Animal models of efficacy to accelerate drug discovery in malaria

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SUMMARY

The emergence of resistance to artemisinins and the renewed efforts to eradicate malaria demand the urgent development of new drugs. In this endeavour, the evaluation of efficacy in animal models is often a go/no go decision assay in drug discovery. This important role relies on the capability of animal models to assess the disposition, toxicology and efficacy of drugs in a single test. Although the relative merits of each efficacy model of malaria as human surrogate have been extensively discussed, there are no critical analyses on the use of such models in current drug discovery. In this article, we intend to analyse how efficacy models are used to discover new antimalarial drugs. Our analysis indicates that testing drug efficacy is often the last assay in each discovery stage and the experimental designs utilized are not optimized to expedite decision-making and inform clinical development. In light of this analysis, we propose new ways to accelerate drug discovery using efficacy models.

Key words: Drug discovery, malaria, animal models, critical path, humanised mouse.

INTRODUCTION

The asexual multiplication of protozoa of the genus *Plasmodium* in human erythrocytes causes malaria (Fig. 1). This is still the most prevalent parasitic disease on earth with *ca*. 225 million cases and 0·8 million deaths annually (WHO, 2010*b*). The number of deaths, mostly children under 5 years infected by *Plasmodium falciparum* in sub-Saharan Africa, has steadily declined in many endemic countries since the introduction of artemisinin combination therapies (ACT) as first line treatment for uncomplicated malaria (WHO, 2010*b*; O'Brien *et al*. 2011). However, the emergence of resistance against artemisinins in South East Asia has raised general concern because of the lack of effective alternative treatments in case of spread to other geographical areas (WHO, 2010*a*; O'Brien *et al*. 2011).

Since the call by The Bill and Melinda Gates Foundation in 2007, malaria eradication has gained momentum as a public health objective. Nowadays, eradication is perceived as an attainable objective based on the lessons learned from previous control, elimination and eradication programmes, particularly from the Global Malaria Eradication Programme between 1955 and 1969 (Nájera *et al*. 2011). As drugs are the mainstay for malaria eradication (The malERA Consultative Group on Drugs, 2011), several target product profiles (TPP) have been proposed for drugs aimed at specific indications (Burrows *et al*. 2011; The malERA Consultative Group on Drugs, 2011). The specifications of each TPP define the characteristics of a new medicine for a therapeutic indication. Currently, the most ambitious TPPs look for radical cure and transmission blocking of *P. falciparum* and *P. vivax* infections (Burrows *et al*. 2011; The malERA Consultative Group on Drugs, 2011). These requirements call for drugs that kill liver stages, gametocytes and hypnozoites, which are the dormant liver stage responsible for the characteristic relapses of *vivax* malaria (see Fig. 1) (Galinski and Barnwell, 2008).

Animal models of malaria are key tools for drug discovery (Fidock *et al*. 2004). These tools provide integrated systems in which the efficacy of drugs is assessed in a physiological context. Efficacy essentially depends on drug disposition (e.g. absorption, distribution, metabolism and excretion, abbreviated as ADME), the toxic effects elicited in the host and the intrinsic anti-parasitic activity of drugs. Both disposition and anti-parasitic activity may be substantially different in animal models and humans because of dissimilarity in body size, physiology and susceptibility to different *Plasmodium* spp. However, many pharmacological interactions between drugs and pathogens are independent of the host involved. As such, animal models allow gaining insight into the *in vivo* pharmacological properties of drugs and their combinations.

