Activity-Based Protein Profiling for the Study of Parasite Biology

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Abstract Parasites exist within most ecological niches, often transitioning through biologically and chemically complex host environments over the course of their parasitic life cycles. While the development of technologies for genetic engineering has revolutionised the field of functional genomics, parasites have historically been less amenable to such modification. In light of this, parasitologists have often been at the forefront of adopting new small-molecule technologies, repurposing drugs into biological tools and probes. Over the last decade, activity-based protein profiling (ABPP) has evolved into a powerful and versatile chemical proteomic platform for characterising the function of enzymes. Central to ABPP is the use of activity-based probes (ABPs), which covalently modify the active sites of enzyme classes ranging from serine hydrolases to glycosidases. The application of ABPP to cellular systems has contributed vastly to our knowledge on the fundamental biology of a diverse range of organisms and has facilitated the identification of potential drug targets in many pathogens. In this chapter, we provide a

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comprehensive review on the different forms of ABPP that have been successfully applied to parasite systems, and highlight key biological insights that have been enabled through their application.

1 Background

Parasites comprise a large biologically diverse group of eukaryotes that exploit the resources of a host organism to facilitate their own survival and propagation. Broadly, parasites can be taxonomically described as being either microparasitic unicellular protozoa (Kingdom: *Protista*), or complex macroparasitic multicellular helminths and ectoparasites [*Animalia*; (Simner 2017)]. The life cycles of these parasites display remarkable diversity in form and complexity, infecting different host species with a range of host-pathogen interactions, transmission routes and tissue tropism. For instance, while some species exhibit strong preference for specific host cell types, others are more generalist, or exist extracellularly (Fig. 1) (McCall et al. 2016).

Human parasitic infections are a leading cause of morbidity and mortality worldwide. Correspondingly, they represent a major global health and economic burden. In the latest 'Global Burden of Disease' study, parasite-related diseases were estimated to account for over 800,000 deaths in 2016, with most being attributed to protozoan infections (G. B. D. Causes of Death Collaborators 2017). By far the most prevalent of these protozoan infections are those caused by members of the phylum *Apicomplexa,* such as *Plasmodium* spp. and *Toxoplasma gondii*. *Plasmodium* parasites are responsible for malaria, a devastating mosquito-borne disease that threatens around half of the global population (3.2 billion) (World Health Organization 2017). In 2016, 446,000 malaria-related deaths in 91 countries were reported by the World Health Organisation (WHO), with sub-Saharan Africa carrying 90% of the burden (World Health Organization 2017). Regarded as the most successful parasite on the planet, *T. gondii* has the capacity to infect any vertebrate host, and seroprevalence studies indicate that up to 50% of the human population

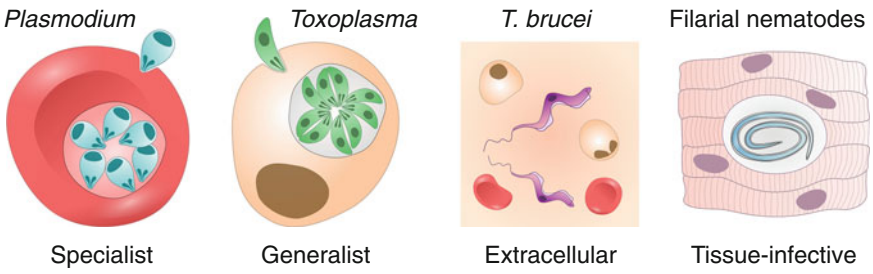

Fig. 1 Major types of infection exhibited by human-infective parasites. Representative parasite species shown are known to infect distinct cell types ('specialists'), a broad range of cell types ('generalists'), reside in extracellular environments (e.g. the bloodstream/lymph), or within tissues

have been infected at some point in their lives (Flegr et al. 2014). *T. gondii* is the aetiological agent of toxoplasmosis, a disease which can lead to life-threatening encephalitis if left untreated. *T. gondii* is also a major veterinary pathogen responsible for the annual loss of over 500,000 lambs, costing the UK sheep industry £12–24 million (Advisory Committee on the Microbiological Safety of Food 2012). Other medically important protozoan parasites include the trypanosomatids, a group of flagellated parasites transmitted by insect vectors and responsible for an array of neglected tropical diseases. Three trypanosomatids of particular concern are *Leishmania* spp., *Trypanosoma brucei* and *Trypanosoma cruzi,* the respective agents of the leishmaniases, human African trypanosomiasis (HAT) and Chagas disease. Infections involving parasitic worms (helminths) are another major contributor to parasite-related deaths. In fact, schistosomiasis caused by trematodes of the *Schistosoma* genus ranks as the second-most-common parasitic disease and claims up to 200,000 lives each year (World Health Organization 2017).

One of the greatest challenges in combating parasitic infection is the development of safe and efficacious anti-parasitic drugs. While chemotherapeutics have been effective in reducing the burden of parasite-related disease, their use has been limited by toxicity, prolonged treatment regimens and/or the emergence of drug-resistant strains (Monzote and Siddiq 2011). For example, resistance to the frontline antimalarial artemisinin has been identified in *Plasmodium falciparum* isolates from across Southeast Asia, compromising the efficacy of well-established artemisinin combination therapies (Haldar et al. 2018). Additionally, drug-resistant *L. donovani* strains in India have limited the effectiveness of pentavalent antimonial compounds for the treatment of visceral leishmaniasis (Croft et al. 2006). On this basis, there is a continuing need to investigate parasite biology and identify novel drug targets for which new efficacious therapeutics can be developed.

