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Target assessment for antiparasitic drug discovery

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Abstract

Drug discovery is a high-risk, expensive and lengthy process taking at least 12 years and costing upwards of US\$500 million per drug to reach the clinic. For neglected diseases, the drug discovery process is driven by medical need and guided by pre-defined target product profiles. Assessment and prioritisation of the most promising targets for entry into screening programmes is crucial for maximising chances of success. Here we describe criteria used in our drug discovery unit for target assessment and introduce the ‘traffic light’ system as a prioritisation and management tool. We hope this brief review will stimulate basic scientists to acquire additional information necessary for drug discovery.

Drugs for neglected tropical diseases

Approximately 1 billion people – one sixth of the world’s population – suffer from neglected tropical diseases (NTDs) including the vector-borne parasitic diseases: filariasis, onchocerciasis, schistosomiasis, African sleeping sickness, Chagas disease and leishmaniasis [1]. Unlike the “big three” infectious diseases (HIV/AIDS, tuberculosis and malaria), NTDs receive comparatively little international attention [2]. Precise figures for the annual death toll and disease burden, measured as disability adjusted life years (DALYs) for NTDs are not available. Estimates vary from 137 to 534 thousand deaths per year and 12.8 to 56.6 million DALYs [2-5]. Many of the existing drugs used to treat NTDs have serious limitations, including: cost; difficulties in administration; poor safety profile; and lack of efficacy e.g. due to drug resistance [6,7]. We understand the biology of these parasites well, yet this knowledge has not been translated into modern therapeutics. In fact, of all new drugs to reach the market in the past 25 years, only 1% was for neglected disease [8]. Together, NTDs account for 5% of the global disease burden; yet, in a typical year, only about 0.1% of global investment in research is devoted to drug discovery for such diseases.

Historically, antiparasitic drug discovery has been conducted through low-cost, low-risk strategies by pharmaceutical companies, and many such drugs were first developed for other indications. These companies have produced therapeutics either via combination therapy using existing drugs, by realising a new indication for an existing drug or through label extensions from veterinary products [9]. Whilst there is some merit in this ‘piggy-backing’ approach many such drugs have proved too expensive or inappropriate for use in real-life settings to have any substantial health impact [10]. Indeed, only 4 out of 17 antiparasitic drugs developed from 1974-2004 were judged to be entirely suitable for use in resource-poor settings [10,11]. Moreover, evaluating patented compounds or drugs for a non-commercial patient class is perceived by some companies as a serious potential threat to future commercial revenue due to the possibility of revealing toxicities that were unknown previously [12].

The withdrawal of many large pharmaceutical companies over the past 25 years from anti-parasitic programmes as part of their core strategies, plus the unyielding need for the discovery of new therapeutics against these diseases means that significant gaps have emerged, particularly in early drug discovery [9]. Fortunately, a number of recent developments have resulted in credible mechanisms through which validated targets for NTDs can be progressed into and through a drug discovery pipeline (see Box 1;).

Therefore, the purpose of this review is to illustrate how molecular targets are assessed for entry into a drug discovery process, and to encourage groups to generate the necessary information for target assessment as a matter of course during their research, thereby producing a plethora of potential anti-parasitic drug targets for the future.

The review will focus on the molecular target approach to drug discovery. Nevertheless, the authors recognise that *in vitro* screening against whole parasites is also a valuable alternative strategy for anti-parasitic drug discovery [13-16].

Goals of a drug discovery programme

The ultimate goal of a discovery programme is the development of a new therapeutic with substantial benefits over existing therapies. To ensure the requirements of a new drug are clearly established and that they drive the process of discovery and development, a Target Product Profile (TPP) is established at the beginning of the programme. The TPP is a list that defines and prioritises the key attributes of the intended new agent (Box 2). The full range of attributes need to be carefully considered, prioritised and agreed upon in advance by all stakeholders, including patients, medical personnel, regulatory authorities and policy makers in disease endemic countries. A TPP evolves and matures as a project advances and thus needs to be periodically reassessed with regard to meeting essential, preferred or minimal acceptable criteria.