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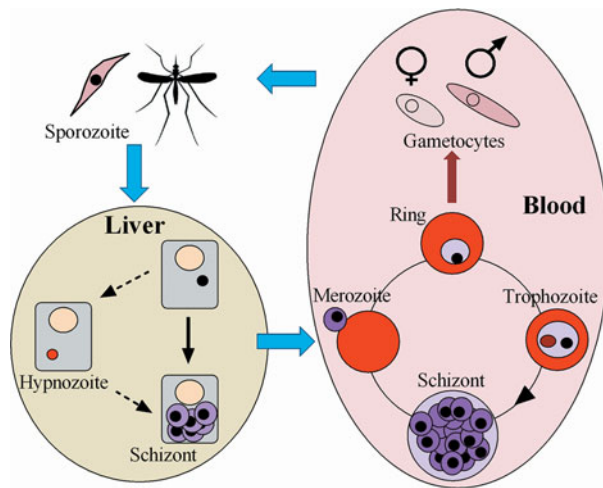
Fig. 1. Plasmodium spp. cycle. (A) *Anopheles* spp. female mosquitoes inoculate sporozoites, present in their salivary glands, in the host. Upon invasion of hepatocytes, sporozoites differentiate and replicate to produce liver schizonts. These cells contain specialized cells (merozoites) capable of infecting erythrocytes once released into bloodstream. The liver infection is asymptomatic. In a few species, including *P. vivax* and *P. ovale*, a fraction of parasites enters a quiescent stage (hypnozoite). Reactivated hypnozoites are thought to be the cause of the characteristic cyclic *vivax* and *ovale* malaria relapses that happen months after infection; (B) Released liver merozoites invade erythrocytes, mature and undergo endomitotic divisions to produce blood schizonts, which contain merozoites infective to new erythrocytes upon schizont rupture. The erythrocyte stage causes the clinical symptoms and is the target of chemotherapy of malaria. The different *Plasmodium* spp. show marked differences in preference to infect reticulocytes or mature erythrocytes and on the erythrocytic cycle duration. For example, *P. vivax* selectively invades reticulocytes whereas *P. falciparum* is not selective. The erythrocytic cycle is of about 24 h in rodent malarial species and *P. knowlesi*, 48 h for *P. falciparum, P. vivax* and *P. ovale* whereas *P. malariae* shows a cycle of 72 h; (C) A low percentage of parasites per erythrocytic cycle differentiate into male and female gametocytes. These gametocytes are specialized sexual cells taken up in mosquitoes' blood meals. Upon gametocyte activation and fertilization in the mid-gut of mosquitoes, new infective sporozoites that reach their salivary glands are produced.

In this review, we analyse the use of animal models of malaria in current drug discovery programmes whose objective is to develop drugs against the erythrocyte stage of *Plasmodium* spp. This paradigm of animal model use could be applied to drug discovery programmes aiming at different TPPs or other infectious diseases.

EFFICACY MODELS IN MALARIA DRUG DISCOVERY

Malaria is caused by pathogens that infect humans and some non-human primates (White, 2008;

Prugnolle *et al*. 2011). This host selectivity has limited the number and type of animal models available for drug evaluation (Table 1).

Models using human plasmodial pathogens

The *Plasmodium* species that cause malaria in humans are *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* (White, 2008). At pre-clinical stages of drug discovery, the efficacy of drugs against human malaria can only be tested using susceptible non-human primates or humanised mice (huMouse) as hosts.

Several species of New World Monkeys belonging to the genus *Aotus* and *Saimiri* are susceptible to infection by the five species mentioned above (Collins, 2002b). *P. falciparum, P. vivax* and *P. malariae* require a process of adaptation *in vivo* to grow reproducibly in New World monkeys. In addition, splenectomy is often necessary to obtain significant parasitaemias and production of infective gametocytes (Collins, 2002a,b). Conversely, *P. knowlesi*, a natural parasite of monkeys, readily infects *Aotus, Saimiri* and Rhesus monkeys (*Macaca mulatta*). As a major advantage of non-human primates, all stages of the biological cycle of the human parasites can be reproduced for drug evaluation by choosing appropriate host-parasite pairs (Collins, 2002a; Stewart, 2003). Nonetheless, the use of monkey models is quite limited due to ethical concerns and experimental complexity because highly specialized facilities are required.

The humanised mouse (huMouse) model is the only pre-clinical *in vivo* system in which parasites grow in human cells. Humanised mice are generated by engraftment of human tissues into immuno-deficient mice (Shultz *et al*. 2012). These mice lack T and B lymphocytes thereby enabling reproducible engraftment of human erythrocytes by intraperito-neal (Angulo-Barturen *et al*. 2008) or intravenous injections (Arnold *et al*. 2011). The erythrocyte stages of *P. falciparum* can grow reproducibly inside engrafted human erythrocytes (Angulo-Barturen *et al*. 2008; Arnold *et al*. 2011) and produce infective gametocytes (Moore *et al*. 1995). Using the *P. falciparum* huMouse (PfhuMouse) a number of standardized assays are available to evaluate the efficacy of antimalarial drugs targeting erythrocytic stages (Moreno *et al*. 2001; Angulo-Barturen *et al*. 2008; Jiménez-Díaz *et al*. 2009b).

