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Chemical Biology Approaches for the study of Apicomplexan Parasites

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Abstract

Chemical biology and the techniques the field encompasses provide scientists with the means to address biological questions in ever evolving and technically sophisticated ways. They facilitate the dissection of molecular mechanisms of cell phenomena on timescales not achievable by other means. Libraries of small molecules, bioorthogonal chemistries and technical advances in mass-spectrometry techniques enable the modern chemical biologist to tackle even the most difficult of biological questions. It is because of their broad applicability that these approaches are well suited to systems less tractable to more classical genetic methods. As such, the parasite community has embraced them with great success. Some of these successes and the continuing evolution of chemical biology applied to apicomplexans will be discussed.

Keywords

Apicomplexan; chemical biology; small-molecule; bioorthogonal

1. Introduction

The field of chemical biology evolved from the overlap between the disciplines of chemistry and pharmacology, and as such can be considered as an extension of those ideas and principals. Broadly, chemical biology can be defined as the application of chemical techniques, and small molecules synthesized using those techniques, to biological systems in order to manipulate them. Through careful observation of these perturbations it is possible to gain unique insights into the system being manipulated. The speed with which these chemical tools can be applied and a biological effect manifest makes chemical biological approaches ideally suited to interrogate systems refractory to more traditional approaches. Apicomplexan parasites are one such example, where despite recent advances the continuing lack of robust, rapid and broadly applicable techniques for the conditional regulation of genes highlights the power of studying these parasites through the lens of chemical biology.

This review aims to provide a brief overview of the application of chemical biology tools and techniques to the study of apicomplexan parasites, focusing on *Plasmodium falciparum* and *Toxoplasma gondii*. Due to space limits, this review will highlight specific examples rather than providing a comprehensive overview of all available literature. Additional reviews are available that more broadly describe the application of small-molecules in

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cellular microbiology [1]. As a conclusion, a view of how this field will continue to evolve with regard to the study of these parasites and other organisms will be presented.

2. Small-molecule tools

Many of the successes of chemical biology in *P. falciparum* and *T. gondii* have been achieved through the use of small molecules to study basic biological processes. In many respects, therapeutic-drug treatment of parasitic infections represent the first forays of the parasite field into chemical biology approaches, some of which pre-date the birth of chemical biology as a recognized discipline. In the 17th century Cinchona bark was used for the treatment of malarial fevers, and later chemical extractions characterized the active anti-malarial component as quinine [2]. Further efforts identified a structurally related molecule, chloroquine, as a cheap and efficacious anti-malarial, and it is arguably the most successful anti-malarial drug identified to date [3]. Despite its success and global application, its precise mode of action remains elusive, with best evidence supporting a molecular mechanism whereby chloroquine interacts with heme in the digestive food vacuole of the parasite and blocks its detoxification [4]. Similarly, artemisinin extracted from Chinese wormwood has rapidly emerged from local use in China as a potent anti-malarial, and is now a global front-line drug for the treatment of the disease. However, once again, the mechanism of action remains a matter of some debate [5]. Although these are examples of classical pharmacology, they can now be recognized as also being chemical biology studies containing many unanswered questions. Application of the chemical biology approaches outlined below may provide alternative ways to dissect the molecular mechanisms of these drugs, which in turn could indicate novel avenues for therapeutic intervention.

In terms of modern chemical biology, the use of small-molecules fall into two distinct types of approach: (1) the application of a small-molecule with a previously described target/mechanism of action, and (2) phenotypic screening of small-molecule libraries to identify compounds of previously unknown mechanism. Figure 1 provides an overview of where such approaches have provided unique insights into parasite biology, some of which will be addressed in greater detail below.

