requires 850.4715.

NaphAlaBes (34): colourless solid; 13% yield after HPLC purification; ESI-MS: 934.3 \([M+H]^+\); HRMS: found \([M+H]^+\) 934.4702. \(C_{51}H_{64}N_7O_{10}^+\) requires 934.4715.

BipAlaBes (35): colourless solid; 16% yield after HPLC purification; ESI-MS: 960.2 \([M+H]^+\); HRMS: found \([M+H]^+\) 960.4862. \(C_{53}H_{66}N_7O_{10}^+\) requires 960.4871.

Tyr(Bzl)AlaBes (BTA) or (36): colourless solid; 15% yield after HPLC purification; ESI-MS: 990.3 \([M+H]^+\); HRMS: found \([M+H]^+\) 990.4944. \(C_{54}H_{68}N_7O_{11}^+\) requires 990.4977.

Synthesis of LysBiotinacetic azide (37):

The synthesis of the LysBiotinacetic azide was performed on Rink resin. Fmoc deprotection on the Rink resin was carried out using 20% Piperidine in DMF for 20 minutes. The free amine was treated with Fmoclys(biotin)OH under standard
coupling conditions (3 eq. HBTU, 3 eq. HOBt, 6 eq. DIEA). Fmoc deprotection followed by coupling with Bromoacetic acid using HATU and DIEA provided the precursor bromo derivative. This on treatment with sodium azide in DMF at 60 °C for 48 h generated the azide. Cleavage of the resin using 95%TFA:2.5%TIS:2.5%H2O and the extract was concentrated, precipitated using cold ether and purified by HPLC (Scheme 4).

ESIMS of LysBiotinacetic azide found [M+H]+ 455.2. C18H31N8O4S+ requires 455.21.

Scheme 4: Synthesis of LysBiotinacetic azide.

III) Synthesis of BiotinBTA click probe (38):

The BiotinBTA click probe was synthesized by reacting the BTA alkyne (1.2 eq.) with LysBiotin acetic azide 37 (1 eq.) using copper sulphate (0.05 eq.), sodium ascorbate (0.15 eq.) in dichloromethane and water (1:1) at 55 °C for 12 h. After 12 h the solvent was evaporated to dryness and the residue was purified by reverse phase HPLC to yield the BiotinBTA click probe 38 in 42% yield as
colourless solid after HPLC purification. ESIMS: 1444.4 [M+H]^+; HRMS: found [M+H]^+ 1444.7054. C_{72}H_{98}N_{15}O_{15}S requires 1444.7088.

**Synthesis of BodipyLysacetic azide (40):**

We commenced the synthesis of BodipyLysacetic azide by initially making the Lysacetic azide 39 using solid phase peptide synthesis as represented in the scheme below, and then coupling the free amine from the lysine residue with the BodipyOSu (1.1 eq.) using triethyl amine in DMF at room temperature for 24 h. The resin was cleaved using 95% TFA:2.5%TIS:2.5%H_{2}O. The cleavage fractions were collected, concentrated and purified by reverse phase HPLC (Scheme 5).

**Scheme 5:** Synthesis of BodipyLysacetic azide.
ESIMS of BodipyLysacetic azide C$_{24}$H$_{33}$BF$_2$N$_8$O$_3$ Calculated Mass 530.27, found 553.1 (M+Na$^+$).

**IV) Synthesis of BodipyBTA click probe (41):**

The synthesis of the BodipyBTA click probe was done employing the same protocol for the click reaction as for the BiotinBTA click probe using BTA alkyne and BodipyLysacetic azide 40. The product 41 was obtained in 45% yield as a colourless solid. ESIMS: 1520.5 [M+H]$^+$; HRMS: found [M+H]$^+$ 1520.7716. C$_{78}$H$_{101}$BF$_2$N$_{15}$O$_{14}^+$ requires 1520.7714.

**V) Synthesis of BodipyPheNaph click probe (42):**
The synthesis of the BodipyPheNaph click probe was synthesized employing the same procedure for the click reaction, as for the BiotinBTA click probe using PheNaph alkyne 23 and BodipyLysacetic azide 40. The product 41 was obtained in 42% yield as a colourless solid. ESIMS: 1540.7 [M+H]⁺; HRMS: found [M+H]⁺ 1540.7800. C₈₁H₁₀₁BF₂N₁₅O₁₃⁺ requires 1540.7759.
Supplementary references