Review

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Review

The Importance of Murine Models and Their Resultant In Vivo Pharmacokinetics, Safety, and Efficacy Assessments for Antimalarial Drug Discovery

Glory Adebayo [1,2,6], Opeyemi I. Ayanda [2,*], Matthias Rottmann [5], Olusola Ajibaye [6], Gbolahan Oduselu [1,4], Julius Mulindwa [3], Olayinka O. Ajani [1,4], Oluwagbemiga Aina [6], Pascal Mäser [5] and Ezekiel Adebiyi [1,7,8,9,*]

1 Covenant University Bioinformatics Research (CUBRe), Covenant University, Ota, Ogun State, Nigeria
2 Department of Biological Sciences, College of Science and Technology, Covenant University, Ota, Ogun State, Nigeria
3 Department of Biochemistry and Sports Science, College of Natural Sciences, Makerere University, Kampala, Uganda
4 Department of Chemistry, College of Science and Technology, Covenant University, Ota, Ogun State, Nigeria
5 Swiss Tropical and Public Health Institute, Kreuzstrasse 2, CH-4123 Allschwil, Switzerland
6 Biochemistry and Nutrition Division, Nigerian Institute of Medical Research, Yaba, Nigeria
7 Division of Applied Bioinformatics, German Cancer Research Center (DKFZ), Heidelberg, Germany
8 African Centre of Excellence in Bioinformatics & Data Intensive Science (ACE)—Uganda
9 Infectious Diseases Institute Makerere University, Uganda
* Correspondence: opeyemi.ayanda@covenantuniversity.edu.ng (O.I.A.); e.adebiyi@dkfz-heidelberg.de (E.A.); Tel. +2349027230460 (O.A): +496221545831(E.A)

Abstract: New chemical entities are consistently being investigated in antimalarial drug discovery and they require animal models for toxicity and efficacy testing. Murine models in searching for novel antimalarial drugs are inevitable because they show unique similarities to human physiology during malaria pathogenesis. Therefore, they provide a preclinical basis (following in vitro assessments of newly identified lead compounds) for further assessment in the drug development pipeline. Specific mouse strains, non-humanized and humanized, have successfully been infected with rodent *Plasmodium* species and the human *Plasmodium falciparum* respectively. Infected mice provide a platform for the assessment of treatment options being sought. In vivo pharmacokinetic evaluations are necessary when determining the fate of new lead compounds in addition to the efficacy assessment of these chemical entities. This review highlights specific murine models important for antimalarial drug discovery and their resultant critical in vivo pharmacokinetic, safety, and efficacy assessments necessary for making appropriate choices of lead compounds.

Keywords: antimalaria; drug discovery; efficacy assessments; murine models; pharmacokinetics

1. Introduction

The Global Technical Strategy for Malaria has maintained a responsibility to facilitate the global eradication of malaria through salient measures [1]. A major driver of malaria elimination is the control efforts galvanized into achieving great strides in the global eradication pursuit [2]. Essential to global control efforts are the tools embraced for the fight, including modern diagnostic, prophylactic, and treatment measures [2]. As a key factor of the treatment measure, the effectiveness of existing medications has seen a decline over the years as a result of drug resistance [3]. This has

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posed a notable challenge and has influenced unrelenting continuous efforts towards the discovery and development of new antimalarial drugs [4]. The discovery and development of new antimalarial entities focuses on evading the drug resistance hurdle and embracing increased effectiveness with easily suitable dosage regimens [5]. Novel drug entities are also being discovered based on specific targets and newly identified mechanisms of action [6,7]. Potent novel entities are prioritized based on their fast antimalarial action and capacity to evade drug resistance hurdles determined by their pharmacodynamic and pharmacokinetic profiles [8].

Due to the significant amount of time required to begin the antimalarial drug discovery process from natural sources, modern antimalarial drug discovery has employed computational and technological tools to develop computer-aided potential antimalarial compounds by virtual screening as an initiation into the drug discovery pipeline [8,9]. The discovery of new chemical entities through high throughput screening of thousands of potentially active compounds is also an initiation into the drug discovery pipeline [8–10]. In silico-based predicted ligands are prioritized based on their predicted binding affinity to specific target proteins and the molecular dynamics associated with the ligand interactions [11]. Predicted pharmacokinetic and physicochemical properties aid in filtering through identified hits that should proceed to be active early lead compounds [9]. Early leads then undergo lead optimization procedures, and the successful ones proceed to late leads. Once optimized and passing all required tests as outlined by the Medicine for Malaria Ventures (MMV) conducted in vivo, the resulting compounds may proceed as antimalarial preclinical candidates (Figure 1) [5].