2.1 Small molecules with known targets/mechanism of action

Protease inhibitors have been used with great success to probe parasite specific processes such as egress (host-cell escape) and host-cell invasion [6, 7]. Typically, in the case of covalent inhibitors, these compounds contain electrophilic traps that specifically modify reactive nucleophiles such as those found in the active sites of enzymes. These covalent modifications can be both reversible and irreversible, depending upon the electrophilic trap present on the inhibitor. The amino acid environment surrounding the catalytic residue in the active site of the enzyme contributes to both the nucleophilicity of the catalytic residue, and to the recognition of substrates. As a result, some protease inhibitors are built around a peptidic scaffold that serves to provide a specificity component to the inhibitor. The additional benefit of covalent inhibitors is that they facilitate downstream identification of targets, a point that will be returned to later. The mechanism of protease inhibitors can also be non-covalent, where they often function as competitive inhibitors of an enzyme/substrate interaction. Also, the interaction between the inhibitor and the enzyme need not occur at the active site as exemplified by non-competitive inhibitors. The potential to interact with and block exosites required for substrate recognition are under active investigation [8].

Broad-spectrum inhibitors have been developed for most enzyme classes, though inhibitors specific for a single target remain a largely theoretical ideal. Starting with a compound with a known mechanism of action provides important insight into possible targets in a system to which it is applied. An excellent example of the benefit of knowing the mechanism of action

is the natural product E-64. This compound contains an epoxide electrophilic trap that effectively inhibits cysteine proteases whose catalytic domain architecture adopts a papain-type fold, such as the cathepsins and calpain [9, 10]. Treatment of intra-erythrocytic *P. falciparum* parasites with E-64 results in two distinct phenotypes. A swollen food-vacuole defect and an inhibition of host cell egress [11, 12]. The molecular basis of each phenotype has subsequently been determined; the swollen food vacuole defect arises from an inability of the parasite to digest hemoglobin imported to the food vacuole from the host cell for detoxification [13]. Subsequent work validated the targets of the inhibitor as being members of a papain-fold family of cysteine proteases called the falcipains. These proteases localize to the digestive food vacuole, and are essential components of the hemoglobin degradation pathway [14]. For the E-64 inhibition of egress, a detailed chemical biology study took advantage of an activity-based probe (see below) similar in structure to E-64 to identify other potential targets of the compound, and thereby implicate these proteins as transducers of the compound effect [15]. This work demonstrated that inhibition of host calpain by E-64 blocked parasite egress, and the authors hypothesize that activation of calpain prior to egress is required to degrade the erythrocyte cytoskeleton and facilitate efficient escape from the host cell, adding weight to the idea that parasites hijack host-cell components for their own benefit.

An alternative strategy is to identify small-molecule inhibitors through target-based screens [16]. For these studies a target protein of interest is expressed and purified from a heterologous expression system, and an assay is developed to monitor a predicted or known function of the protein target. Often, a recombinant protein of interest can be difficult to express or purify (especially for *P. falciparum* which has an A/T rich genome and asparagine-rich inserts in proteins). As a result, target-based screens have also been developed using crude cell-preparations such as whole cell lysates where the specific activity of a protein of interest can be resolved from other related activities present in the sample [17]. In the case of proteases activity, assays are often substrate based, with the readout being a change in either fluorescence or absorbance of a reaction mixture. For example, monitoring the release of a fluorescent group from a peptide substrate upon its recognition and cleavage by an enzyme target. Once established, the ease of set-up and simple readout means these screens are suited to automation and can be performed in medium or even high-throughput formats. Libraries consisting of hundreds of thousands of small molecules of defined classes can be tested with minimal effort. In an excellent example of this type of approach, high-throughput screening of compound libraries identified a small-molecule inhibitor of *P. falciparum* subtilisin-like protease 1 [18]. Recombinant PfSUB1, expressed and purified from insect cells, was screened in a fluorogenic substrate assay using a library of over 170,000 compounds. The screen identified one molecule, MRT12113, which was shown to be a potent inhibitor of PfSUB1 inhibitor and to have no activity against related proteases or general toxicity against mammalian cells. At low concentrations, treatment of intra-erythrocytic parasites resulted in the egress of merozoites that could not reinvest erythrocytes, while high concentrations of MRT12113 inhibited egress. Moreover, each aspect of the phenotype tracked with changes in the proteolytic maturation of egress- and invasion-associated proteins, such as SERA5 and MSP1, and the specific role of PfSUB1 in directly processing these substrates was then confirmed *in vitro*. Thus, the identification and subsequent application of the PfSUB1 inhibitor allowed for the protease to be ascribed a function as a maturase responsible for proteolytically processing parasite surface proteins required for invasion, and for triggering a proteolytic cascade that culminates in the escape of the parasite from its host cell niche [18, 19].