While genetic engineering approaches have been powerful for dissecting the basic biology of prokaryotic systems, eukaryotic parasites have historically been less amenable to such modification. For instance, some species exhibit poor transfection efficiencies, or reduced homologous recombination rates due to AT-rich genomes (as is the case with *P. falciparum*; (Suarez et al. 2017). As an alternative to genetic approaches, small-molecule-based chemical biology techniques have provided significant insight into the molecular basis of many essential parasite processes. Activity-based protein profiling (ABPP) has emerged as one particularly versatile strategy for characterising enzyme function on a global scale and has facilitated rapid identification of novel druggable nodes in a range of biological systems, including parasites (Cravatt et al. 2008). Central to this approach is the use of activity-based probes (ABPs). ABPs are small molecules functionalised with fluorescent reporter or affinity-based tags that can be used to profile the activity of specific enzymes classes or residues via covalent interaction with their target(s) (Cravatt et al. 2008). This chapter aims to provide an overview of the diverse applications of ABPP that have advanced our understanding of parasite biology over the last 10 years. For the purpose of clarity, we will focus on a selection of the major biological discoveries that have been made using ABPP, rather than providing a detailed account of all available literature.

2 Activity-Based Probes for Target Discovery in Parasites

2.1 Profiling Protease Activity

Perhaps the most widespread use of ABPP in parasites has been for the study of proteases. Proteases are enzymes that catalyse the proteolytic processing of protein substrates via the hydrolysis of peptide bonds (López-Otín and Bond 2008). Based on their catalytic residues or mechanism, proteases are categorised as cysteine, serine, threonine, aspartyl and metalloproteases. Together, they are responsible for a diversity of molecular functions including the regulation of protein activity and localisation, modulation of protein–protein interactions (PPIs) and generation of new active biomolecules (López-Otín and Bond 2008). In parasites, proteases display remarkable functional diversity in order to carry out essential processes such as immune modulation and host cell invasion (McKerrow et al. 2006) with much of our understanding of their contributions to these processes coming through the application of protease-directed ABPs.

2.1.1 Cysteine Proteases

One of the first successful applications of parasite-based ABPP includes the identification and characterisation of the *P. falciparum* papain family of cysteine proteases known as the falcipains (Greenbaum et al. 2002). In two separate studies, it was demonstrated that treatment of intraerythrocytic *P. falciparum* parasites with E-64 inhibits parasite egress from red blood cells (RBCs) and induces the enlargement of parasite food vacuoles (Greenbaum et al. 2002; Salmon et al. 2001). E-64 is an epoxide-containing natural product derived from fungi (*Aspergillus* spp.) and a known covalent inhibitor of clan CA cysteine proteases. Given the clear potential for cysteine proteases to be antimalarial drug targets, a series of E-64-based ABPs were synthesised to profile the proteins associated with the egress and enlarged food vacuole phenotypes (Chandramohanadas et al. 2009; Greenbaum et al. 2000, 2002). Using a biotinylated E-64 derivative, DCG-04 (Fig. 2a), four proteases were identified and found to have distinct activities in the different life cycle stages of the parasite (Greenbaum et al. 2002). For instance, falcipain-1 was shown to be upregulated during the invasive extracellular merozoite stage, highlighting a potential role for this protease in RBC invasion and/or rupture (Greenbaum et al. 2002). To validate a role for falcipain-1 in parasite invasion, Bogyo and colleagues used competitive ABPP of a broader epoxide-based peptide library, identifying several falcipain-1-specific inhibitors (Greenbaum et al. 2002). In this work, merozoite lysates were treated with the epoxides before profiling global cysteine protease activity with a radiolabelled version of DCG-04 (^{125}I-DCG-04; Fig. 2a). The most selective inhibitor, YA29-Eps(S, S), prevented merozoite invasion of erythrocytes without any discernible effects on other parasite processes, indicating that falcipain-1 activity is associated with invasion. Adopting