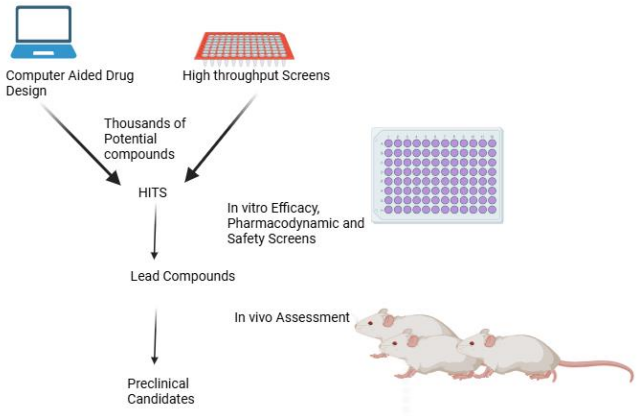

Figure 1. Antimalarial Drug Discovery Workflow.

Animal models (in vivo) are part and parcel of the antimalarial drug discovery venture [5]. Hamsters (*Mesocricetus auratus*), mice (*Mus musculus*), rats (*Rattus norvegicus*), dogs (*Canis lupus familiaris*), and monkeys (*Macaca mulatta*) (Figure 2) are commonly employed as animal models during the drug discovery process for pharmacokinetic studies and humanized mice for efficacy studies [5,12,13]. The animal model of choice for in vivo evaluations depends on the similarity of the target protein between the animal model parasite and the human parasite [14]. These models are necessary to predict drug responses in humans [15]. Nevertheless, murine (mice) models are considered more accessible and versatile, hence their preference over other animal species in early drug discovery [14,16].

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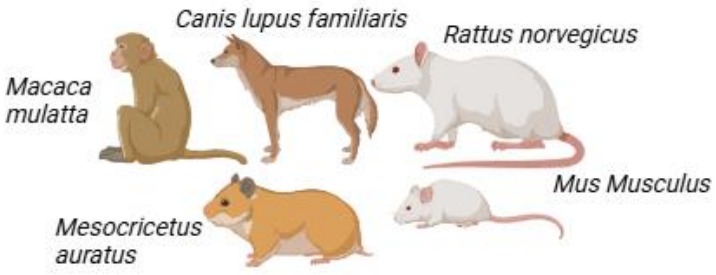

Figure 2. Animal species used as in vivo models in Antimalarial Drug Discovery.

The discovery of new antimalarials employs the use of murine models for investigative purposes, including efficacy, vaccine development, safety, and so on; this program dates back decades ago [17]. Human physiology and genetics are closely related to murine models providing many opportunities to explore malaria research [18]. Humans and mice share 99% similar conserved regions of their genome [19]. Understanding murine responses in host-pathogen interactions during malaria infection or drug discovery provides insights into the possible parallel outcomes in humans especially when murine strains are passaged with the matching *Plasmodium strain* [18]. Genetic engineering also easily manipulates rodents to produce transgenic strains, especially humanized mice models which are easily adaptable to different experimental designs in malaria research [20]. Despite the similarities between humans and murine, there are specific differences such as *P. berghei* hepatocytic development occurring faster than *P. falciparum* hepatocytic development [21].

Some of the advantages of murine models over other animal species used in antimalarial drug discovery include:

1. Mice genes can be easily manipulated [18].
2. Tissue sections are easily accessible for examination in case of histopathology [14,22].
3. This animal model is miniature-sized and can be handled easily [23].
4. Mouse models are easily affordable [23].
5. Immune responses to *Plasmodium* infection in murine models is widely used to understand that in human *Plasmodium* infection owing to their well-defined immune system [20].

Four *Plasmodium* species central to murine infection are *Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium vinckei*, and *Plasmodium yoelii*. These parasite species share certain conserved regions with *Plasmodium falciparum*, although these parasites share stage-specific differences in their intraerythrocytic development within the host [24]. *P. berghei* and *P. yoelii* are phylogenetically related and infect mature erythrocytes [24,25]. *P. berghei* and *P. yoelii* infect murine through the invasion of reticulocytes [26,27]. *P. berghei*. *P. vinckei* and *P. yoelii* are lethal murine parasites unlike *P. chabaudi* [25,28]. *P. yoelii* exterminates its host within one week of infection but the murine mortality rate of *P. vinckei* infection varies according to the strain involved [25,29]. *P. yoelii* is commonly used to evaluate liver/pre-erythrocytic stage *Plasmodium* activities in rodents [30,31]. Overall, the rodent malaria models are more straightforward to use for drug discovery than for vaccine development, as it is easier to protect mice from malaria infection than humans [32].