To initiate a drug discovery programme, a pool of putative targets will require assessment. The process thereafter is very much a voyage of discovery, and the chances of ultimate success can be increased by intelligence-based assessment and selection of these targets. This assessment needs to remain in the context of the TPP. By way of example, the TPP for two contrasting parasitic diseases are shown in Box 2.

Molecular Target Assessment

The assessment and prioritisation of targets is well established in commercial drug discovery, and this article will illustrate how this process can be appropriately applied to target assessment for entry into a parasitic disease discovery pipeline. The criteria of key importance against which each target is assessed are shown in Table 1. Each criterion has an associated scoring system depicted in the familiar colours of a traffic light. Once a series of targets have been scored, the traffic light assignment provides an initial, global view of the pipeline being considered, highlighting the overall most mature targets and any key areas of weakness. In most cases, a red assignment is not a complete 'stop', it simply represents the current status of the target and indicates where further research is required in order to progress a target towards entry into a pipeline.

Critical areas for target assessment

Attrition rates for drug discovery are high, in fact only 1 in 5 projects survives through preclinical development and less than 1 in 10 makes it through clinical development; hence, less than 1 in 50 make it to the clinic [17]. Most projects fail through problems in either biology (selection of targets often subsequently revealed as poorly validated) or chemistry

(failure to identify suitable drug-like lead compounds for optimization, lack of efficacy, toxicity or drug metabolism and pharmacokinetic (DMPK) issues). It is thus imperative that antimicrobial drug targets are fully characterised and shown to be essential for either growth or (preferably) survival of the pathogen. Wherever possible, this should be demonstrated in the host vertebrate stage(s) rather than the vector stage(s). Ideally, essentiality should be extended to an appropriate animal model of infection, since host environmental factors can have a profound effect on gene expression and susceptibility to drugs. It is also of crucial importance that targets are selected which have binding sites that can accommodate drug-like molecules i.e. are “druggable”. There has been much debate about what proportion of the human genome is druggable, currently estimated at 8-12% of all genes [18]. This statistic is currently unknown for pathogens, but is likely to be similar.

Target validation: two complementary approaches

The two principal approaches to target validation can be broadly categorised as ‘chemical methods’ and ‘genetic methods’. As discussed below, both methods have their strengths and weaknesses and, whenever possible, both approaches should be employed as they can yield valuable complementary information.

Chemical validation involves the use of drugs or experimental compounds to provide evidence that specific inhibition of a target results in inhibition of growth or death of the parasite. The value attached to such information increases in the following order: (i) *in vitro* activity against vector stages; (ii) *in vitro* activity against host stages; (iii) *in vivo* activity in animal models; and, best of all, (iv) clinical activity in humans. The major advantages of chemical validation are listed in Table 2. Only a few of the current drugs for NTDs meet all of these requirements: eflornithine used for the treatment of the chronic (*Trypanosoma gambiense*) form of human African trypanosomiasis is arguably the best example. With less-specific compounds or those that bind weakly, it is sometimes possible to show a correlation between inhibitory potency *in vitro* against the target (IC₅₀) and the whole cell (EC₅₀). Although such correlations are rarely perfect because of differences in cellular uptake, metabolism and other ‘off-target’ effects, they nonetheless can serve as a useful indicator of druggability.

Genetic methods are regarded by some as the most definitive method for target validation [19,20]. However, essentiality does not mean that the target is sufficiently different from that of the host to allow selective inhibition, underlining the need for additional chemical validation. The precise techniques employed depend on the genetic tools available for any given parasite (e.g. availability of inducible or non-inducible expression vectors; choice of drug-selectable markers) and the genetic and physiological properties of the organism under study (e.g. gene copy number, ploidy; ease of culture in defined media; susceptibility to drug selection; ease of transfection). In this respect, genetic manipulation of *Leishmania* and *Trypanosoma* [21,22] is generally easier than that of *Plasmodium* [23], but genetic manipulation of the related apicomplexan parasite, *Toxoplasma gondii* [24], can provide invaluable information about probable outcomes in malaria.

At the DNA level, targeted gene deletion by homologous recombination with a gene conferring resistance to a toxic drug or experimental compound will completely ablate expression of that particular allele. If the organism is diploid, or haploid with more than one genetic locus for the potential target, then multiple rounds of transfection with gene-deletion constructs and selection with multiple drug-selectable markers will be required. Various outcomes of such experiments have been observed and, as discussed below, must be interpreted with caution.

