Malaria is a parasitic disease that results in significant morbidity and mortality, primarily in sub-Saharan Africa. The principal means of control of malaria remains chemotherapy. However, multidrug resistant *Plasmodium falciparum*, the organism that causes most malaria-associated deaths, has now become a major problem, necessitating the discovery of novel antimalarial drugs. Unfortunately, systematic genetic approaches for the validation of drug targets including such techniques as RNA interference, antisense technology (i.e., preventing protein synthesis from a given gene), and genetic mutations are either not possible or are insufficiently robust in the *P. falciparum* system. In the absence of these genetic approaches in *P. falciparum*, small molecules can be used to investigate the therapeutic potential of target protein families and provide clues about protein function. Small molecules can directly block the activity of proteins, disallowing them to perform their function. The consequences of this effect can be studied and can allow insight into the role that that protein target plays. The feasibility of using small molecules to complement genetic methods of analyzing protein function has been demonstrated in a number of recent studies in *P. falciparum* (1).

One class of proteins with particular interest for drug target validation in *P. falciparum* is proteolytic enzymes, which normally cleave proteins into smaller protein fragments or release amino acids. Proteases are known to participate in several critical pathways during the life cycle of *P. falciparum* and likely play crucial roles in other uncharacterized processes. Inhibition of PfA-M1, which is localized to the digestive vacuole, prevented the degradation of oligopeptides derived from hemoglobin and likely starved the parasite. Specific inhibition of Pf-LAP had no effect on DV function; however, parasite death ensued by an unknown mechanism.

For these enzymes have been unsuccessful. Although there are several inhibitor classes that target aminopeptidases, no specific inhibitors have been developed for any of these enzymes in *P. falciparum*.

In this study, we used a small molecule-based approach to elucidate the function of these two essential malarial MAPs (Fig. P1). The basic chemical method we employed is a variant of a forward chemical-genetics strategy that utilizes inhibitors, to which fluorescent or trackable tags are attached, which can be to characterize the function of enzymes like PfA-M1 or Pf-LAP within complex proteomes. These inhibitors are called activity-based probes (ABPs) and typically possess two main structural components: (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
apart the enzymes
different specificities for Pf-LAP and PfA-M1 could help tease
early evidence that the use of different inhibitors with
parasites died late in the life cycle. These findings provided
parasites to develop a swollen digestive vacuole, and these
swelling of the digestive vacuole during the lifestage in which
treated with BTA, the PfA-M1 inhibitor, showed progressive
lifecycle in the presence of either BTA or PNAP. Parasites
inhibition, we followed parasites throughout the erythrocytic
compounds for either PfA-M1 or Pf-LAP.

proteins provided a molecular basis for the selectivity of these
cocrystal structures of the probes bound to their respective
show specificity of the probes in parasite lysates. Finally,
used fluorescent versions of each probe in labeling assays to
than 15-fold more specific for PfA-M1 than Pf-LAP. We then
specific for Pf-LAP than PfA-M1, while BTA had a greater
further experiments showing that PNAP was over 10-fold more
lifecycle; (iii) probes more specific for PfA-M1 caused
parasites to develop a swollen digestive vacuole, and these
parasites died late in the life cycle. These findings provided
early evidence that the use of different inhibitors with
different specificities for Pf-LAP and PfA-M1 could help tease
apart the enzymes’ roles.

Specificity of the probes for the MAPs was confirmed by
further experiments showing that PNAP was over 10-fold more
specific for Pf-LAP than PfA-M1, while BTA had a greater
than 15-fold more specific for PfA-M1 than Pf-LAP. We then
used fluorescent versions of each probe in labeling assays to
show specificity of the probes in parasite lysates. Finally,
cocrystal structures of the probes bound to their respective
proteins provided a molecular basis for the selectivity of these
compounds for either PfA-M1 or Pf-LAP.

To better understand the effects of Pf-LAP and PfA-M1
inhibition, we followed parasites throughout the erythrocytic
lifecycle in the presence of either BTA or PNAP. Parasites
treated with BTA, the PfA-M1 inhibitor, showed progressive
swelling of the digestive vacuole during the lifestage in which
the majority of hemoglobin digestion occurs. The expansion of
the digestive vacuole was dose-dependent, suggesting that this
morphology was mediated by targeting of BTA for a single
molecule. In contrast, parasites treated with PNAP died prior
to the initiation of hemoglobin degradation and formation of
the digestive vacuole. This suggested to us that the essential
function of Pf-LAP likely does not occur during hemoglobin
degradation. Due to the lack of distinct changes in parasite
morphology upon its inhibition, it is quite difficult to
formulate testable hypotheses about its role. Alternatively, the
observation of marked digestive vacuole swelling upon PfA-M1
inhibition by BTA immediately led us to ask whether the role
of PfA-M1 is within the digestive vacuole as a hemoglobinase.

falciparum is able to utilize the amino acids from
hemoglobin for its own metabolism and is thus able to survive
in media lacking exogenous amino acids, with the exception of
isoleucine, the only amino acid absent from hemoglobin. We
reasoned that if PfA-M1 did indeed play a role in hemoglobin
proteinolysis, parasites forced to rely on amino acid acquisition
solely from hemoglobin would be more susceptible to PfA-M1
inhibition than would parasites with an exogenous source of
amino acids. Indeed the susceptibility of the parasites to BTA
was increased almost threefold when parasites were made to
depend on Hb digestion for amino acid acquisition.

The evidence described thus far strongly suggested that
PfA-M1 is an essential component of the parasite’s DV-
localized hemoglobin degradation pathway. To confirm this
finding, we needed to identify hemoglobin peptides as
substrates for PfA-M1. Therefore, we undertook a proteomics
approach to identify peptides that became more abundant
when PfA-M1 was inhibited in parasites. Our findings revealed
that a number of hemoglobin peptides were elevated in BTA-
treated parasites versus controls.

This study has illustrated the power of using small
molecules to investigate the therapeutic potential of proteins
in systems where genetic techniques are difficult, if not
impossible, to perform. We used two probes specific for two P.
falciparum MAPs to discern the proteins’ function in different
stages of the parasite’s erythrocytic life cycle. Specifically, we
identified a role for PfA-M1 in hemoglobin degradation and a
role for Pf-LAP unrelated to hemoglobin degradation, much
earlier in the parasite’s life cycle. The susceptibility of
parasites to these inhibitors highlights these proteases as
potential antimalarial targets.

1. Puri AW, Bogyo M (2011) Using small molecules to dissect mechanisms of microbial