2. Current Murine Models Used in Antimalarial Drug Discovery

Most rodent species used in malaria research are inbred strains owing to the uniformity obtained from individual experimental outcomes, especially in antimalarial drug discovery [33,34]. Although outbred strains are widely used, their diversity from humans continues to inspire more adventure in the use of humanized mice strains [20].

Mice are either susceptible or resistant to *Plasmodium* infections and their resistance may indicate non-lethality of the parasite to mice or late mortality [35]. Screening of novel compounds that consider the fatality of cerebral malaria also requires the use of murine models susceptible to cerebral malaria pathogenesis [36]. Different strains of mice important to antimalarial drug discovery are highlighted in this review.

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2.1. Inbred Mice

Plasmodium-infected Bagg Albino c (BALB/c) mice are widely used in malaria research, from understanding the gut microbiome and its relationship with malaria pathogenesis over vaccine development to the discovery of new chemical entities in ethnomedicine and more [37–39]. BALB/c mice are albino and inbred strains, photophobic, and possess a lengthy reproductive threshold [40,41]. Mortality in these mice is delayed until high parasitemia is reached, and they are resistant to cerebral malaria [20,42]. These mice can be infected with the different rodent *Plasmodium* species and could be used in the efficacy studies of new chemical entities for antimalarial drug discovery (Table 1).

AKR/J inbred mice are resistant to *P. berghei* infection therefore, they are rarely considered models for in vivo antimalarial efficacy testing in drug discovery [43,44].

C3H/HeJ is an immunocompetent inbred strain susceptible to both *P. berghei* and *P. chabaudi* malaria that has displayed reduced responses to chloroquine treatment but efficacious responses to dihydro triazines and biguanides in the treatment of babesiosis and malaria [45–47]. This strain of mice is more commonly associated as a model for babesia research than malaria [48].

CBA mice are an inbred strain susceptible to *P. berghei* infection. They are commonly used as a model for cerebral malaria because they are genetically predisposed to it at hypoparasitemic conditions even when infected with other rodent strains including *P. chabaudi* and *P. yoelii* [43,49].

SJL/J is an inbred mouse strain susceptible to *P. berghei* and *P. chabaudi* . *P. berghei* infection may lead to severe malaria and, consequently, cerebral malaria but in an asymptomatic condition [36,50]. This strain may be considered a murine model for screening of lead compounds in antimalarial drug discovery.

C57BL/6 mice are commonly used inbred strains in antimalarial drug discovery programs. They have a competent immune system, and they are also called ‘B6” [51–54]. C57BL/6 were successfully inoculated with *P. falciparum*. Hence, they are recommended for use in antimalarial drug discovery [52]. They are also widely used as a model of human cerebral malaria and immunological responses to malaria. They are resistant to *P. chabaudi* malaria [20,24,55,56].

DBA/2J mice are inbred strains rarely used as models in antimalarial drug testing but they have been explored for neurological experiments. However, they are resistant to cerebral malaria but susceptible to *P. berghei* infection [42,43,45,57]. This mouse strain has also been infected with other rodent parasite strains, including *P. yoelii, P. chabaudi, and P. vinckei* making it suitable for drug screening [43,45].

2.2. Outbred Mice

Swiss Webster is a widely used outbred mouse susceptible to *P. berghei*, *P. chabaudi* and *P. yoelii* infection and is currently still used in the determination of in vivo efficacy of new chemical entities [58]. This outbred strain also manifests cerebral malaria in severe parasitemia cases which is also considered in treatment development [59]. Transgenic parasite lines of *P. berghei* have also been explored in this mouse model [60].

Institute of Cancer Research (ICR) mice are outbred and highly susceptible to *P. chabaudi* infection as they are to *P. berghei* [56,61]. They are commonly used as a model for the in vivo screening of potential antimalarial molecules obtained from plant sources [62–64]. These mice also manifest cerebral malaria in severe cases [36].

CD1 mice are Swiss-based outbred strains susceptible to multiple rodent *Plasmodium* species [13]. This mouse strain is employed to evaluate the pharmacokinetic properties and the safety of novel lead compounds [13,65].