In addition to proteases, other proteins have been targeted with small-molecule functional modifiers. In particular, actin modifiers such as cytochalasin D have played a key role in

confirming the essential role of this protein in virulence-associated cellular events. Cytochalasin D binds to G-actin and prevents polymerization of actin monomers. Treatment of extracellular *T. gondii* tachyzoites with this mycotoxin was shown to inhibit motility and block the ability of the parasite to invade host-cells [20]. This small-molecule was critical to advancing our understanding of how extracellular parasite motility is driven by an actinomyosin motor, and how invasion of host cells by both *P. falciparum* and *T. gondii* is also actin dependent [21-23].

Small-molecules can also modify biological processes without necessarily having to directly interact with a protein target. These types of small molecules have served to dramatically increase our understanding of basic biological phenomena such as calcium signaling. The calcium ionophore A23187 is a lipid-soluble ion transporter, with selectivity for calcium. It actively transports ions across cell membranes, and is used to artificially increase the intracellular concentration of calcium. A23187 is often used in conjunction with the cell-permeable calcium chelator, BAPTA [24], which in its methyl-ester form (BAPTA-AM) is retained in cells by the activity of endogenous esterases. Artificial induction of calcium signaling triggered by A23187 demonstrated an essential role for this secondary messenger in triggering egress from host cells [25]. Similarly, A23187 was used to show that calcium was required for the secretion of invasion-associated apical organelles called micronemes [26], a process that could be ablated by pretreating the cells with BAPTA-AM.

More recently, protein engineering has been employed as an alternative strategy to achieve small-molecule selectivity. The underlying rationale being that a protein of interest can be modified such that it becomes uniquely sensitive to a small molecule inhibitor [27]. The Shokat laboratory at UCSF pioneered the specific application of the technique to kinases [28]. The so-called “bump-and-hole” methodology takes advantage of naturally occurring conserved bulky amino acids found in the ATP binding pocket of the kinase that can be replaced by a smaller residue to create the “hole”. The activity of the mutant kinase is assessed to ensure it is indistinguishable from wild-type. Existing kinase inhibitor scaffolds are then evolved to take advantage of the new physical space provided by the hole; the structure activity relationship between the protein/drug interaction surfaces is advanced to a point where the interaction of the drug with the kinase is critically dependent upon the hole incorporated by the mutation. Moreover, in the wild-type kinases the natural bulky residue now excludes binding of the optimized inhibitor, resulting in a unique kinase/inhibitor pair. In work by Lourido *et al* [29] the technique was applied to the calcium-dependent protein kinase 1 (CDPK1) of *T. gondii*. By replacing the native gene with a bump-and-hole mutant they were able to specifically target this kinase, and demonstrated that CDPK1 activity was required for microneme secretion. Lourido and colleagues have now further advanced the technique by modifying another kinase, calcium-dependent protein kinase 3 (CDPK3) such that it is sensitive to a separate, unique compound [30]. Combining the two mutants in a single strain has enabled the authors to dissect the specific contributions of CDPK1 and CDPK3 to the processes of egress, gliding motility and host-cell invasion. Moreover, they have managed to determine where there is functional redundancy and where the activity of each respective kinase is unique.

2.2 Phenotypic screening of small-molecule libraries

A powerful alternative to using small-molecules of known function, or target-based screens as a starting point for a chemical biology investigation is a phenotypic screen [1, 16, 31]. For these screens a measureable phenotype is chosen as the metric to quantify. Ideally, this is something that can be quantitatively assessed in a high-throughput format. Such screens have been successfully applied to host-cells invasion by *T. gondii* [32, 33], and the entire intra-erythrocytic developmental cycle of *P. falciparum* [34]. The principal advantage of

phenotypic screens is that if a biological phenomenon is observed, the drug has succeeded in penetrating the parasite and interacting with target(s) that mediates that effect. Thus, the identified small-molecule can be immediately considered as being bioactive. Phenotypic screens have the additional advantage of removing bias from the observation. By selecting a parameter (phenotype) to observe and recording all outputs, it is possible to identify unexpected phenomena.