Although their use in new drug discovery pro-grammes has not been reported, the development of liver stages of *P. falciparum* is also supported in different strains of immunodeficient mice engrafted with human liver cells in ectopic (Morosan *et al*. 2006; Sacci *et al*. 2006) or orthotopic (Vaughan *et al*. 2012) locations. Of note, Fah–/–, Rag2–/–, Il2rg–/– (FRG) immunodeficient mice can be used to serially expand functional human liver cells *in vivo* from

Table 1. Models for malaria drug discovery

	Mouse	Humanised mouse	Non-human primates
Plasmodium spp.	*P. berghei, P. yoelii, P. chabaudi, P. vinckei*	*P. falciparum*	*P. falciparum, P. vivax, P. malariae, P. ovale, P. knowlesi*, and infectious for Old World and New World monkeys (*P. cynomolgi*)[a]
Parasite long-term culture *in vitro*	No	Yes	*P. falciparum, P. knowlesi*
Parasite cycle stage for assay	Full cycle No surrogate for hypnozoite stage	Erythrocyte stage Liver stage[b] Gametocyte[b]	Full cycle *P. cynomolgi* hypnozoite stage
Parasite maintenance	*In vivo* passage	*In vitro/in vivo* passage	*In vitro/in vivo* passage
Host immunity	Immunodeficient Immunocompetent	Immunodeficient	Immunocompetent
Complexity	+	+++	+++++
Facilities	+	+++	+++++
Accessibility	+++++	+++	Highly restricted
Animal handling	+	++++	++
Cost	++	+++	+++++

[a] Strains of *P. falciparum, P. vivax, P. malariae, P. ovale* adapted to grow in monkeys.
[b] No reports on use for chemotherapy studies in drug discovery programmes.
Adapted from Angulo-Barturen and Ferrer (2012).

primary donors (Azuma *et al*. 2007). The development of FRG mice is a breakthrough that has been used to demonstrate for the first time the production of viable liver merozoites on *P. falciparum* sporozoite infection in huMouse (Vaughan *et al*. 2012). This study is particularly relevant because the quality of the *P. falciparum* liver stages found in FRG mice suggests its suitability to support *P. vivax* as well.

Models using surrogate plasmodial species

The mouse is the most widespread used host in malaria drug discovery owing to its versatility and accessibility. There are four major *Plasmodium* species adapted to grow in mice: *P. berghei, P. yoelii, P. chabaudi* and *P. vinckei* (Landau and Gautret, 1998). Of these, *P. berghei* is the most widely used rodent species whereas *P. yoelii, P. vinckei* and *P. chabaudi* are used to a lesser extent. The severity of the disease in mice, which ranges between lethal infections and self-limited disease, depends on the strain of parasite species and the murine genetic background (Landau and Gautret, 1998). The choice of plasmodial species is often based on empirical basis because not all rodent *Plasmodium* spp. are equally susceptible to drugs (Landau and Gautret, 1998). The rodent *Plasmodium* spp. have biological cycles of about 24 h, which is a major difference from the classical human pathogens (Landau and Gautret, 1998; Sanni *et al*. 2002). In spite of these differences, the rodent-adapted parasites can reproduce the full cycle of malaria in mammals (Scheller *et al*. 1994). As a downside, the evaluation of drugs is performed with parasites that show significant evolutionary divergence from *P. falciparum* and *P. vivax* (Prugnolle *et al*. 2008).

Macaques are the natural host of *P. cynomolgi*, a malaria parasite that has a biological cycle similar to *P. vivax* and can infect humans (Coatney *et al*. 1961, 1971). *P. cynomolgi* has a cycle of maturation in blood of 48 h with reticulocytes being the preferred cell target of infection. The blood-stage infection is self-limited but presents characteristic relapses (Kocken *et al*. 2009). *P. cynomolgi* is also noteworthy as it can also form hypnozoites upon liver infection. All these characteristics make *P. cynomolgi* an interesting surrogate model for *P. vivax* infection (DiTusa *et al*. 2010).

ROLE OF EFFICACY MODELS IN CURRENT DRUG DISCOVERY

The drug discovery process

Modern drug discovery is structured as a series of sequential iterative steps in which the properties of drugs as potential medicines are investigated (Payne *et al*. 2007). The drug discovery paradigm is typically divided in four stages: hit to lead, lead optimization, preclinical development and clinical development. At each step, test compounds are evaluated in different assays to understand risk and assess efficacy, toxicity, DMPK and physicochemical properties as indicators of their potential as future drug molecules. Ultimately, progression from one stage of discovery to the next indicates that drugs have no overt issues or the risks identified can be managed at a later stage in the development process (see Fig. 2).