Naval Medical Research Institute (NMRI) mice are outbred strains susceptible to *P. berghei* infection [66,67]. In NMRI mice, *P. berghei* parasite development occurs exponentially. Therefore, the efficacy of new chemical entities can be estimated suitably in this mouse model [67]. Hepatic-stage *Plasmodium* infection is also assessed using this model which could mean inoculation of transgenic

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parasites [68]. NMRI mice are also models for cerebral malaria pathogenesis from *P. berghei* or *P. yoelii* infection [36].

2.3. Humanized Mice

Humanized mice are now the order of the day in antimalarial drug discovery because of their potential for being engrafted with human cells at immunocompromised conditions while also knocking in human genes into the mouse genomes. Therefore, more effectively representing human physiology for treatment purposes [69].

NOD/SCID/gamma (c) (null), known as NOG mice, are immunocompromised and void of essential lymphocytes like the B and T cells although macrophages remain present [70]. Meanwhile, macrophages have emerged as critical natural protective agents in malaria pathogenesis in humans which can now be assessed in this mouse strain [71]. These human model mice can be engrafted with human *Plasmodium* parasites for novel therapeutic studies, as well as the evaluation of pharmacological parameters [72].

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mouse strain, similar to NOG, is susceptible to both *P. falciparum* and *Plasmodium vivax*. They are helpful for the efficacy determination of new chemical entities against *P. falciparum.* Efficacy can also be evaluated against liver-stage hypnozoites characterized by *P. vivax* infection [73,74].

FRG NOD huHep mouse is a model for the human chimeric liver in *P. falciparum* malaria research. They have aided the understanding of *P. falciparum* pathology transiting from hepatic to erythrocytic stage development, which presents a platform for novel antimalarial screening [75].

5xfad mice is a transgenic mouse model that is predisposed to Alzheimer's disease as they are characterized by the presence of some amyloid plaques [76]. Cerebral malaria has been hypothesized to be associated with apolipoprotein-mediated amyloidosis, whose pathogenicity may also be observed in Alzheimer's disease [77]. Elevated levels of apolipoprotein are observed in the mouse brain during cerebral malaria pathogenesis, that could result in neurological dysfunction, as was observed in mice [77]. Artesunate is reported to alleviate the amyloidosis pathology in 5xfad mice [78]. This suggests that 5xfad mice could be explored for antimalarial drug development in treating cerebral malaria.

Table 1. Current List of Murine Strains as Used in Previous Studies and Their Excellent Inputs to Antimalarial Drug Discovery.

Murine Model (Mice)	Inbred/Outbred	Preclinical Assays Conducted	Resistant/Susceptible to *P. berghei*	Murine *Plasmodium* Species Assessed	References
BALB/c	Inbred	Efficacy, Pharmacokinetics, Safety,	*Susceptible*	*P. yoelii*, *P. chabaudi*, *P. vinckei*	[33,37,45,79,80]
AKR/J	inbred	Pharmacokinetics	Resistant	-	[45]
C3H/HeJ	Inbred	Efficacy	Susceptible	*P. chabaudi*	[45,47]
CBA	Inbred	Efficacy	Susceptible	*P. yoelii*, *P. chabaudi*, *P. vinckei*	[43,49,81]
SJL/J	Inbred		Susceptible	*P. chabaudi*	[45,50]
C57Bl/6	Inbred	Efficacy, Safety	Susceptible	*P. yoelii*, *P. chabaudi P. falciparum*, *P. vinckei*	[20,24,43,55,56].

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DBA/2J	Inbred		Resistant	*P. yoelii* , *P. chabaudi*, *P. vinckei*,	[82–86]
Swiss Webster	Outbred	Efficacy, Toxicity	Susceptible	*P. yoelii* *P. chabaudi*	[28][25,28,87–89]
ICR	Outbred	Efficacy, Pharmacokinetics, Safety	Susceptible	*P. yoelii* , *P. chabaudi, P. vinckei*	[13,90–93]
CD1	Outbred	Efficacy, Pharmacokinetics, Safety	Susceptible	*P. chabaudi, P. yoelii*	[36,66–68,94]
NMRI	Outbred	Efficacy, Pharmacokinetics, Safety	Susceptible	*P. chabaudi, P. yoelii*	[72,95–98]
NOD/SCID/γc null (NOG)	Humanized	Efficacy, Pharmacokinetics		*P. falciparum*	[73,74,82]
NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ	Humanized	Efficacy, Pharmacokinetics		*P. falciparum*	[99,100]
FRG NOD	Humanized	Efficacy		*P. falciparum*	[78]
huHep5xfad	Transgenic	-	-	-	

Specific non-clinical assessments are conducted to identify or validate new chemical entities from ethnobotanical sources or synthetic approaches. Pharmacokinetic and pharmacodynamic properties inform the process of drug dosage optimization [101].