In an early example of applying this technique to *T. gondii*, Ward and colleagues screened a library of approximately 12,000 small molecules against tachyzoites in a host-cell invasion assay [32]. In addition to monitoring invasion, the group included a range of secondary screens to more precisely define the stage of invasion where inhibitors exerted their effect. With these combined primary and secondary screens, the authors identified specific inhibitors of gliding motility, microneme secretion and conoid extrusion. These small molecule tools allowed the authors to determine the contribution of specific cellular events to an invasion process that were thought to be inextricably linked, yet shown by these studies to be distinct. For example, inhibition of conoid extrusion was shown to block invasion, without affecting microneme secretion or motility.

Similarly, Bogyo and colleagues screened a targeted library of 1500 protease-directed small molecule inhibitors containing covalent electrophilic traps against blood-stage *P. falciparum* [34]. For the *P. falciparum* replication assay, effects on parasite intracellular growth, egress and invasion were quantified as the change in total DNA content of the parasite population over time. The screen identified two compounds that independently inhibited parasite egress through the inhibition of two distinct targets: dipeptidyl-aminopeptidase 3 (DPAP3) and PfSUB1, complementing target-based screen undertaken by Yeoh *et al* and described above [18]. This study confirmed the role of PfSUB1, and implicated the function of another previously unidentified target (DPAP3) in the regulation of parasite egress. The function of DPAP3 is the focus of continuing studies.

For a similar phenotypic screen against *T. gondii* the Bogyo laboratory assayed the process of host-cell invasion by extracellular tachyzoites. Using the same protease-directed library two distinct classes of compounds were identified: those that inhibited invasion, and those that enhanced it. Although unexpected, enhancement has been observed in similar screens [32, 35]. With regard to one inhibitor, a peptidic alpha-beta unsaturated ketone called WRR-086, a combination of genetic, biochemical and chemical biology mass-spectrometry techniques (see techniques section below) were used to validate the phenotypically relevant target of the compound as the *T. gondii* orthologue of the Parkinson's disease-associated protein, DJ-1, a redox-dependent chaperone [33]. Not only did this study and the identification of this target indicate that calcium alone is insufficient to trigger microneme secretion, it also implicated intracellular redox potentials, interpreted by TgDJ-1, as an additional regulator of invasion. In a separate study, the Bogyo laboratory investigated a panel of the enhancer compounds. These substituted chloroisocoumarins were shown to trigger microneme secretion and invasion through the inhibition of a previously uncharacterized acyl-protein thioesterase (TgPPT1) [36]. Once again, a combination of classical genetic and biochemical approaches and modern chemical biology techniques were used to validate TgPPT1 as the target, and confirm that it functioned as a depalmitoylase in the parasite. This confirmed dynamic palmitoylation as a critical regulator of host-cell invasion in this parasite, highlighting the power of phenotypic screens for identifying biological pathways of unexpected and unexplored importance.

A key conceptual advance in the use of small molecules was their conversion into activity-based probes (ABPs) [37]. ABPs can simply be considered as a covalent small-molecule inhibitor that has been chemically modified such that it now contains a fluorescent moiety or

other reporter molecule or handle such as biotin. Alternatively, an ABP can contain a latent functionality that allows for later addition of reporters by orthogonal chemical approaches (see below for bioorthogonal chemistries) [38]. These probes covalently label protein targets in such a way as to allow for their visualization across multiple complementary biological platforms such as SDS-PAGE, fixed and live cell microscopy, and even *in vivo* imaging in animal models (Figure 2). Some of these molecules, such as fluorophosphonate-rhodamine are broad-spectrum probes for serine enzymes [39, 40], with the particular electrophile present on the probe providing selectivity for a given nucleophile. Cysteine probes are widely available, but higher basal reactivity of free-cysteines irrespective of catalytic function means that broad-spectrum cysteine probes do not exist. Instead, cysteine-targeted probes tend to hit a specific enzyme class, such as DCG-04 that targets papain-fold proteases such as cysteine cathepsins [39]. These broad-spectrum probes can then be used in competition assays [41]. In these assays intact cells or cell lysates are treated with a compound of interest, and then with the ABP. If the test compound binds to a target that is also a target of the probe, then it will compete with the ability of the probe to bind to the protein target by occupying the binding site. These competition assays provide information regarding compound selectivity, and generate leads for downstream target identification and validation studies. Additionally, conversion of a selective small molecule into an ABP creates a powerful tool that can be used in further drug screening efforts.