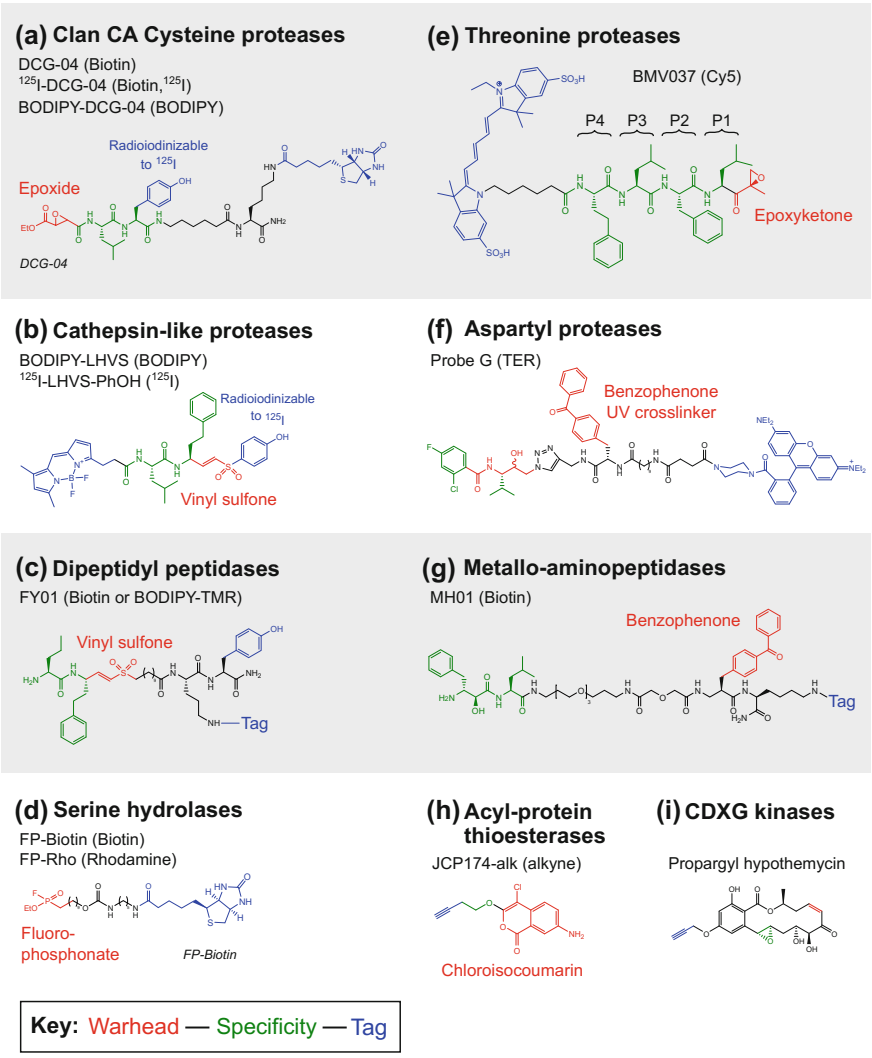

Fig. 2 Representative activity-based probes (ABPs) used to interrogate the function of major enzyme classes in parasites. ABPs have been used for the characterisation of cysteine (**a**–**c**), serine (**d**), threonine (**e**), aspartyl (**f**) and metalloproteases (**g**) of parasites, as well as acyl-protein thioesterases (**h**) and protein kinases that contain a CDXG motif (I). Specific probes are listed under the enzyme class they target, with their associated fluorescent reporter or affinity tag(s) bracketed

a similar ^{125}I-DCG-04 labelling approach, Eksi et al. (2004) later revealed that falcipain-1 is also upregulated in late-stage gametocytes. Here, genetic perturbation of falcipain-1 was shown to ablate oocyst production in the mosquito, suggesting it also plays a role in the sexual stage of the malaria life cycle.

Since its initial use in *Plasmodium*, DCG-04 has been broadly applied to other parasite systems. In *T. gondii*, a fluorescently conjugated variant of DCG-04 (BODIPY-DCG-04) was used to show that *Tg*CPL, a cathepsin L-like cysteine protease required for the maturation of microneme adhesins, also acts as the maturase for *Tg*CPB (a related cathepsin B-like protease (CPB) implicated in parasite invasion and replication) (Chaparro et al. 2018; Dou et al. 2013; Parussini et al. 2010; Que et al. 2004). In the parasitic helminth *Schistosoma*, CPBs are secreted from the acetabular glands and are thought to play a central role in the invasion of larvae by degrading structural proteins of the host skin (Dvořák et al. 2008). To characterise any species differences in terms of the abundance or complement of secreted proteases, Dvořák et al. (2008) used ^{125}I-DCG-04 to compare the activity of CPBs in the pathogenic secretomes of several *Schistosoma* species. Competitive ABPP using a selective inhibitor of CPB activity revealed that CPBs are much more abundant in the cercarial extracts of *Schistosoma japonica* than in *Schistosoma mansoni* (Dvořák et al. 2008). Fluorogenic peptide cleavage assays later demonstrated that the CPB activity in the secretions of *S. japonica* is 40-fold greater than *S. mansoni* (Dvořák et al. 2008). By contrast, serine proteases, which are also associated with schistosomal invasion, were found to be the most abundant protease class in the secretome of *S. mansoni* yet were absent in *S. japoncium*. These findings suggested that significant differences can be found in the secreted protease repertoire of schistosomes and provided insight into the different virulence profiles of these species. For instance, the high potency of CPBs to degrade collagen was proposed as an explanation for why *S. japonicum* larvae traverse the host dermis and epidermis at significantly higher rates than *S. mansoni* larvae (He et al. 2005). DCG-04 labelling was also used to identify 'cruzain' as the major active cysteine protease in the insect-form epimastigotes of *T. cruzi* (Doyle et al. 2011). Subsequent genetic knockout experiments demonstrated that cruzain inhibits the activation of infected macrophages by interrupting signalling pathways relying on NF-κB P65, demonstrating a previously unknown role for this protease in immune evasion (Doyle et al. 2011). This probe has been applied to *Cryptosporidium parvum,* the apicomplexan responsible for cryptosporidiosis, which further emphasises the broad utility of DCG-04 in less tractable parasite systems. *C. parvum* expresses a cysteine protease called cryptopain-1, which is essential for parasites survival in vivo. Through competitive inhibition studies with ^{125}I-DCG-04, the clan CA cysteine inhibitor K11777 was shown to both inhibit recombinant cryptopain-1 in vitro, and cryptopain-1 activity with negligible toxicity in vivo, highlighting cysteine protease inhibitors as potential anti-cryptosporidials (Ndao et al. 2013).