3. In Vivo Pharmacokinetic (PK) Studies

Several factors, including excellent pharmacokinetic properties, increased half-life, and appropriate metabolic distribution, can influence the potency of a compound [102]. Pharmacokinetic properties such as in vivo bioavailability are considered a significant complement to the in vitro efficacy assessment in the identification of lead compounds [103]. Drug metabolism and pharmacokinetic (DMPK) profiling are essential to the optimization of lead compounds in the antimalarial drug development program, and it determines how far lead compounds can get on the drug discovery pipeline [104]. DMPK profiling involves both in vitro and in vivo experiments. In vitro experiments require the use of cells in a controlled environment, but In vivo experiments require the use of animal models, of which murine models are critical and therefore emphasized here [104].

3.1. Oral Bioavailability

In vivo, oral bioavailability of drugs is associated with oral absorption [105]. Oral bioavailability in rodent models may be used to predict that in humans [105,106]. Oral bioavailability is the percentage of administered test compound/drug that ends up in the bloodstream (Figure 3). In contrast, oral absorption is the amount of test compound taken up by the gastrointestinal system

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[105,107]. Oral bioavailability influences the decision on drug exposure and dosage regimen of test compounds. Low oral bioavailability may lead to the failure of lead compounds despite in vitro potency, which must be prevented in clinical trials to avoid the wastage of resources [108,109]. Although several in silico tools have been designed to predict oral bioavailability, actual in vivo experiments do not always correlate [105,108]. Nevertheless, predictive tools can guide the decision processes for potent test compounds' experimental oral bioavailability determination that should be selected as acceptable lead compounds [105,108]. In vivo, oral bioavailability is usually estimated from calculations after the experiment has been conducted in rodent models using high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) [105,110]. The oral bioavailability of a test compound can be elevated by increasing the solubility and reducing the compound's melting point [109,110]. Excellent oral bioavailability represents good drug gastrointestinal permeability and absorption [109,110].

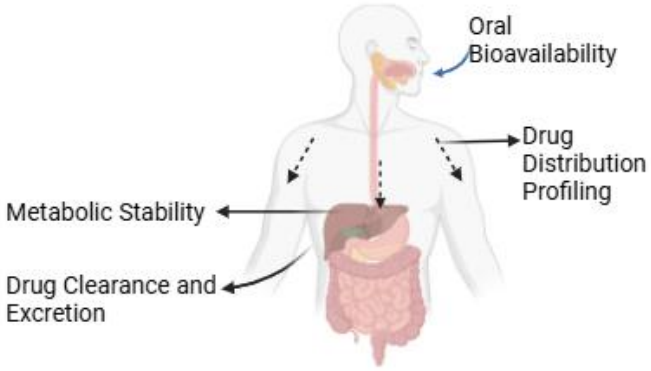

Figure 3. In vivo Pharmacokinetic Parameters in Antimalarial Drug Discovery.

In the selection process of potent hits traveling from phenotypic screening, it is of essence to first consider their in vivo pharmacokinetic profile [111]. If the pharmacokinetic properties are undesirable due to challenges occurring from oral bioavailability, other routes of administration are best employed, such as the intravenous route [111].

3.2. Drug Distribution Profiling

Drug distribution in vivo is determined by evaluating how barriers such as the blood-brain barrier (BBB) are penetrated and the volume of compound molecules that traverse the membrane to reach their target as a measure of plasma protein binding (PPB) as well as the apparent volume of distribution of the drug [112,113]. Drug distribution is a reversible process in pharmacokinetic profiling that begins with the process of drug dilution to plasma binding through absorption, and then drug molecules are dispersed to other parts (Figure 3) [114].

The volume of distribution (Vd) of a test compound or drug is the apparent volume of the drug or test compound in plasma, it measures the extent of distribution of the compound [111,115]. Vd is very important because it influences the half-life of the test compound as well as the dosage frequency [115]. When the Vd of a test compound is low, the half-life of that compound will be low [116]. The apparent volume of distribution of a test compound is a pharmacokinetic property required when a decision is to be made on the choice of lead compounds, especially if they are considered in combination with other compounds or existing antimalarials such as artemisinin-based treatment [112,117,118]. The apparent volume of distribution is usually determined after intravenous administration [119,120]. Dosage optimization of drugs is also influenced by Vd, amongst other pharmacokinetic parameters [121].