Hit to lead

Hits are drugs that have shown specific activity *in vitro* against an essential molecule of *Plasmodium*
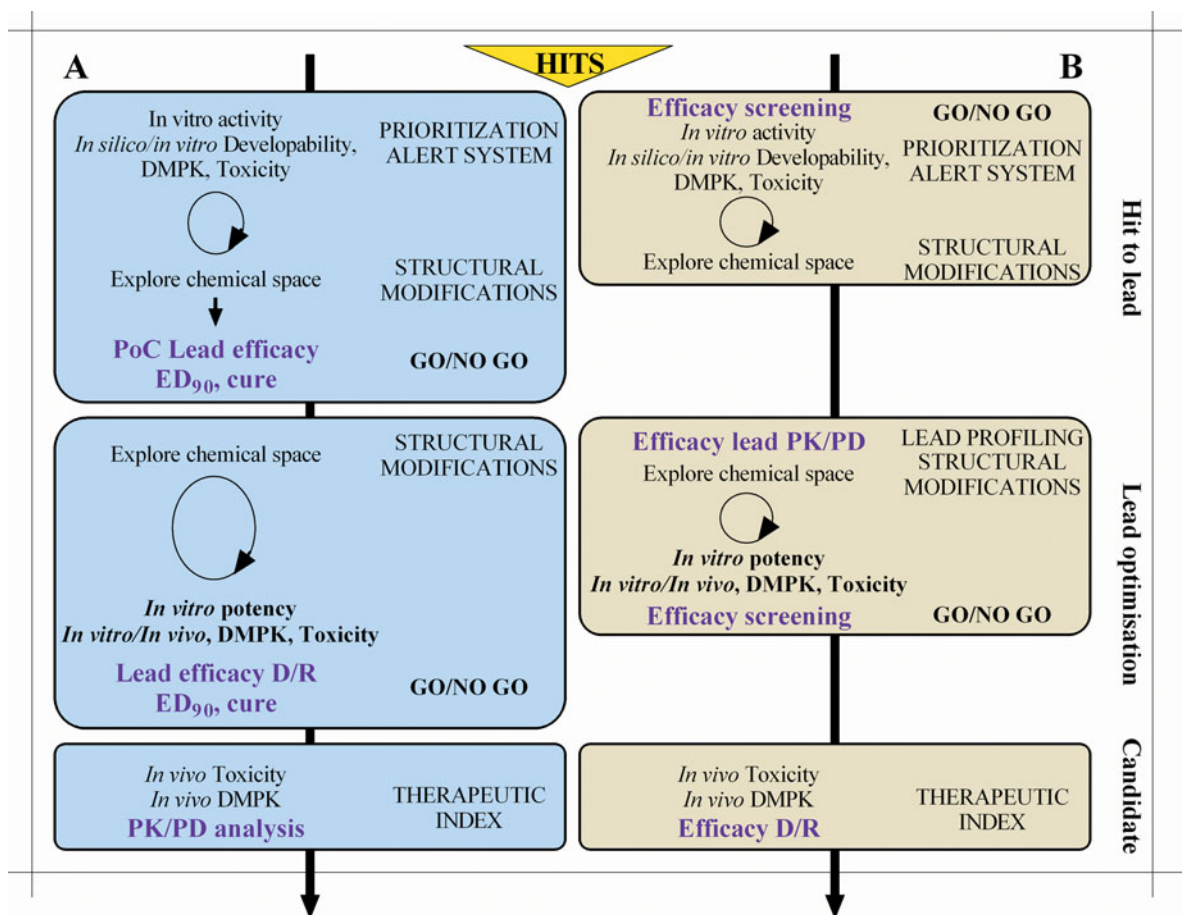
Fig. 2. Comparison of efficacy evaluation in critical pathways. The Fig. summarizes two strategies of evaluation *in vivo* in hit-to-lead, lead optimization and candidate selection. (A) Efficacy evaluation is performed at the end of each progression step. This strategy of evaluation is followed by most current drug discovery programmes. In general, efficacy evaluation relies on relatively large experiments (dose–response, abbreviated as D/R, >10 mice per compound) designed to estimate the potency of compounds (e.g. ED_{90}, etc.). The compounds tested are generally selected after extensive filtering using *in silico/in vitro* filtering; (B) Efficacy is performed at the beginning of each evaluation step. Efficacy evaluation relies on screening format assays involving small number of mice per compound ($\leqslant 3$ per compound) aiming at detecting improvement or not over a reference compound of the series. In this strategy all *in silico, in vitro* and *in vivo* data are integrated in decision-making on compounds at early stage.