Each of the small-molecule approaches outlined above has associated caveats and weaknesses. For all, the levels of evidence required to validate a target and to confidently attribute a biological phenomenon to a drug-effect are the same. We have attempted to outline distinct criteria that we believe should be at least considered and at best rigorously adhered to whenever small molecules are used to investigate a biological phenomenon. Similar to the chemical biology “pillars” suggested to be a foundation for such studies [42] and experimental ideals alluded to in other excellent recent reviews [31, 43, 44], we present these criteria as four individual requirements for small-molecule studies:

1. Observation of bioactivity (phenotype) in an intact biological system.
2. Target identification and confirmation of target engagement.
3. Biochemical determination of functional consequence of compound/target interaction.
4. Genetic validation to demonstrate phenotypic relevance of the target.

The descriptions of the uses of small molecules provided above address the first aim. The use of broad-spectrum ABPs and the conversion of a compound of interest into a probe can satisfy the second, and will be supplemented with many of the chemical biology techniques described below. Aims 3 and 4 require more traditional biochemical and genetic approaches; putative targets such as enzymes can be heterologously expressed and tested with a compound for direct binding. Mutation of the key enzyme nucleophile can confirm the dependence of the compound/protein interaction upon the reactive catalytic amino acid in the enzyme active site. Biochemical assays can then confirm the functional consequence of compound binding upon enzyme activity. In tractable systems, genetic manipulations provide a powerful tool to further validate a putative target. Genes can be knocked out to demonstrate the direct dependence of a phenotype upon the presence of the gene-product, and also illustrate the loss of sensitivity to the compound in the absence of the relevant target. Similarly, over-expression and knockdown approaches can result in a shift in the EC₅₀ for a given effect, correlating target abundance with compound sensitivity. Together, biochemical and genetic approaches are powerful tools for target validation and dissection of the molecular mechanisms underlying a compound effect.

3. Chemical biology techniques

The following are examples of specific chemical biology techniques applied to the study of parasite biology that highlight the versatility of the field, and the power of the toolbox it provides to researchers. Using chemical biology techniques small-molecules can be tagged (bioorthogonal chemistry), biological processes tuned (targeted degradation), proteins manipulated (non-natural amino acid incorporation) and biological processes profiled (metabolic labeling with bioorthogonal metabolic intermediates). Some of these techniques that have been applied to parasites will be addressed in more detail below.

3.1 Bioorthogonal chemistries

Orthogonal chemistry for biological systems remains at the forefront of modern chemical biology. The ability to perform chemical reactions in cells and cell preparations that are completely orthogonal to the endogenous chemical reactions of a cell provides unique chemical tools to manipulate biology without altering native chemistries [45]. Figure 3 illustrates some of these bioorthogonal reactions where latent chemical handles, with the most widely used being alkyne and azido functionalities, are incorporated into molecules of interest. In the case of alkynyl and azido functionalities, copper-catalysed cycloaddition creates a stable 1,2,3-triazole group, which covalently links the two molecules together. This is commonly referred to as Click chemistry, though it should be noted that the phrase is typically used to describe a more limited set of chemical reactions than Sharpless and colleagues originally intended [46]. An alkynylated small-molecule covalently associated with a protein via an electrophilic trap can have an azido-fluorophore “clicked” on to it, enabling visualization of the molecule interacting with discrete protein targets [38]. Alternatively, by clicking on an azido-biotin the protein targets of a particular compound can be selectively isolated and identified/quantified by mass-spectrometry. This technique has been further modified for tandem orthogonal proteolysis activity-based protein profiling (TOP-ABPP) [47], where the specific residue modified by the covalent inhibitor on the protein targets can be identified. This methodology is incredibly powerful, and was key to the identification of TgDJ-1 and TgPPT1 as putative targets of the small-molecule inhibitor and enhancer respectively [33, 36].