Drugs or test compounds in vivo are either bound to plasma or unbound. When bound in the blood, they are bound to proteins and lipid molecules, hence plasma protein binding (PPB) [122].

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Determining PPB for lead compounds is critical to inform further decisions in the drug development pipeline; If it is reversible, PPB does not influence the efficacy of the test compound in vivo [123,124]. High PPB means that the apparent volume of distribution and lipophilicity is high. Still, the elimination of the test compound is reduced, which is advantageous in the context of the extended half-life [114,125,126]. PPB can be determined using ultracentrifugation or equilibrium dialysis [126]. The increased half-life of lead compounds may be traced to high PPB. It influences the raising of the apparent volume of distribution and may prosper the course of a single dose regimen of the lead compound being validated in vivo [126]. Albumin and alpha-1-acid glycoprotein (AAG) are two common plasma proteins measured when evaluating the PPB property of a test compound [116,127]. Both plasma proteins are produced in the liver, and plasma albumin exists in higher concentrations than plasma AAG. Abnormal physiological conditions, therefore, alter the functioning of the plasma proteins [125,127]. PPB can be assessed in vivo in rodents [128]. Albumin in plasma binds to both acidic and basic compounds. Meanwhile, AAG binds to more basic, neutral lead compounds [125]. It is worth noting that children have reduced plasma protein binding, and pregnant women also display reduced levels of plasma protein binding to albumin. Therefore, the apparent volume of distribution will be reduced, and this should be considered in the drug development plan for neonates [101,121,125].

Blood Brain Barrier (BBB) penetration is an important in vivo pharmacokinetic parameter for consideration during antimalarial drug development. BBB penetration is a parameter for the representation of the drug distribution profile of test compounds [113,129]. It is a measure of the concentration of test compounds present in the brain against the concentration found in the blood [113]. BBB is of particular interest when treating cerebral malaria. A dysfunctional BBB is significantly associated with cerebral malaria [130]. *Plasmodium* parasites compromise the BBB, leading to neurological dysfunction in the current mouse model (C57BL/6) for cerebral malaria infected with *P. berghei*. Artesunate is currently recommended as a treatment for cerebral malaria [131,132]. Novel treatments tailored toward cerebral malaria are encouraged. In vivo BBB integrity is determined through spectrophotometry, having extracted the brain from experimental mice injected with Evans blue [130,131]. Intravenous administration of lipid-carrying lead molecules could elevate BBB integrity [132]. Intranasal delivery of nanostructured lipid carriers has also been reported to effectively traverse the blood-brain barrier for the transport of treatment for cerebral malaria [132,133].

3.3. Metabolic Stability

Microsomal stability assay is a test conducted to evaluate the metabolism rate of lead compounds undergoing optimization. It measures the metabolic stability of the test compounds in both in vitro and in vivo hepatocytes. The metabolism of test molecules is usually a function of hepatic processes facilitated by the liver cytochrome enzymes [134].

Microsomes are packed with cytochrome P450 enzymes that metabolize most antimalarials in the liver [28,135]. Microsomal stability of several potential antimalarials has been tested in rats, mice, dogs, and human microsomes[53,136]. Nevertheless, the metabolic rate of microsomal enzymes in *Plasmodium*-infected mice is reported to be lower than in the uninfected counterparts, and the measurement of how low this clearance would be is yet unclear [28]. Metabolic stability is measured as a function of the half-life of the test compounds, the level of liver microsome proteins present, and the intrinsic clearance (in vitro) [28,136]. The half-life explains the removal of 50% of the test compound, while intrinsic clearance describes the hepatic activity (as a function of the microsome protein content) against the test compound minus the influence of other hepatic factors such as the blood flow in the liver [137]. During the assay, samples of the test compound in the presence of extracted liver microsomes are collected at different time points for the estimation of intrinsic clearance [138]. Increased clearance rates insinuate decreased half-lives of the test compounds in the liver, indicating a large volume of drug distribution [139,140]. Microsome stability assays are evaluated for blood stage, liver stage, and transmission-blocking potential antimalarials [141–144].

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Lead compounds displaying excellent metabolic stability proceed further in the antimalarial drug development pipeline [144]. In vitro microsomal stability assays are conducted more frequently than in vivo microsomal assay in antimalarial drug development [138,141–145].