At first sight, the ability to obtain viable organisms in which expression of a target is completely abolished is suggestive that the target is not essential for growth or survival and therefore probably not a drug target. However, such phenotypes must be demonstrated in the appropriate life-cycle stage and life-cycle environment. Indeed, if the phenotype of such a null mutant can be predicted, it may be possible to obtain viable cells by growth in medium containing an appropriate supplement (e.g. ornithine decarboxylase deficiency rescued by putrescine [25,26] or thymidylate synthase deficiency rescued by thymidine [27]). In situations where chromosomal null mutants cannot be obtained, this negative result is suggestive, but not absolute proof, of the essential nature of the target. Further evidence can be obtained by ‘rescue’ of chromosomal null mutants by expression of another copy of the target (sometimes from a related species) either on an episomal vector or at another chromosomal locus [21-24]. Unfortunately, this reveals no information as to precisely what level of enzyme activity is compatible with growth or survival and therefore what level of inhibition has to be achieved by drug treatment. Inducible or repressible gene-expression system(s), such as the tetracycline-inducible systems for trypanosomes are useful in this regard [28-30]. In some circumstances, a genetic KO of an essential target, for example dihydrofolate reductase (DHFR) [31], may nonetheless yield viable organisms due to compensatory genetic changes. This further underlines the importance of both chemical and genetic evidence of essentiality.

At the RNA level, expression of a target can be modulated by RNA interference (RNAi) using double-stranded RNA (dsRNA) [32]. Stable expression of dsRNA using inducible systems is preferable to constitutive expression, since transgenic organisms lacking an essential biochemical component generally cannot be selected – only ‘escape mutants’ can be recovered making interpretation difficult. However, even with inducible systems, insufficient expression of dsRNA may fail to knock down target expression to levels required to reveal a phenotype (Table 2). Thus a “negative” result in the absence of careful phenotypic characterization of target expression is almost without value. A “positive” result, where growth inhibition correlates with decreased target production, can be quite helpful. However, even positive results can be problematic, if RNAi causes “off-target” effects.

Modulation of target levels by overexpression or knockdown by any of the above genetic methods is also useful in mode of action studies of lead compounds. Elevated target expression leads to decreased drug sensitivity in whole cells and, conversely, decreased target expression can lead to hypersensitivity.

Druggability

For a target to be ultimately validated by successful clinical trials, it must be both essential to the organism, and its function be appropriately modulated, such as inhibition of an enzyme, by compounds capable of achieving therapeutic concentrations on dosing to patients, i.e. drug-like compounds.

Traditionally, the selection of targets for drug discovery rarely included an assessment of the likelihood of discovering drug-like ligands [17]. This omission has contributed to the failure of screening campaigns, as the binding sites of many targets are too large or too small, too polar or lack sufficiently deep binding pockets to potently bind drug-like compounds (Figure 1). Therefore, the concept of target druggability has come to the fore to help identify targets with the greatest chance of success in the hit and lead identification process.

Druggability of a target has been described as the presence of protein folds that favour interactions with drug-like chemical compounds. The druggability of a target is not an absolute, but more of an assessment of the probability of finding potent drug-like inhibitors. Drug-likeness of a compound, which is a combination of physicochemical properties,

DMPK and toxicological profile, determines whether the compound can be formulated and delivered by the required route and, when dosed, affords efficacy with an acceptable safety profile [33]. A number of approaches are available to identify potential druggable targets [33,34] (see Box 3).

These new paradigms of assessing the suitability of targets for entering hit discovery represent potentially exciting ways of reducing the current high rates of attrition.

Assay feasibility

The traffic light scoring system for assay feasibility is an assessment of 'readiness' for the process at a given time point. The overall tractability of screening an isolated molecular target *in vitro* is dependent upon a number of other criteria (for ideal requirements see Box 4).