Peptide-based probes bearing vinyl sulfone (VS) reactive groups have also been valuable in delineating the function of parasite papain-family cysteine proteases. In a phenotypic screen using a series of known cysteine protease inhibitors, Teo et al. (2007) identified morpholinourea-leucyl-homophenolalaninyl-phenyl-vinylsulfone (LHVS) as an inhibitor of *T. gondii* host cell invasion, with a half-maximal inhibitory concentration (IC_{50}) of 10 μM. Subsequent biochemical and cell-based studies revealed that LHVS specifically impedes parasite attachment and gliding

motility by blocking the secretion of adhesion proteins from specialised apical organelles known as micronemes (Teo et al. 2007). To identify the proteases targeted by LHVS and associated with this phenotype, Carruthers and colleagues performed competitive ABPP using a fluorescently tagged inhibitor derivative (BODIPY-LHVS; Fig. 2b). This study revealed a cathepsin P-like protease (*Tg*CPL) as the principle target of LHVS (Larson et al. 2009). Fluorescence microscopy of BODIPY-LHVS labelling in live extracellular parasites later showed that *Tg*CPL localises to two discrete structures at the apical end of *T. gondii*. While this pointed to a possible role in the proteolytic maturation of invasion-associated microneme proteins, the molecular function of *Tg*CPL remains unclear. Nevertheless, recent pharmacological studies have highlighted *Tg*CPL as a promising therapeutic target in chicken embryo models of acute toxoplasmosis (Chaparro et al. 2018). In similar studies, McKerrow and colleagues used a radioactively iodinated derivative of LHVS (^{125}I-LHVS-PhOH; Fig. 2b) to profile the activity of cysteine proteases in *T. brucei* bloodstream form parasites (Caffrey et al. 2001). Here, ^{125}I-LHVS-PhOH labelling of cell lysates identified brucipain (a cathepsin L-like protease) as an abundant protease in this life cycle stage, which was confirmed through competitive inhibition with two known trypanosomal cysteine protease inhibitors, Z-Phe-Ala-CHN_2 and *N*-Me-pip-Phe-homoPhe-VSPh. Follow-up genetic studies using RNA interference (RNAi) subsequently validated brucipain as an essential secreted virulence factor required for *T. brucei* traversal of the host blood-brain barrier (Abdulla et al. 2008; Nikolskaia et al. 2006).

In *P. falciparum*, an alternative VS-based probe, FY01 (Fig. 2c), was developed and used alongside DCG-04 to characterise a group of CPL proteases known as the dipeptidyl peptidases (DPAPs) (Arastu-Kapur et al. 2008). In this study, Bogyo and colleagues employed competitive ABPP to identify selective inhibitors against DPAP1 and 3, two variants predominantly expressed in the intraerythrocytic merozoite stage. While DPAP1 had previously been associated with the degradation of host haemoglobin in the parasite food vacuole, the precise function of DPAP3 remained unknown. Compared to the broad-spectrum cysteine protease probe DCG-04, FY01 was determined to have high selectivity for DPAP3, as visualised by in-gel fluorescence following labelling with BODIPY TMR-conjugated FY01. Here, selective labelling of DPAP3 was confirmed by mass spectrometry (MS) following affinity purification of proteins with a biotinylated version of FY01. Owing to its inability to efficiently label DPAP3, DCG-04 could be used to assess the specificity of inhibitors against DPAP1. Using competitive ABPP to screen a library of small-molecule irreversible protease inhibitors, vinyl sulfones SAK1 and SAK2 were identified as specific inhibitors of DPAP3 and DPAP1, respectively. In an elegant series of small-molecule inhibition assays, it was demonstrated that DPAP3, but not DPAP1, is required for the rupture of late-stage schizonts during egress, highlighting a novel role for this protease in the asexual phase of the malaria life cycle.

2.1.2 Serine Proteases

Another important class of parasite-derived protease that has been well characterised using ABPP are the serine proteases. Belonging to the serine hydrolase family, such proteases depend on a conserved nucleophilic serine residue positioned within the active site to attack the carbonyl group of an amide bond, forming an acyl-enzyme intermediate that is subsequently hydrolysed to break the bond (Long and Cravatt 2011). The inherent nucleophilicity of the catalytic serine renders it susceptible to covalent modification by a range of broad-spectrum serine hydrolase ABPs including the fluorophosphonates (FPs), aryl phosphonates, isocoumarins, sulfonyl fluorides and carbamates (Cravatt et al. 2008).

In parasites, serine protease activity was first profiled in *Babesia divergens*, a bovine pathogen that can cause lethal babesiosis in immunocompromised humans (Montero et al. 2006). *Babesia* species are apicomplexans with an intraerythrocytic life cycle comparable to that of *Plasmodium*, involving active invasion of RBCs by merozoites. While serine proteases such as *Pf*SUB1 and 2 were thought to play a pivotal role in the entry of *P. falciparum* merozoites (via the proteolytic maturation of parasite and host-derived proteins), whether such enzymes had similar function in *B. divergens* was not known. To identify potential serine hydrolases in *B. divergens*, Montero et al. (2006) labelled the lysates of extracellular merozoites with a biotinylated derivative of the FP probe (FP-biotin; Fig. 2d). Western blot analysis of FP-biotin labelled proteins with an avidin-conjugated secondary antibody identified two distinct protein species. Using antibodies against a related homologue present in *P. falciparum* (*Pf*SUB1), immunoprecipitation experiments demonstrated that these FP-biotin labelled species were the active and precursor forms of a serine subtilisin-like protease, subsequently denoted *Bd*SUB1. Further work found that *Bd*SUB1 localises to invasion-associated secretory organelles known as dense granules and is essential for *B. divergens* invasion, indicating functional conservation with *Pf*SUB1. ABPP has been further used to delineate the roles of several *Plasmodium* serine proteases expressed in the intraerythrocytic parasite stages. In the same chemical biology study that characterised the DPAP1/3 cysteine proteases, Bogyo and co-workers used an isocoumarin-based probe to identify *Pf*SUB1 as a novel regulator of *P. falciparum* merozoite egress (Arastu-Kapur et al. 2008). In this study, a biotinylated chloroisocoumarin from the same protease inhibitor library was identified as an inhibitor of schizont rupture, with a half-maximal effective concentration value (EC_{50}) of 22 μM. This biotinylated inhibitor, JCP104, was used to label protein targets in both intact and permeabilised schizont samples lacking an RBC membrane and with reduced human protein contamination. Western blotting and MS analysis of proteins covalently modified by the biotinylated inhibitor revealed *Pf*SUB1 as the primary target. Furthermore, fluorogenic peptide substrate cleavage assays demonstrated that JCP104 inhibits the proteolytic activity of recombinant *Pf*SUB1 with an IC_{50} consistent with the EC_{50} reported for the schizont rupture assay. These data suggested that JCP104 likely impairs parasite egress by blocking the activity of *Pf*SUB1, supporting a role for this serine protease in the parasite's intraerythrocytic