3.2 Mass spectrometry profiling

Chemical biologists have embraced mass-spectrometry as a powerful tool to profile targets of small-molecules inhibitors. They have also advanced such methodologies from a tool to simply identify protein targets to methods that can be used to systematically profile complex cellular functions. A recent example makes use of a palmitic acid analogue containing a latent alkyne functionality. Cells are metabolically labeled with this fatty-acid analogue, 17-octadecynoic acid, which is taken up by cells and replaces native palmitate as it cycles on and off protein substrates during the process of dynamic palmitoylation [48]. Following metabolic labeling all palmitoylated protein species have a clickable moiety associated with them via the alkynyl-palmitate group. Cell membrane fractions can be isolated, and following a click reaction with a retrievable group such as biotin, the global complement of palmitoylated proteins in an organism can be profiled by mass spectrometry. Such a palmitoylated proteome (sometimes referred to as the palmitome) was recently generated for *P. falciparum* [49], with the authors of the study confirming the association of this post-translational with a broad range of protein targets involved in diverse functions such as cytoadherence, drug resistance, signaling, development, invasion and metabolism. Multiple groups are currently working to generating similar lists for *T. gondii* where palmitoylation has been shown to play a key role in the regulation of motility and invasion associated proteins [36, 50-52].

In principal this approach can be extended to any fatty acid, with the only technical limitation being the generation of the alkynylated acyl analogues, a challenge being tackled by a number of groups [53]. With these in hand, global proteomic descriptions of the entire acylome should be readily achieved.

3.3 Chemical genetics for non-covalent small molecules

Identification of targets of non-covalent reversible inhibitors remains a technical challenge; the lack of a stable covalent compound/target complex means that the targets cannot be selectively isolated with the approaches described above. Technical advances in whole genome sequencing, and the general reduction in cost, has created the opportunity for large-scale chemical genetics [54]. These approaches take advantage of the natural propensity of both *P. falciparum* and *T. gondii* to become resistant to an inhibitory small molecule following prolonged periods of culture with that compound. Mutant populations can be isolated and sequenced, and the resistance-associated mutations mapped to specific loci in the genome. Mutated genes can then be verified as targets of the drug, if the mutation provides protection by interfering with the ability of the drug to interact with the target. Incorporating an additional mutagenesis step in the protocol can increase the speed with which mutants are selected for. Chemical mutagenesis immediately increases the diversity of mutations in the population, and thereby increases the likelihood of selecting for a resistant. This approach has proven successful in *T. gondii*, where chemical mutagenesis was used to isolate parasites resistant to the egress and death inducing effects of the calcium ionophore A23187 [55]. Although generated some time ago, recent whole genome sequencing of mutant strains identified the site of mutation as being within the locus encoding TgCDPK3 [56]. The mutations were shown to inactivate the kinase, and confirmed an essential role for this kinase in ionophore-induced egress.

3.4 Chemical rescue

A landmark recent study found that *in vitro* cultured blood-stage *P. falciparum* only maintain an ancient plastid-like organelle called the apicoplast for production of isopentenyl pyrophosphate (IPP), a key metabolic precursor for isoprenoid biosynthesis [57]. Previous work had shown that the antibiotic fosmidomycin, an inhibitor of non-mevalonate isoprenoid precursor biosynthesis, inhibited parasite growth. Surprisingly, the authors of this study discovered that supplementation of *P. falciparum* cultures with IPP chemically rescued fosmidomycin treated parasites. Over time, parasites completely lost the apicoplast indicating that at least for *in vitro* cell culture the sole purpose of this highly conserved organelle is to provide IPP for downstream isoprenoid biosynthesis. This is the first example of a chemical rescue resulting in the loss of an entire organelle, and highlights the potential for similar small molecule chemical rescue approaches to uncover new biology.