The guiding principle in all cases is the development of assays which are fit for purpose and are as directly related as possible to the target protein or the pathway concerned. The assessment should be target-driven, not format-driven, and the process should be governed by quality of the output rather than speed of throughput. This is even more important in the context of enzyme targets in infectious diseases, which often come with little previous drug discovery precedent and none of the reagents/technologies which accelerate assay development in the standard world of the druggable gene families i.e. ion channels, kinases and G-protein coupled receptors (GPCRs). A sole focus on homogeneity of assays (performed as simple mix and read protocols) is therefore not appropriate and separation steps can be accommodated if necessary. Sometimes enzymatic coupling of the target enzyme to yield an assayable end product is required; this requires a judgement on whether time is best spent on deconvolution of hit compounds or on novel reagent development programmes to avoid enzymatic coupling. In contrast to the isolated target approach, cell-based screening in parasites has been widely used for identification of cytotoxic agents (phenotypic screening) leading to often complex and time-consuming target identification studies. Whilst it offers the advantage of identifying only cell penetrating agents, it is often limited to medium throughput, small scale screening and generates a more complex and ambiguous environment for the rational development of structure-activity relationships (SAR) in a compound series (see review [35]). Exploitation of a specific molecular target using whole organism assays represents a useful compromise between isolated target and phenotypic screening but does require technologies such as target-linked reporter systems or comparative screening in wild-type and target-deleted organisms to be available in the organism of interest.

The decision on assay format is often therefore based on the proposed scale of the compound screen, assay performance and, in the case of cellular screening, how directly linked the read-out is to the target of interest.

Structural information

The structural information available for targets can range from none for a novel target with no close homologues, through limited information from homology models, to full protein-ligand structures derived from NMR or X-ray crystallography. Even when no direct structural information is available SAR and/or homology models can be used to predict the binding mode of compounds, allowing the design of further analogues to test the model and help to direct subsequent rounds of chemistry. Although obtaining a structure of a target protein can require considerable effort, the detailed structural information provides advantages over programmes carried out without this knowledge. The use of structure-based drug design has been extensively reviewed [36-39], and some benefits are listed below.

- In silico docking can be used to identify alternative compound series for screening and in the optimisation of leads.
- Protein-ligand complexes can validate hits from biochemical screening campaigns by confirming that they bind the target with a defined mode.
- Lead optimisation can be driven by rational design of ideal drug candidates rather than easily achievable chemistry.
- The identification of “hot spots” of binding from an analysis of the binding mode across compound series, especially fragments, can be used to optimise ligand efficiency.
- Structural information can be used to understand and exploit factors such as changes in compound-binding mode, conformational changes of the target protein, and effects on the ligand due to binding, to optimise potency or selectivity.
- Understanding of selectivity compared to other targets and how activity against these targets could be tuned.
- An understanding of resistance caused by mutations within the target protein and the design of compounds active against these resistant mutants.

Used intelligently, structural information can reduce the number of compounds needed to be made to identify pre-clinical candidates; a particularly important requirement in the field of NTDs due to the limited resource available.

Toxicity

Target-based toxicity can arise due to inhibition of human homologues of the parasite candidate target. In theory, for inhibition of a molecular target that is unique and essential to the pathogen but absent in the human host, this would not be an issue. In practice, not only are unique targets rare, but also targets might display co-enzyme or substrate binding sites common to human homologues. These can be identified through comparison of human and pathogen genomes [40,41] and the human counterpart used in a counterscreen. More commonly a human homologue is present, but there are often significant differences between the pathogen and the human enzymes. A prime example is dihydrofolate reductase (DHFR), where structural differences between species allows the development of exquisitely selective inhibitors [42]. The evidence for selectivity is often chemical. Unfortunately, this information is not always available at the stage of selecting a molecular target for a high-throughput screen, although structural data on the molecular target and human homologue can give very useful information as to the possibility of selective inhibition. When an essential human homologue is present with little difference to the parasite enzyme, then toxicity is likely to be a significant problem. A notable exception is the case of inhibition of ornithine decarboxylase (ODC) by difluoromethylornithine (DFMO) where selectivity is attributed to more rapid turnover of human ODC and other factors [43,44]).

Resistance potential

An organism has many possible mechanisms of generating resistance to a drug, including point mutations, over-expression of the molecular target, gene amplification, reduced uptake of drug, increased efflux, metabolic by-pass and enzymatic inactivation of drug.