life cycle. To define the mechanism by which *Pf*SUB1 regulates merozoite egress, JCP104 was then used to assess whether inhibition of this protease affects the proteolytic processing of *Pf*SUB1 substrates. For this experiment, Arastu-Kapur et al. focused on the serine repeat antigen 5 (SERA5). SERA5 is an essential pseudoprotease with a role in schizont rupture, and proteolytically processed by *Pf*SUB1 within the parasitophorous vacuole (PV), the membrane-bound structure in which the parasite develops (Collins et al. 2017; Yeoh et al. 2007). As with DPAP3 inhibition, JCP104-mediated inhibition of *Pf*SUB1 resulted in a dose-dependent accumulation of unprocessed SERA5 within intact schizonts concurrent with a reduction in the amount of processed SERA5 in the cell culture media following egress. Intriguingly, the processing of SERA5 was dependent on the presence of functional DPAP3, as small-molecule inhibition of DPAP3 blocked the production of the mature form of *Pf*SUB1 and subsequent merozoite egress. These findings led to the development of a new model for RBC rupture during egress, whereby *Pf*SUB1 is proteolytically matured in exoneme organelles by DPAP3, before being released into the PV where it then processes SERA and matures other merozoite surface proteins including MSP1. This study demonstrated the power of ABPs to characterise the synergy between two mechanistically different proteases in a critical and complex parasite cellular process.

Another *P. falciparum* serine protease that has been characterised with the aid of ABPP is *Pf*ClpP, an orthologue of the cyanobacterial caseinolytic protease P (Rathore et al. 2010). In 2010, Rathore et al. (2010) screened a series of synthetic β-lactone probes to identify compounds that inhibit the activity of *Pf*ClpP. Their best inhibitor, compound U1, had an IC_{50} of 8.4 μM and was converted into an ABP to determine the specificity of this compound in *P. falciparum* proteomes for subsequent use in cell-based studies. Here, a terminal alkyne group was introduced to allow for functionalisation of the probe with an azide-linked rhodamine (Az-Rho) fluorescent reporter by copper-catalysed azide-alkyne cycloaddition reaction, click chemistry (Kolb et al. (2001). Following intact labelling of intraerythrocytic *P. falciparum* trophozoites, in-gel fluorescence analysis of labelled proteins revealed high selectivity of this probe for *Pf*ClpP. Having validated the specificity for the probe, cell-based inhibition studies were conducted with the parent inhibitor (U1) to characterise the function *Pf*ClpP throughout the parasite's intraerythrocytic life cycle. Treatment of *P. falciparum* with U1 resulted in a parasite growth arrest approximately 96 h post-drug treatment, consistent with the well-documented delayed-death phenotype that is associated with the loss of the apicoplast (an essential organelle situated at the anterior of the parasite where isoprenoid, fatty acids and haem are synthesised). Here, U1-treatment induced the formation of abnormally shaped and non-replicative apicoplasts during parasite schizogony. Together, these findings suggested that *Pf*ClpP plays a key role in the functional biogenesis of the apicoplast during intraerythrocytic asexual development.

2.1.3 Threonine Proteases

The proteasome is a large macromolecular protein complex composed of multiple proteolytic subunits that are dependent upon an N-terminal threonine nucleophile for their catalytic activity. As with other eukaryotic systems, the parasite proteasome is critical for a range of essential processes that depend on the proteolytic degradation of polyubiquitinated proteins, such as cell differentiation and replication (Munoz et al. 2015). Small-molecule inhibition studies have highlighted the parasite proteasome as a promising anti-parasitic drug target. For instance, selective inhibition of the catalytic β2 and 5 subunits of the *P. chabaudi* proteasome results in parasite clearance in a mouse model of malaria (Li et al. 2016). Indeed, ABPP of threonine proteases has been integral to the characterisation of the proteasome in various parasites, and its validation as a drug target.