or the whole parasite itself. The sources of hits are usually high-throughput molecular or phenotypic screenings, depending on whether the objective is to inhibit a specific molecular target or the intact parasite/host cell, respectively. The availability of the full genome sequence for *P. falciparum* and, more recently, *P. vivax*, had raised hopes for the identification of hundreds of new targets (Gardner *et al.* 2002; Carlton *et al.* 2008). However, no new class of antimalarials has been discovered in the last twenty years using target-based approaches (Chatterjee and Yeung, 2012; Guiguemde *et al.* 2012). These negative results are in close agreement with the experience accumulated in anti-bacterial drug discovery (Payne *et al.* 2007) and highlight the importance of testing compounds in systems in which the target is in its native functional context.

Recently, a series of phenotypic screens have delivered more than twenty thousand compounds that have activity *in vitro* against the asexual erythrocyte stage of *P. falciparum* (Chong *et al.* 2006; Weisman *et al.* 2006; Baniecki *et al.* 2007; Plouffe *et al.* 2008; Gamo *et al.* 2010; Guiguemde *et al.* 2010). This work has identified an unprecedented number of potential starting points for drug discovery. The prioritization of hits according to their physicochemical and ADMET (absorption, distribution, metabolism, excretion and toxicity) properties in *in silico* and *in vitro* assays is employed to select the most promising new scaffolds to start drug discovery programmes (Keldenich, 2009; Gleeson *et al.* 2011). Selected scaffolds are then modified to improve their physicochemical and DMPK properties, identify early toxicological flags and increase their antimalarial potency.

The demonstration of efficacy *in vivo* for a new scaffold is a major goal of the hit-to-lead stage. Murine models are the most widely used to achieve this proof of concept because they require low amounts of compound (tens of mg of solid) and are

accessible to many laboratories. While *P. berghei* is still the most widely used model, other rodent-infecting species have also been employed across a number of drug discovery programmes (Jain *et al.* 2004; Chong *et al.* 2006; Bhattacharjee *et al.* 2007; Coslédan *et al.* 2008; Kelly *et al.* 2009).

The choice of model may depend on the genetic similarity at the level of the molecular target. For example, *P. yoelii* was chosen to test 4-(1H) pyridones because of the high sequence homology with *P. falciparum* for the target cytochrome *bc1*, which is a key protein in the mitochondrial respiratory chain (Yeates *et al.* 2008). In other cases, the choice is guided by empirical considerations based on the relative sensitivity of each model to the drugs tested. Thus, diamidines have been tested in the *P. vinckei* model because *P. berghei* is almost insensitive to these drugs (Angulo-Barturen *et al.* 2008).

To address the efficacy proof of concept two different assays, or variants of them, are typically employed: the Thompson test and the Peters' suppressive 4-day test (Thompson and Werbel, 1972; Peters and Robinson, 1999). In the Thompson test, mice having patent infections with *P. berghei* are treated with a drug for three days and the survival time of treated mice is compared to vehicle-treated controls. In contrast, in the Peters' 4-day test, the administration of compounds starts one to three hours after infection with parasitized-erythrocytes and the resulting parasitaemia is measured on the fifth day, that is 24 h after the last dose administration, and compared to vehicle-treated controls. Both assays are robust and reproducible, as demonstrated by the numerous projects that rely on them (Fidock *et al.* 2004).

Assessing the pharmacokinetics of compounds tested *in vivo* before evaluation enhances the power of discrimination of the efficacy study. Prior knowledge of exposure enables researchers to select those compounds that have the greatest potential of showing *in vivo* efficacy (Lowes *et al.* 2012; Nagle *et al.* 2012; Zhang *et al.* 2012). This strategy is particularly important in cases in which drugs fail to inhibit rodent *Plasmodium* spp. even though sufficient drug exposure is achieved in the blood. A differential susceptibility to the drug between the human pathogen *in vitro* and the rodent surrogate *in vivo* is inferred in these situations. In these cases, evaluating the efficacy of drugs either in non-human primates or humanized mouse models is the only realistic alternative.