However, when attempting to assess a molecular target for drug discovery, it is possible to make some predictions about resistance. Thus, the presence of isoforms of the enzyme within the pathogen leads to possibilities of resistance; if there are several enzymes that appear to carry out the same (or very similar) metabolic roles, there is a possibility of one

substituting for another. Similarly resistance can occur by pathways that could by-pass the molecular target. An interesting example is the pteridine reductase, PTR1; this enzyme is able to act as a by-pass for DHFR in *Leishmania* when DHFR is inhibited [45]. The use of pathogen genome data can assist in the prediction of the presence of possible by-pass mechanisms.

Within the laboratory setting, it is almost always possible to develop pathogens that are resistant to particular agents, by culturing the parasites in sub-lethal concentrations of inhibitors. This can be a useful technique to indicate possible mechanisms of action and modes of resistance, although there are many instances where mechanisms of resistance seen *in vitro* in the laboratory have not been found during clinical use [46,47]. Within a clinical setting, mechanisms of resistance are often more difficult to deduce, due to the large number of factors that may select for drug resistance.

With any pathogen, resistance is likely to occur eventually, emphasising the need for a full drug-discovery pipeline to provide alternative drugs. However, one way to slow down the emergence of drug resistance is by use of combination therapy, such as is seen in the development of recent anti-malarial [48-50] and tuberculosis therapies [51].

CONCLUSION

The emergence of new alliances between academic and industrial partners committed to drug discovery offers exciting new prospects for drug discovery against neglected diseases. The focus of this discussion has been on the molecular target approach to drug discovery and the key criteria necessary to commit valuable resources to a drug discovery campaign. Of all criteria considered here, those of target validation and druggability are thought to be of paramount importance to the probability of success of a drug discovery programme. It is hoped that this review will encourage basic scientists to generate this information as a matter of course during their research and thereby produce a robust pipeline of potential anti-parasitic drug targets for the future.

Box 1. Recent Developments Facilitating Drug Discovery for Tropical Diseases^a

- Whole genome sequencing of several protozoan and helminth parasites, in some cases multiple species (see <http://www.sanger.ac.uk/pathogens/>)
- Establishment of new public-private partnerships (PPP) and non-profit pharmaceutical companies such as:
 - Medicines for Malaria Venture (see <http://www.mmv.org>)
 - Drugs for Neglected Diseases initiative (see <http://www.dndi.org/>)
 - TB Alliance (see www.tballiance.org/)
 - Institute for One World Health (see <http://www.oneworldhealth.org/>)
- Injection of funds by philanthropic organisations such as the Bill and Melinda Gates Foundation and the Wellcome Trust into PPPs leading to new neglected disease drug discovery initiatives expanding out of academic institutions
- In-kind contributions from large pharmaceutical organisations using a no-profit, no-loss model in which scientists from these organisations work within dedicated institutes and in partnership with relevant Public Health bodies.

^aFurther details available from [2,6,7,10] and websites listed above.

Box 2. Parasitic Disease Target Product Profiles for Human African Trypanosomiasis and Malaria ^a

The target product profile (TPP) is an important strategic planning and decision making tool in drug discovery and development [52]. It is used to define the desired features for the end-product including:

- Therapeutic area
- Spectrum of activity (e.g. active against all species including drug resistant isolates)
- Target population (e.g. pregnant women and children)
- Dose, frequency and route of administration (e.g. once a day, oral route)
- Safety and efficacy (better than existing treatments)
- Toxicity (minimal side effects, better than existing treatments)
- Potential for use in drug combinations
- Contraindications (e.g. minimal drug-drug interactions; suitable for use in HIV/AIDS or TB co-infections)
- Low potential of developing parasite resistance
- Stability under tropical conditions (i.e. > 2 years shelf life at 40 °C and 75% relative humidity)
- Cost of goods (i.e. equivalent to or cheaper than existing treatments)

A TPP for uncomplicated falciparum malaria includes:

- Oral (ideally once per day for not more than three days)
- Low cost of goods (~US\$1 per full course of treatment)
- Effective against drug-resistant parasites (e.g. those that have developed resistance to chloroquine or Fansidar)
- Fast acting and curative within three days
- Potential for combination with other agents
- Paediatric formulation should be available
- Stable under tropical conditions

A TPP for human African trypanosomiasis (HAT) includes:

- Active against *T.b.gambiense* and *T.b.rhodesiense*
- Active against melarsoprol refractory strains
- Efficacy against early and late-stage disease desirable
- Formulation (oral against early stage desirable; parenteral against late stage acceptable)
- Curative in 14 days (late stage) or less (early stage)
- Cost less than current treatment for early stage disease (\$100-140)

- Safe in pregnancy
- Stable under tropical conditions

^a Additional TPPs for neglected diseases are available elsewhere [6]

Box 3. Identifying potential druggable targets

- Targets can be selected on the basis of having clinically validated homologues from other species. This assumes that if one member of a gene family binds drug-like compounds, other members will do so, as binding-site architecture is generally conserved between gene family members.
- Identification of parasite targets that have been shown to be inhibited by drug-like compounds as opposed to non drug-like molecules, e.g. inhibition of isocitrate lyase (ICL) with the highly charged 3-nitropropionic acid does not demonstrate druggability [53].
- Computational definition and characterisation of active sites or binding sites of known druggable proteins can be used to assess potential targets for druggability. Since drugs have defined property parameters, it follows that druggable protein binding-site properties must be complementary with a definable set of characteristics. In addition, as most drugs bind to discrete binding sites, it should be possible to identify druggable binding sites from the abundance of protein structure data available in the Protein Data Bank and in the future from structural genomics projects [34].
- A simple model derived using nuclear magnetic resonance (NMR)-based screening data has been used, including terms such as polar surface area, surface complexity, and pocket dimensions, which accurately predicts experimental screening hit rates. The model correctly predicted (94%) the druggability of protein targets not used in the training set of the proteins for which high-affinity, non-covalent, drug-like leads have been reported. Therefore the model potentially allows the quantitative comparative analyses of protein binding sites of uncharacterized targets derived from genomics research [54].

Box 4. Ideal Criteria for Developing an Enzyme Assay ^a Amenable to Screening

Target

- Target protein must be available in batch quantities of sufficient size to support an entire campaign of primary, re-test and potency screening; typically ~1.5 fold the initial number of compounds to be screened equates to the numbers of data points required
- A standard inhibitor(s) must be available against which performance of the assay can be benchmarked; the developed assay is required to return potency values within 2 fold of an established mean between assay days
- Assay performance: optimised to achieve the following minimum metrics: Z' score ^b >0.5; intra-plate CVs <10%, signal to background >3:1.
- Assay format: 384 plate formats are preferred but 96 can often be accommodated

- Assay Readout: in a typical screening facility the following readouts are considered standard: absorbance; variety of fluorescence modalities; radiometric, luminescence. Non-radiometric platforms are preferred for larger scale screening (>10,000 compounds).

Assay Conditions

- Dimethyl sulphoxide (DMSO) tolerance of at least 1% required to accommodate compound addition
- End-point assay preferable to maximise throughput, with read-time well within time linearity to avoid underestimation of inhibitor potency
- Automated liquid handling robots require component reagents to be as non-viscous as possible with minimal levels of detergent.
- Enzyme load should be well within linearity with respect to enzyme concentration to ensure that initial velocity reduction by inhibitors correlate with the formation of enzyme-inhibitor complex
- Enzyme load should be within low nanomolar range to ensure sensitivity of compound inhibitor potency, the lower limit of which is regarded as 10-fold the enzyme concentration for IC₅₀ determinations
- Pre-incubation of target with compound can ensure that even slow binders are captured in the hit finding process
- Small but liquid handler-tolerable levels of detergent in the assay can assist in dissolution of compound aggregates and thereby minimise the identification of non-specific or 'promiscuous' inhibitors
- In the case of enzyme targets, substrate concentration should be at or preferably below K_m ; this maximises hit finding for competitive agents.
- If an uncompetitive agent is sought the substrate concentration should exceed K_m .