To profile the activity of individual proteasome subunits, Nazif and Bogyo (2001) developed a range of peptidic ABPs that target the catalytic threonine via a vinyl sulfone or epoxyketone warhead. These probes contain variations of a four amino acid specificity sequence, which interact with the specificity pockets of the catalytic β subunits downstream of the site of peptide bond hydrolysis (position P1). Altering the amino acid sequence of the probe's peptide specificity element provided insight into the substrate recognition preferences of each subunit. This approach was used to identify unique substrate recognition properties of the parasite proteasome that can be exploited in drug development. For instance, Wang et al. (2003) used a series of ^{125}I-labelled peptide vinyl sulfones that target the β1, 2 and 5 subunits of the human proteasome to determine the specificity of the equivalent subunits in *T. brucei* (Wang et al. 2003). SDS-PAGE analysis of products radiolabelled with a general probe, ^{125}I-YL$_3$-VS, revealed that β2/5 has similar specificity for hydrophobic residues in the P1 to P4 positions of the substrate. Unlike its human orthologue, the *T. brucei* β1 subunit was not labelled despite its primary sequence being predictive of catalytic activity, suggesting that this subunit has significantly altered peptide specificity in *T. brucei* or may be catalytically inactive. Furthermore, substitution of the P1 leucine to an asparagine in a related probe (^{125}I-NP-L$_2$N-VS) resulted in selective labelling of β2 that was not seen in humans, indicating that this subunit may have different substrate specificity compared with the human proteasome. To define the catalytic profile of the trypanosomal proteasome competitive ABPP was performed, testing the effect of small-molecule inhibitors on subunit labelling with the general ^{125}I-YL$_3$-VS probe. Leupeptin, a specific inhibitor of the trypsin-like activity of the mammalian proteasome, prevents labelling of β2 but not β5, suggesting these subunits are responsible for the trypsin and chymotrypsin-like activity of the *T. brucei* protease, respectively. Bogyo and colleagues screened a positionally scanned library of peptide substrates (P1 to P4) to investigate substrate specificity differences between the *T. brucei* and human proteasome. Using a fluorogenic peptide cleavage assay, they found that the *T. brucei* 20S proteasome has an overall preference for hydrophobic residues in the P1 to P4 positions like its human counterpart. However, unique to the *T. brucei* proteasome was a preference for peptides containing a P1 glutamine, thus

identifying a difference in substrate specificity and a possible site to therapeutically target.

Threonine protease-directed ABPs have also been used to validate the specificity of inhibitors against the *P. falciparum* proteasome (Li et al. 2016). Probing substrate specificity with a diverse range of polypeptides, Li et al. (2016) first reported that the *P. falciparum* 20S proteasome, unlike the human proteasome, has strong preference for cleavage of tryptophan residues at P1 and P3. Subsequently, competitive ABPP was used to assess the specificity of inhibitors that contain tryptophan at one or both positions for the different β subunits. The parasite 20S proteasome was treated with the modified inhibitors before labelling with a fluorophore (Cy5)-tagged epoxyketone ABP (BMV037; Fig. 2e) (Li et al. 2014) to detect residual activity. Excitingly, substitutions at both positions resulted in selective inhibition of the parasite β2 catalytic subunit compared to the equivalent human subunit. Structural analysis of the inhibitor-bound proteasome revealed an unusual open conformation around the active site of the *P. falciparum* β2 subunit, providing a basis for future structure-guided drug design. Despite likely having some off-target activity with the P3 position of the human β5 active site, this parasite β2-selective inhibitor effectively perturbed parasite growth with minimal host toxicity in a mouse model of malaria. Overall, this study demonstrated the power of ABPP for the development of selective inhibitors of the *Plasmodium* proteasome, a proven chemically tractable target that could be exploited for future antimalarials.

2.1.4 Aspartyl and Metalloproteases

In contrast to cysteine, serine and threonine proteases, aspartyl and metalloproteases lack a nucleophilic residue and instead depend on the activation of water molecules for their proteolytic activity. Hence, ABPs directed towards these protease classes are typically potent inhibitors of a given target that feature a tag and a photo-crosslinking group for covalent modification (Cravatt et al. 2008). Activity-based profiling of the aspartyl and metalloproteases in parasite systems has been largely restricted to *Plasmodium*. Nevertheless, these proteases are critical for a range of essential processes in diverse macro- and microparasites including food digestion in *Schistosoma* (Goupil et al. 2016), and immune modulation by *Toxoplasma* (Hammoudi et al. 2015).

The plasmepsins (PMs) represent a family of approximately ten functionally-diverse aspartyl proteases expressed in both intra- and exoerythrocytic stages of the *Plasmodium* life cycle (Banerjee et al. 2002; Coombs et al. 2001; Nasamu et al. 2017; Russo et al. 2010). Of these, four (PM-I/II/IV and the histoaspartic protease, HAP) have well-established roles in the digestion of host haemoglobin in the RBC and are essential for parasite growth (Banerjee et al. 2002; Coombs et al. 2001). While PMs have long been considered promising antimalarial targets, their functional redundancy has suggested that each must be simultaneously inhibited in order to effectively clear parasitaemia. To enable assessment of

inhibitors that target all four PMs, Liu et al. (2009) developed a series of broad-spectrum hydroxyethyl probes bind to the active site of these proteases adjacent to catalytic aspartic acid residue(s). These probes also contain a terminal azide chemical handle, enabling click-conjugation of alkyne-linked benzophenone (BP) photocrosslinkers and affinity/reporter tags such as the tetraethylrhodamine (TER) fluorophore (Fig. 2f). For one particular probe, consistent labelling of the PMs was confirmed in the lysates of several intraerythrocytic parasite stages by two-dimensional gel electrophoresis (2DGE)-MS and western blotting. Here, the activity of the PMs was present in the insoluble and soluble fractions of trophozoite and schizont stages, respectively. These data supported a change in the subcellular localisation of the PMs from the membrane-bound parasite food vacuole to the soluble compartment of that digestive organelle, consistent with previous reports that PM-II is released in a soluble form from the vacuole during development (Klemba et al. 2004). Competitive ABPP was then used to assay a small library of 152 hydroxyethyl-based inhibitors against PM activity. One compound, G16, caused a dramatic reduction in the fluorescence labelling intensity by the probe (IC_{50} = 0.84 μM) and was selected for growth inhibition studies. Treatment of late-stage schizonts with G16 resulted in a decrease in newly formed ring-stage parasites and an accumulation of extracellular merozoites, indicating that this compound affects the development, egress and/or reinvasion of *P. falciparum* erythrocytic stage parasites.