3.4. Drug Clearance and Excretion

The rate at which an efficacious lead molecule is eliminated from the animal describes the excretion process. Meanwhile, the total clearance involves the disappearance of the drug molecules from the plasma-compound-bound complex at a given time [146]. The metabolic activities in the liver and kidney marshal this plasma clearance (Figure 3). Clearance from the liver is termed 'hepatic intrinsic clearance,' and from the kidney, it is called 'renal clearance' [10,134]. In vitro, hepatic intrinsic clearance of free unbound molecules is evaluated using liver microsomes. High intrinsic clearance is directly proportional to a rise in the octanol/pH 7.4 buffer partition coefficient. Nevertheless, reduced levels of intrinsic clearance of unbound molecules increase the half-life of the molecules [126,134]. This intrinsic clearance provides a basis for the prediction of in vivo total clearance of the test molecule [134]. The transition from in vitro to in vivo hepatic intrinsic clearance requires the use of scaling factors that consider the weight of the in vivo mammalian species to be used [134].

The excretion of lead compounds as a pharmacokinetic property incorporates the half-life or elimination of the drug in vivo [146]. Renal excretion provides the means for eliminating unbound test molecules and can be evaluated in vivo in rodent models [53]. Renal clearance is facilitated by glomerular filtration and active transport (both can be extrapolated using the rodent model for evaluation) [147].

4. In Vivo Safety Studies

Due to the desired extended half-life of potential antimalarial drugs, toxicity outcomes of lead molecules must be considered at an early stage of the drug discovery program using pre-clinical testing such as mouse models [148]. Hewitt et al., [148] provided a guideline for safety tests to be conducted on lead compounds in vivo before they are decided as fit to proceed for further drug development. Some in vivo toxicity assays include cardiotoxicity, genotoxicity, phototoxic potential, Good Laboratory Practice (GLP) toxicology studies, combination toxicity studies, cumulative exposure studies, and developmental and reproductive toxicology testing [5,148,149].

4.1. Cardiotoxicity (hERG)

Cardiotoxicity as a detrimental side effect has been associated with certain classes of antimalarials, including the quinolines. This is a critical subject as new molecules are being investigated for antimalaria development [150,151]. At an early stage of preclinical studies, assessing toxic effects of lead compounds on cardiomyocytes (Figure 4) are recommended (World Health Organization) for investigation with measurements of the molecular marker human ether-ago-go related gene (hERG) [148,150–152]. Siqueira-Neto et al., [152] recommend an absence of toxicity against hERG at 1 μm during the early stage of preclinical tests. Meanwhile, at the late stage, the threshold for hERG toxicity must be further reduced when evaluated in vivo. In vivo, cardiotoxicity of antimalarials and potential antimalarials has been determined in rats and zebrafish [151,153].

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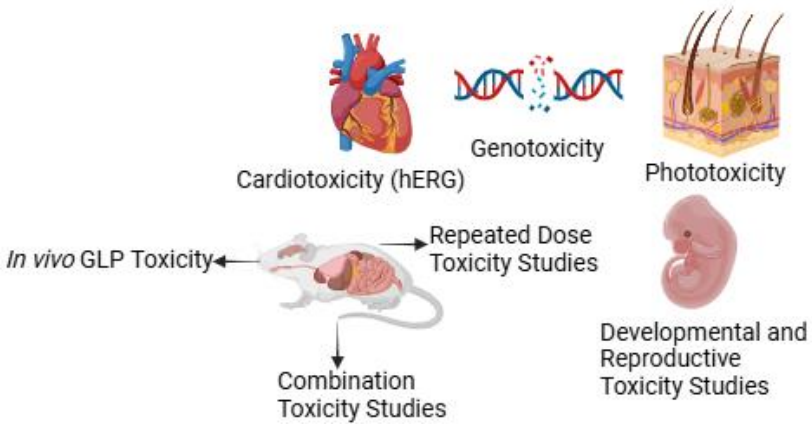

Figure 4. In vivo Safety Parameters in Antimalarial Drug Discovery.

4.2. Genotoxicity

Genotoxicity assays are designed to measure the level of genetic damage conferred by a test molecule (Figure 4), and the odds of the damage caused are the risk of transmission from one generation to another. There are both in vitro and in vivo assays conducted to determine the genotoxic status [154,155]. It is essential to conduct in vivo genotoxicity experiments in addition to in vitro experiments in order to obtain reliable results [155]. Drug candidates with genotoxic potential are excluded during early preclinical screening [155,156]. In vivo genotoxicity has been conducted in rodent models and dogs in the early stages of antimalarial drug discovery [149,157]. Carcinogenicity studies are not considered during antimalarial drug discovery because the dosage regimen is for a short period [148].