^a See [55] for a useful review of the basics of enzyme kinetics in the context of drug discovery

^b The Z' statistic gives an assessment of the robustness of the assay [56] and is determined using the following equation:

$$Z' = 1 - \frac{3\sigma_H + 3\sigma_L}{\mu_H - \mu_L}$$

Where: μ_H and μ_L are the mean high (full) and low (background) signals of the assay, respectively, and σ_H and σ_L standard deviations. A "perfect" assay would have a Z' value of 1.0.

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Figure 1. ‘Good’ and ‘bad’ pockets for binding of drug-like molecules

(a) An example of a poorly druggable protein (Hepatitis C serine protease) with a shallow featureless active site (PBD code 2A4R) [57]. **(b)** An example of a highly druggable active site (spleen tyrosine kinase) with a deep pocket allowing an inhibitor (Gleevec) to be significantly buried (PBD code 1XBB) [58]

Table 1
Traffic Light Definitions for Target Assessment

Criterion	Red	Amber	Green
Target validation	No or weak evidence that the target is essential for growth or survival	Either genetic or chemical evidence that target is essential for growth or survival ^a	Genetic and chemical evidence that target is essential for growth or survival ^a
Druggability	No drug-like inhibitors are known and active site of target is not druggable	Drug-like inhibitors are known or active site potentially druggable	Drug-like inhibitors are known and druggable active site (i.e. clinical precedent within the target family)
Assay feasibility	No <i>in vitro</i> assay developed and / or significant problems with reagents (cost or supply)	<i>In vitro</i> assay exists, development into plate format feasible, but not achieved	Assay ready in plate format and protein supply assured within appropriate timelines
Toxicity	Human homologue present and little or no structural or chemical evidence that selective inhibition is possible	Human homologue present, but some structural or chemical evidence that selective inhibition is possible	No human homologue present or human homologue known to be non-essential
Resistance potential	Target has multiple gene copies or isoforms within same species and is subject to escape from inhibition ^b	Target has isoforms within same species or may be subject to escape from inhibition ^b	Target has no known isoforms within same species and is not subject to escape from inhibition ^b
Structural information	No structure of target or closely related homologue	Structure without ligand available and / or poor resolution (> 2.3 Å) or opportunity to build a good homology model	Ligand bound structure of target (or ligand in closely related homologue) available at high resolution (< 2.3 Å)

^aSee Table 2 for the relative merits of genetic and chemical validation.

^bPossible resistance mechanisms include: accumulation of substrate that could reverse inhibition; or target can be deleted, readily modified by point mutation, readily amplified or by-passed.

Table 2**Strengths and Weaknesses of Different Target Validation Methods**

Method	Strengths	Weaknesses
Chemical validation	<ul style="list-style-type: none"> Addresses the key druggability issues of cell permeability (<i>in vitro</i> whole cell assays); selective toxicity and drug metabolism (<i>in vivo</i> animal models); safety and efficacy (clinical) Identifies non-protein targets Identifies pro-drugs and compounds acting by lethal synthesis 	<ul style="list-style-type: none"> Highly specific inhibitors frequently not available Lack of specificity or variable cellular pharmacokinetics may lead to poor structure-activity-relationships Correlation between target Inhibition and predicted molecular or biochemical phenotype sometimes difficult to demonstrate <i>in vitro</i> or <i>in vivo</i>
Genetic validation	<ul style="list-style-type: none"> Complete genomes available Suitable for genes of unknown or uncertain function 	<ul style="list-style-type: none"> Cannot identify non-gene targets (e.g. haemozoin) Does not address key druggability issues Does not identify drugs acting via lethal synthesis
Knockout methods	<ul style="list-style-type: none"> Definitive, 'clean' phenotype Few or no off-target effects 	<ul style="list-style-type: none"> Laborious (usually requires multiple transfections in diploid organisms) Null mutants for essential genes require genetic or nutritional rescue Multicopy genes can be problematic Compensatory (suppressor) mutations can occur
RNA interference (RNAi)	<ul style="list-style-type: none"> Rapid and easy to perform Suitable for multicopy gene families 	<ul style="list-style-type: none"> Not possible in many parasite species No phenotype due to insufficient silencing Off-target effects due to unintentional silencing 'Escape' mutants with essential genes