4. Future evolution of chemical biology for apicomplexans

4.1 Diversity-oriented synthesis

Small molecules have been broadly applied to the study of parasite-specific basic biological processes, and often researchers are only limited by the variety of compounds available. Traditional chemical synthetic routes have their own limitations, and one approach that has been used to increase the diversity of available compounds is diversity-orientated synthesis (DOS) [58, 59]. DOS strategies such as those pioneered by Stuart Schreiber enable chemists to rapidly generate libraries of novel chemical structures that sample unexplored regions of chemical space. Diversity-oriented synthetic routes are typically designed such that an initial reaction generates a novel skeletal scaffold containing multiple reactive functionalities. These core functionalities are then expanded using other chemical building blocks to generate diversity within the library pool. Moreover, DOS allows chemists to pursue

multicomponent coupling reactions, where multiple chemical building blocks are coupled to a poly-functionalized scaffold in a single reaction. Existing knowledge of biologically active chemical groups enables DOS strategies to focus on biologically relevant chemical space [60], and thereby increase the likelihood of generating libraries of compounds with biological activity. The ability to build these libraries using solid-phase synthesis not only increases the ease with which they can be generated, but also provides the opportunity to incorporate electrophilic traps to facilitate later downstream target identification. Despite such libraries being available, there has been little application to parasites, with groups tending to favor compound libraries targeted at a specific biological activity, such as kinases and proteases. With target identification approaches now within reach of most laboratories, we expect the use of these novel DOS generated scaffolds to investigate potentially unknown regions of biological space to become more prevalent through increased application in phenotypic screening.

4.2 Reactive cysteine profiling

New chemical biology techniques continuously being developed for all aspects of biology are one of the reasons why the field is so exciting. Many of these offer the prospect of novel insights into unexplored areas of parasite biology. One particular technique the Bogoy laboratory is currently pursuing is to globally profile reactive cysteines [47]. This mass-spectrometry approach uses a titration of iodoacetamide to rank cysteine reactivities across an entire proteome, and as such has the power to identify reactive cysteines of unknown function. Cell lysates are treated with a click-able iodoacetamide at either a high concentration or a low concentration. At the high concentration iodoacetamine (IA) will indiscriminately alkylate and label all free cysteines. An azido-biotin is clicked onto the probe, targets pulled out and mass-spectrometry used to identify all labeled proteins. At the low concentration, reactive cysteine's will be preferentially targeted by the probe. Again, these targets are profiled by mass-spectrometry. Comparing the two datasets (high versus low) allows for a given target cysteine on a protein to be given a ratio score. If identified at both concentrations of IA, the ratio score is low and the cysteine classified as "reactive". By combining the method with specific cell treatments it is possible to monitor how the reactivity profiles change and thereby implicate specific cysteines in a given cellular response [61]. Not only does this advance the discovery of new biological functions for a given protein, such reactive cysteines are readily targetable by small-molecules and as such are ideal candidates for therapeutic intervention.

4.3 Chemical genomics

Genetic approaches for both *P. falciparum* and *T. gondii* continue to become more sophisticated; the most recent advances for conditional knockouts in *T. gondii* have questioned the essential nature of many genes [62]. This study by Meissner and colleagues affirmed the growing belief that a simple yes/no determination of whether or not a gene is essential is insufficient, as it neglects the possibility of conditionally essential genes, or genes that are *almost* essential but whose function can be compensated for upon deletion. This has been elegantly highlighted in recent studies showing overlapping functions between two *Toxoplasma* kinases: TgCDPK1 and TgCDPK3 (see section 2.1) [30]. One exciting future prospect would be to combine chemical biology and chemical genetics to study overlap between different regulatory pathways and functions. Genetic knockouts could be screened in conjunction with panels of small-molecule inhibitors to identify unique or co-regulated pathways; selective inhibition of a bump-hole modified kinase could be simultaneously profiled with a compound library of interest, and a given the phenotypic effect quantified in the presence and absence of the selective kinase inhibitor. Such an approach would be informative as a reduction in compound effect (shifted effective concentration – EC₅₀) in the absence of kinase activity could be indicative of putative

targets functioning in the same pathway or signaling module as the kinase. This would help to position potential targets within discrete signaling pathways, provide further clues to their likely identities, and dissect the molecular mechanism underlying the compound effect.