Lead optimization

In lead optimization, efficacy studies seek to detect small differences in potency between compounds that show small structural differences. As in hit-to-lead, the Thompson test and the Peters' suppressive 4-day test are the most widely used assays. The parameters of efficacy are essentially measurements of potency, that is, the mg per kg of body weight necessary to achieve a specific biological endpoint (Fidock *et al.* 2004). The usual biological endpoints are the number of days until recrudescence (Saenz *et al.* 2012; Anderson *et al.* 2013) and the reduction in parasitaemia with respect to vehicle-treated mice (e.g. ED_{50} and ED_{90}, defined as the effective dose levels that reduce parasitaemia by 50 or 90%, respectively) (Coslédan *et al.* 2008; Khan *et al.* 2009; Booker *et al.* 2010; Barker *et al.* 2011; Biagini *et al.* 2012; Brunner *et al.* 2012; Younis *et al.* 2012). Although it should be noted that assessments of the minimum number of animals necessary to estimate the parameters of efficacy at a predetermined power and confidence level are scarce and typically not calculated.

The drugs that selectively inhibit human pathogens represent a challenge during the lead optimization. Although non-human primates have been utilized for profiling of efficacy of choline analogues (Salom-Roig *et al.* 2005), these hosts are mostly used in projects seeking drugs for radical cure (Lin *et al.* 2009). In contrast, the PfhuMouse model offers a practical alternative that has been exploited by several recent drug discovery projects. For example, the poor activity of some triazolopyrimidines against *P. berghei* dihydroorotate dehydrogenase (DHODH) compared to the *P. falciparum* enzyme prompted researchers to use the PfhuMouse Model to measure efficacy *in vivo* (Coteron *et al.* 2011). Noteworthy, an increasing number of projects employ the PfhuMouse model at some point during the lead optimization process irrespective of the difference in susceptibility between *P. falciparum* and *P. berghei* (Coslédan *et al.* 2008; Jiménez-Díaz *et al.* 2009*b*; Booker *et al.* 2010; Barker *et al.* 2011; Sanz *et al.* 2011; Brunner *et al.* 2012; Nilsen *et al.* 2013).

Candidate selection

Candidate selection is a milestone in the progression of a project and represents a commitment to clinical development of a specific asset. At this stage, the candidate molecule is thoroughly evaluated for DMPK, toxicity, efficacy and physicochemical properties. The difference between the levels of compound in blood that are efficacious and those at which toxicity is observed in animals is known as the therapeutic index (TI) or therapeutic window. The TI is an important parameter in order to decide whether a drug should be progressed to clinical development.

Efficacy animal models should provide estimates of the efficacious levels to support TI calculations. Ideally, this should be addressed through detailed pharmacokinetic/pharmacodynamic (PK/PD) studies linking drug exposure and parasitological inhibition or cure. Even though detailed PK/PD studies are hardly addressed in recent literature

describing new candidates, some valuable dose-fractionation studies assessing cure of mice have been performed. For example, the candidate drugs MK-4815, NITD609 or OZ439 were shown to cure Balb/c mice infected with *P. berghei* (Rottmann *et al*. 2010; Charman *et al*. 2011; Powles *et al*. 2012).

A proof of concept in an animal model of infection with human plasmodial pathogens is also often included in the candidate evaluation package. For example, efficacy against *P. falciparum* and *P. cynomolgi* in non-human primates has been tested in choline analogues that inhibit phosphatidylcholine biosynthesis (Wengelnik *et al*. 2002). However, as described above, the PfhuMouse model is an alternative test system that has been successfully exploited to estimate drug efficacy in an increasing number of projects (Coslédan *et al*. 2008; Jiménez-Díaz *et al*. 2009*b*; Booker *et al*. 2010; Barker *et al*. 2011; Coteron *et al*. 2011; Skerlj *et al*. 2011; Nilsen *et al*. 2013).

Pre-clinical and clinical development

Drug development starts after candidate selection. At this stage, pre-clinical studies aim at preparing Phase I (first time in human) and Phase II (proof of concept in humans). Efficacy experiments in animals are rarely performed during the pre-clinical or clinical development phases before the drug has demonstrated efficacy in humans. Once this proof of concept in humans has been achieved, efficacy models can be used to find new therapeutic indications for known drugs as exemplified by the repositioning of azithromycin (Andersen *et al*. 1995) and iron chelators (Ferrer *et al*. 2012) as antimalarials. Particularly important is the use of animal models to study the optimal partnering of marketed drugs to create new antimalarial combinations. Examples of this type of study include characterization of new combinations of artemisinin derivatives in *P. berghei* (Guo *et al*. 2012), the evaluation of artemisone and mefloquine in *Aotus* monkeys infected with *P. falciparum* (Obaldia *et al*. 2009) and the evaluation of chloroquine and azithromycin in combination with *R*-amlodipine to reverse chloroquine resistance in *P. yoelii* (Pereira *et al*. 2011).