P. falciparum also contains three metallo-aminopeptidases (MAPs) thought to contribute to the proteolytic degradation of host haemoglobin during intraerythrocytic development: aminopeptidase N (*Pf*A-M1), aminopeptidase P (*Pf*APP) and leucyl aminopeptidase (*Pf*-LAP) (McGowan 2013). To elucidate the role of MAPs in the biology of *Plasmodium*, Greenbaum and colleagues established a novel ABPP platform using an ABP scaffold derived from the natural product, bestatin (Harbut et al. 2011). Bestatin is known to inhibit multiple families of MAPs and has been shown to disrupt the growth of *P. falciparum* both in vitro and in vivo, potentially by disrupting the haemoglobin digestion pathway (Naughton et al. 2010). To identify the molecular targets of bestatin, a bestatin-based affinity probe (MH01; Fig. 2g) containing a BP crosslinker and biotin moiety was synthesised and used to label the lysates of asynchronous *P. falciparum* cultures. Western blot detection of probe-labelled biotinylated proteins revealed *Pf*A-M1 and *Pf*-LAP as the principle targets of MH01, with probe labelling successfully outcompeted following pre-treatment of the lysates with the bestatin parent molecule. To gain further insight into the specific functions of the MAPs and thus the mechanism underlying bestatin's effect, the authors generated ABPs specific to *Pf*A-M1 and *Pf*-LAP. This was achieved by screening the inhibitory activity of bestatin-based ABP libraries against each protease, with each probe containing a variation of the two amino acids that govern its interaction with the active sites. Inhibition experiments using the most selective *Pf*A-M1 probe, BTA, demonstrated that *Pf*A-M1 inhibition results in parasite death at the trophozoite stage following disruption of proteolytic digestion of haemoglobin and the phenotypically-associated swelling of the food vacuole. By contrast, inhibition of *Pf*-LAP using a *Pf*-LAP-specific

probe (PNAP) correlated with an early death chemotype at the ring-to-trophozoite transition with no obvious morphological features.

2.2 *Profiling Acyl-Protein Thioesterases*

Acyl-protein thioesterases (APTs) are diverse hydrolytic enzymes that catalyse the removal of lipid modifications from protein-associated cysteines through esterase activity. For instance, APTs that specifically cleave palmitate groups are known as palmitoyl-protein thioesterases (PPTs, or depalmitoylases) and play an important role in palmitoylation, a reversible post-translational modification (PTM) that modulates protein function by promoting their membrane localisation, stability and trafficking. As members of the serine hydrolase family, the activity of such enzymes can be profiled using serine hydrolase-directed ABPs, e.g. ABPP was used to characterise the function of a thioesterase in *T. gondii*, *Tg*PPT1 (also known as *Tg*ASH1) (Child et al. 2013; Kemp et al. 2013).

In 2011, Hall et al. (2011) screened a covalent small-molecule library, identifying a related set of substituted chloroisocoumarins that intriguingly enhanced the invasion of host cells by asexual *T. gondii* parasites. Cell-based studies later revealed that these compounds promote an invasive phenotype by inducing microneme secretion and gliding motility, with JCP174 producing the most consistent results (Child et al. 2013). Given the unusual nature of this effect, derivatives of JCP174 were synthesised to facilitate the identification of the targets of this 'enhancer' compound. Using the fluorescently labelled broad-spectrum serine hydrolase probe FP-Rho (Fig. 2d), it was first shown that JCP174 competes for labelling of one specific protein species by in-gel analysis. This ABP competition did not occur when using an inactive analogue of JCP174 that lacked an aromatic amine moiety conserved amongst the enhancer-type compounds. An alkyne-modified variant of JCP174 (JCP174-alk; Fig. 2h) was synthesised to allow probe-labelled proteins to be coupled with a biotin-azide affinity tag and identified by tandem orthogonal proteolysis ABPP (TOP-ABPP) (Weerapana et al. 2007). Mass spectrometry analysis of biotinylated proteins revealed the primary target of JCP174 to be *Tg*PPT1 (Child et al. 2013). A combination of small-molecule and genetic approaches was then used to validate a role for *Tg*PPT1 in microneme secretion and gliding motility, revealing for the first time the importance of dynamic palmitoylation in the regulation of *T. gondii* invasion. The contribution of *Tg*PPT1 to host cell invasion was also demonstrated by Kemp et al. (2013), who used a similar competitive ABPP approach to identify this depalmitoylase as a primary target of various β-lactone-based compounds. These findings initiated the development of a general JCP174-based fluorescent ABP (JCP174-BT) that can be used to profile both parasite and human APTs activity in situ (Garland et al. 2018). Future studies using this probe may provide further insight into the functions of depalmitoylases in other parasite systems and the potential for discovery of novel druggable nodes.

2.3 *Profiling Protein Kinases*

Protein kinases are implicated in essential functions at virtually every stage of the parasite life cycle and are thus widely acknowledged as drug targets (Doerig 2004). Despite their importance, developing ABPs for profiling protein kinase activity has been a long-standing challenge in chemical proteomics (Rosenblum et al. 2013). Protein kinases catalyse the direct transfer of phosphate from ATP to a protein substrate without any covalent enzyme-ligand intermediate and thus do not typically have a defined nucleophilic residue that can be targeted for covalent modification. Further, as the architecture of the ATP-binding site is similar for many protein kinases (and other enzyme classes), developing competitive inhibitors and probes of ATP-binding with selectivity for the target enzyme(s) remains difficult. Nevertheless, most kinases contain at least one conserved lysine within their active sites that interact with the phosphate backbone of the ATP substrate. This interaction has been exploited in the development of several kinase-directed ABPs including acyl-phosphate ATP probes, Wortmannin-based probes and hypothemycin (Cravatt et al. 2008), some of which have been applied to study kinase function in parasites.