4.4 Predictive phenotypic screening

Similar to the way in which chemical biology initially emerged from an overlap in scientific disciplines, the most exciting future developments for the field will likely again emerge from overlap in ways of thinking. Systems biology approaches are generating vast quantities of data, yet using these global descriptions of biological systems to dissect molecular mechanisms remains challenging. Genetic profiling with microarrays was able to identify co-regulated pathways and pathway components through a “guilt by association” approach [63, 64]. We propose that a similar conceptual framework could be successfully applied to mass-spectrometry datasets generated for small-molecule treated samples. Similar integrated approaches to dissect small-molecule perturbation of complex biological systems have been previously described as “systems chemical biology” or “chemoproteomics” [65, 66]. Such an approach could be undertaken in a high-throughput fashion, and would seek to identify a biological effect without *a priori* decisions as to what that effect might be; phenotypic screening without the experimental bias of choosing a phenotype to observe. This specific application of systems chemical biology we term “*predictive phenotypic screening*”. An intact biological system would be treated with a retrievable small-molecule covalent modifier and direct targets of the small-molecule isolated and characterized. Mild retrieval conditions used to isolate direct targets could preserve protein/protein interactions, enabling isolation and profiling of protein complexes targeted by a molecule of interest. These data would be combined with the complete and post-translationally modified proteomes (e.g. phospho-proteome, acylome), profiled in parallel by mass-spectrometry. The identities of direct targets and global cellular response to the treatment would indicate the biological process being perturbed, produce a multi-parameter description of the biological outcome of compound treatment and thus predict the likely phenotype. This would then be confirmed by an assay specifically tailored for visualization of the phenotype, and further dissected using the chemical biology approaches described above.

In summary, the future of chemical biology in apicomplexan parasites is full of potential, with the only limiting factor being the imagination of the research community.

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Highlights

- Small-molecules are powerful tools with which to study parasite biology.
- Phenotypic screens using libraries of small molecules can uncover novel biology.
- Bioorthogonal chemistries expand the toolbox of modern chemical biologists.
- Diversity-oriented synthesis will promote exploration of new regions of biological space.

Figure 1.

Examples of small-molecules used to study apicomplexan biology. Schematic depicting some successful uses of small molecules to target specific proteins and cellular events in *P. falciparum* and *T. gondii*. **1:** WRR-086, a peptidic alpha-beta unsaturated ketone that interacts with TgDJ-1 and blocks microneme secretion in *T. gondii* tachyzoites [33]. **2:** JCP174, a substituted chloroisocoumarin that inhibits the depalmitoylating activity of TgPPT1 and enhances invasive capacity of *T. gondii* tachyzoites [36]. **3:** JCP104, a biotinylated chloroisocoumarin that binds to PfSUB1 and blocks egress of blood-stage *P. falciparum* merozoites [34]. **4:** SAK1, a vinyl sulfone that inhibits PfDPAP3 and blocks *P. falciparum* merozoite egress [34]. **5:** E64, an epoxy succinate that inhibits host calpain to block *P. falciparum* merozoite egress [15], and inhibits the activity of parasite digestive food vacuole falcipains [12].

Figure 2. Applications of activity-based probes. Activity-based probes (ABPs), such as a fluorophosphonate rhodamine (structure in the center of the schematic, with the rhodamine fluorophore highlighted in pink) can be applied to study protease function across multiple biological platforms such as **1**, biochemical *in vitro* labeling of proteins, separation by SDS-PAGE and visualization of labeled target species by fluorescent scanning of the gel; **2**, labeling of intact or fixed cells to visualize the subcellular localization of small molecule targets or specific enzyme activities; **3**, application of ABPs to *in vivo* models of disease to visualize the association of specific enzyme activities with distinct pathophysiological states.

Figure 3. Bioorthogonal chemical reactions. A schematic of commonly used bioorthogonal reactions. Reactions **1-3** are three different types of cycloaddition that enables two biologically orthogonal functionalities to be combined: **1** Copper-catalysed dipolar 1,3-cycloaddition (Click-chemistry) generating a triazole group from an alkyne and an azide. **2** Strain-promoted Click (copper-free Click) using an azide and a cyclooctyne. **3** Tetrazine ligation, where an inverse-demand Diels-Alder reaction drives the cycloaddition of tetrazine and norbornene groups.