Summary

The general pattern of the current use of efficacy models in drug discovery indicates that *in vivo* evaluation is performed with a relatively low number of compounds after extensive testing and selection by *in silico* and *in vitro* assays. The evaluation *in vivo* aims to measure the potency of compounds by estimating ED_{90} or analogous parameters of efficacy utilizing experimental designs widely employed in the scientific community. These experiments are executed using relatively large numbers of animals per compound tested (>10 mice/compound/assay).

NEW DIRECTIONS USING EFFICACY MODELS

The animal models of malaria are unique tools to analyse the properties of drugs as future medicines. Because of their nature, best practices in animal experimentation demand a periodic critical review of the current procedures in the field. As a final objective, only optimally designed experiments necessary to obtain crucial information for drug development should be addressed. Here we propose several lines of improvement that we have started to implement in our strategy of evaluation.

A translational approach

The drug discovery process would benefit from efficacy models able to inform the design of clinical studies. This entails that the parasitological response of infected individuals to therapy is studied using comparable methods and parameters of efficacy in both drug discovery and clinical development.

Efficacy models that employ human pathogens may have a critical role in translational medicine for malaria (Burrows *et al*. 2011; Angulo-Barturen and Ferrer, 2012). Not surprisingly, humanised murine models are being increasingly used in drug discovery programmes (Coslédan *et al*. 2008; Booker *et al*. 2010; Barker *et al*. 2011; Coteron *et al*. 2011; Nilsen *et al*. 2013). One practical advantage of these models is their simplicity compared to non-human primates (Collins, 2002*a*,*b*). Moreover, latest advances in huMouse models suggest that mice engrafted with human bone marrow and liver cells will be commercially available shortly. Nonetheless, surrogate plasmodial species may also have a valuable role in predicting human clinical doses. As an example, a PK/PD analysis using the Peters' 4-day test in *P. yoelii*-infected mice has been employed to estimate the effective daily dose of *R*-amlodipine necessary to overcome chloroquine resistance in humans treated with a combination of chloroquine and azithromycin (Pereira *et al*. 2011).

The efficacy of a drug in humans is measured by the rate at which the parasites are cleared from peripheral blood and the rate of cure after treatment (White, 2011). In experimental models, the parasite clearance rate is not a common measurement of efficacy, although it has been used to assess the onset of action upon treatment (Charman *et al*. 2011). Differences in parasitaemia at a given time of the assay with respect to vehicle-treated controls are the most used parameters of efficacy (Thompson and Werbel, 1972; Peters and Robinson, 1999; Fidock *et al*. 2004). However, a new type of assay based on modelling the treatment of patients in mice and

measuring the parasite clearance rate has been recently developed in a *P. berghei* murine model (Jiménez-Díaz *et al*. 2013). This type of assay allows estimates of treatment duration with new drugs in early drug discovery to be assessed because the elimination of the parasite is a first order process (White, 2011). As a downside, it is still pending the implementation of suitable metrics for the parameters that allow comparisons of parasite clearance rates in animals and humans. On the contrary, the comparison of the rate of cure in mice and humans treated with antimalarials is straightforward. This interspecies comparison is possible by using logistic analysis, which relates a dichotomous response variable (cured/not cured) with continuous explanatory variables (dose level, exposure, time of treatment, etc. . .). Thus, logistic analysis offers a powerful tool to validate murine systems for human dose prediction (Angulo-Barturen and Ferrer, 2012).

Animal models can inform the design of clinical studies by estimating the efficacious exposure of drugs *in vivo*. These estimations have been indirectly addressed by extrapolating data from separated PK and efficacy studies (Barker *et al*. 2011). By blood microsampling of animals employed in efficacy studies, the PK/PD relationships governing the efficacy of compounds can be studied at the desired level of precision (Pereira *et al*. 2011; Nilsen *et al*. 2013). The efficacy-coupled microsampling approach is particularly powerful, because it allows the discrimination between the intrinsic antimalarial potency of compounds and their DMPK liabilities in the host species chosen.