Kinase-based ABPP has been employed to identify the targets of hypothemycin in *T. brucei*, a polyketide natural product inhibitor of CDXG-type kinases with potent trypanocidal activity (Nishino et al. 2013; Schirmer et al. 2006). Kinases play key roles during the life cycle of *T. brucei* and are being increasingly explored as therapeutic targets for African trypanosomiasis (Nett et al. 2009; Parsons et al. 2005). However, while the *T. brucei* kinome comprises 182 potential targets, their interrogation with small-molecule inhibitors has been hampered by the observation that many trypanosomal kinases share high sequence similarity with their human orthologues. Recently, Nishino et al. (2013) produced a semi-synthetic derivative of hypothemycin, a potent inhibitor of CDXG-type kinases, and demonstrated its effectiveness in clearing bloodstream form *T. brucei* parasites in vitro and in vivo. To enable the targets of hypothemycin to be identified, an alkynye-modified hypothemycin-based probe was synthesised for click-conjugation of biotin- or rhodamine-coupled azides (Fig. 2i). Using the biotinylated probe, gel-free competitive ABPP was then conducted with isobaric mass tags to allow for MS-based quantitation of hypothemycin-sensitive proteins in parasite lysates. Using hypothemycin, 11 of 21 total CDXG motif-containing kinases were identified with variable sensitivity to the probe. Here, the previously uncharacterised *Tb*CLK1/2 kinases were shown to have the highest affinity for hypothemycin, followed by other kinases including *Tb*GSK3short and *Tb*MAPK2. Inhibition experiments in live, intact trypanosomes confirmed *Tb*CLK1 as the preferred target of hypothemycin, with its inhibition correlating with loss of cell viability. Although hypothemycin exhibited considerable cytotoxicity in mammalian cells, this study identified *Tb*CLK1 as a potential drug target and introduced hypothemycin-based probes as tools to profile the activity of CDXG-type kinases.

3 Activity-Based Protein Profiling in Parasites: An Exciting Future

Chemical proteomics is a growing field of research that lies at the interface between chemistry and biology. As highlighted in this chapter, the recent development and application of chemical proteomic technologies to parasite systems has contributed vastly to our understanding about the basic biology of a diverse range of pathogens over the last decade. In particular, ABPP has emerged as a powerful technique for functional profiling of diverse enzyme classes and has facilitated the identification of proteins associated with many essential parasitic processes. Additionally, chemical proteomic approaches aimed at profiling PTMs at a systems level have been invaluable for associating PTMs and PTM substrates with critical cellular events. Collectively, these techniques have facilitated the discovery of new potential targets that could be exploited in the development of next-generation therapeutics for many parasite-related diseases. Further, the versatility of ABPP in screening the potency and selectivity of small-molecule inhibitors in complex biological samples has provided a strong foundation for target-based rational drug design. Assessment of the cellular uptake, pharmacokinetic and pharmacodynamic properties of such compounds will provide insight into the full potential of these inhibitors as novel anti-parasitic agents.

Future development of the chemical proteomic technologies described in this review will offer exciting opportunities for parasitologists to explore unchartered territories of parasite biology. Indeed, chemical probes are continually being developed for new enzyme classes, broadening the types of protein that can be experimentally accessed (Chuh et al. 2016; Chuh and Pratt 2015; Yang and Liu 2015). It can therefore be anticipated that further application of these techniques to parasites will aid the functional assignment of previously uncharacterised proteins and thus uncover unique aspects of parasite biology and new drug targets.

This chapter has focused on examples in which chemical biology has been exploited to study parasite proteins with a specific enzymatic activity or PTM. Nevertheless, it is worth mentioning that other chemical proteomic technologies exist, which to our knowledge have yet to be applied to parasites. One well established and rapidly evolving area of ABPP is quantitative reactivity profiling. In this approach, broad-spectrum electrophilic probes are used to quantify the intrinsic reactivity of a specific nucleophilic amino acid type in a complex proteome (Abo et al. 2018). Indeed, this can provide insight into the catalytic and regulatory functions of certain amino acid side chains in proteins, as well as their PTM state and inhibitor occupancy (Abo et al. 2018). While several platforms have been developed for this purpose, perhaps the most widely used is isotopic tandem orthogonal proteolysis activity-based protein profiling (isoTOP-ABPP) (Weerapana et al. 2010). Pioneered by the Cravatt group, isoTOP-ABPP enables the relative abundance of reactive cysteines to be quantified on a global scale using an alkyne-tagged iodoacetamide probe that has specific reactivity towards the cysteine thiol. This technique has been successfully applied to profile cysteine reactivity in

diverse biological systems, and since its development has been adapted to study the sensitivity of cysteines to various thiol-dependent PTMs including *S*-sulfenylation and *S*-nitrosylation (Deng et al. 2013; Martell et al. 2016; Yang et al. 2015; Zhou et al. 2016). Furthermore, this platform has recently been expanded to allow for profiling of other nucleophilic hotspots such as lysine side chains using amine-reactive probes (Anderson et al. 2017; Hacker et al. 2017). Although the concept of reactivity profiling remains largely unexplored in parasite systems, the potential for these technologies to reveal the importance of reactive amino acids in parasite proteomes and identify new druggable targets is an exciting prospect.

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