Methodological improvement

The improvement of the experimental designs employed in efficacy studies can reduce the number of animals required while increasing the translational value of the parameters measured. It is surprising that the statistical techniques used in clinical trials to minimize the number of patients are not widely implemented in experimental efficacy (van der Worp *et al*. 2010). For example, a substantial reduction in the number of animals has been achieved in estimating the *in vivo* potency of ELQ-300 against *P. falciparum* by using individuals as experimental units instead of groups of individuals (Nilsen *et al*. 2013). This approach can also take advantage of advanced statistical methods, for example, non-linear mixed-effect (NLME) models (Paterson and Lello, 2003). A NLME model has been successfully utilized to measure the efficacy of G25, an inhibitor of phosphatidylcholine biosynthesis, in *P. cynomolgi*-infected macaques (Kocken *et al*. 2009). The NLME model controlled the influence of pseudo-replication and auto-correlation on multiple samples taken from the same individual over time and allowed accounting for the individual variability of the course of parasitaemia on the overall effect of drug treatment.

The properties of drugs as antimalarial medicines are established at early stages of drug discovery when the chemical scaffold is chosen. By comparison with drugs of known mechanisms of action, it would be possible to classify the new drugs according to their phenotypic effects on parasites. Among the many techniques available, multiparametric flow cytometry is particularly useful for compound classification (Jiménez-Díaz *et al*. 2009*a*; Apte *et al*. 2011; Malleret *et al*. 2011). Flow cytometry requires very small blood samples (units of μl) that do not interfere with the evaluation of efficacy *in vivo*. Therefore, incorporating high-content experimental designs might increase the decision-making and translational value of *in vivo* models.

Critical pathway

Animal models of efficacy are usually go/no go decision points in critical paths. This means that the sooner the level of *in vivo* efficacy is established for a given compound the quicker it can be eliminated for further progression thus saving resources. Therefore, it seems logical to perform efficacy studies at the earliest possible point in the critical path.

In vivo screening for efficacious compounds can be addressed immediately after the identification of hits (Fig. 2). In support of this contention, all antimalarial families on the market belong to chemical scaffolds identified through large screening campaigns performed during the 20th century in avian or murine malaria models (Kinnamon and Rothe, 1975; Ockenhouse *et al*. 2005; Slater, 2005). Nowadays, the identification of more than twenty thousand compounds that are active *in vitro* against the erythrocyte stage of *P. falciparum* raises the question of how to exploit this knowledge (Guiguemde *et al*. 2012). A detailed consideration of the *in vivo* primary screening addressed in the past comes after the evaluation of *ca.* 800 compounds from the Tres Cantos Antimalarial Collection (TCAMS) against *P. berghei* (Jiménez-Díaz *et al*. 2013). This study indicates that performing *in vivo* screens on a large number of hit compounds is a feasible task whose main bottleneck is compound synthesis. Moreover, about 10% of the compounds tested had some efficacy and about 3% were as efficacious as marketed antimalarials. The main advantage of the *in vivo* screening approach is that the starting points for drug discovery programmes are compounds with sufficient exposure to be efficacious and without overt toxicities. The downside is the risk of losing compounds with bad PK properties that might be improved through rational chemical optimization (Fig. 2).

Full PK/PD analysis of the pharmacological properties of compounds could be addressed as the starting point of lead optimization. The importance of PK/PD studies to understand the efficacy of compounds is widely recognized (Gabrielsson *et al*. 2009). A series of PK/PD studies in *P. berghei*-infected mice on the efficacy of doxycycline alone or in combination with dihydroartemisinin (Batty *et al*. 2007), piperaquine (Moore *et al*. 2008, 2009) and chloroquine (Moore *et al*. 2011) have been published recently. The methodology described in these papers, which address the parasitological response to treatment (e.g. parasite clearance and relapse during and after treatment), might be useful for efficacy studies in late hit-to-lead or early lead optimization. This early PK/PD analysis would also allow a judgment on whether the pharmacological characteristics of the compound series meet the desired efficacy endpoints.

CONCLUDING REMARKS

Animal models of efficacy play a crucial role in malaria drug discovery. As tools that integrate efficacy, drug disposition and toxicology, efficacy models can provide new insights into the PK/PD properties of antimalarial drugs. No doubt, these studies could improve decision-making and likely inform the design of clinical trials. All these improvements are compatible with a more effective use of the variety of efficacy models available, which offer alternatives to design different critical pathways for drug progression.

There is compelling evidence indicating that the use of animal models in drug discovery can be optimized. Here we propose that performing more informative efficacy studies at an earlier point in discovery screening cascades might dramatically accelerate the development of new antimalarial medicines while reducing the number of animals employed.

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