4.3. Phototoxicity

Phototoxicity occurs when undesirable skin responses (Figure 4) are observed after administering a drug candidate or a test molecule [158,159]. The phototoxic potential of antimalarial drug candidates has recently been considered for assessment during an early preclinical phase. This test is also conducted in vivo [148,152,160].

4.4. Good Laboratory Practice (GLP) Toxicology Studies

Good Laboratory Practice (GLP) toxicology assessment is a regulatory laboratory procedure conducted in vivo (in rodents and dogs) for 2 weeks [148,161]. This toxicity study is a dose range-finding experiment (in rodents) to determine how harmful specific concentrations of the lead molecule will be to rodents according to GLP standards (Figure 4) [162]. One frequently used method is the maximum tolerated dose (MTD) approach that assesses the toxicity and dose range of the lead compound [162,163]. GLP procedures are primarily non-clinical studies conducted before clinical studies in drug development [148,162].

4.5. Combination-Toxicity Studies

Combination therapy in antimalarial drug development is critical to the elimination of malaria due to the challenge of monotherapy resistance, yet the toxicological outcomes are not to be overlooked [164,165]. In the process of discovering new drug combinations of excellent efficacies, the combination of compounds must not be of increased toxicity to get maximum safety [166]. Combination toxicity is a non-clinical study conducted for about three months using rodents (Figure 4). Each compound is evaluated individually (and in combination), especially when each compound is exclusively efficacious as a requirement for the combination of test compounds used [167].

4.6. Repeated Dose Toxicity

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Repeated dose toxicology (RDT) study is a non-clinical approach to evaluating the toxicity of the test compounds to determine safety [168]. RDT for potential antimalarials is determined in both rodent and non-rodent species, but particularly in rats (Figure 4). Usually for a period of 14 days, where the test compound is administered for all the days [148,169,170]. RDT is a significant testing approach for new chemical entities that are not administered as a single dose, and side effects of the test compounds are sufficiently estimated before further development while considering test compounds with increased half-lives [148,171].

4.7. Developmental and Reproductive Toxicity Testing

Artemisinin displayed embryotoxicity in the first three months of pregnant women (Figure 4). Therefore, developmental and reproductive toxicology is paramount in considering lead compounds that should proceed for further development [172–174]. Development and reproductive toxicological studies are preclinical studies conducted to determine the test compound's embryotoxic or teratogenic effects in vivo, informing the decisions of choice and how to improve the selected lead compounds [5,172,173,175].

5. In Vivo Rodent Efficacy Studies

In vivo, efficacy testing in antimalarial drug discovery is an indispensable aspect of the development pipeline [176]. The potency of lead compounds is evaluated at different developmental stages of the parasite in vivo [6]. Rodents are the face of preclinical efficacy testing in antimalarial discovery, following validations from in vitro screenings and the identification of lead compounds [24]. During efficacy studies, infected rodents with matching rodent *Plasmodium* strains are carefully selected based on the class of antimalarial candidate being evaluated or the type of population the drug is designed for [176].

5.1. Prophylactic Test

In vivo prophylactic test is an immediate efficacy evaluation to be conducted, having pinpointed the lead compound [177]. The compound's prophylactic efficacy is the lead compound's capacity to prevent the development of the *Plasmodium* parasite, thereby preventing the progression of the parasite's pathological cycle [178,179]. Prophylactic treatment is administered chiefly to travelers and migrants to and from malaria-endemic countries [178]. To evaluate prophylaxis in animals, rodents are initially administered the test compound and inoculated with the rodent *Plasmodium* strain afterward. The efficacious activity of the test compound is measured by the level of parasite densities or parasitemia at the end of the experiment [180,181]. Therefore, an advantageous edge to new chemical entities considered for prophylactic treatment is their long half-life [181]. Lead compounds may be evaluated for their prophylactic efficacy by hindering the parasite development at the pre-erythrocytic/ hepatic (causal) or erythrocytic (suppressive) stage [179,182]. Suppressive treatment inhibits erythrocytic-stage parasitic infections [183].

5.2. Suppressive Test

The most prevalent in vivo chemo-suppression assay is Peter's four-day suppressive test, which entails a four-day treatment procedure a few hours after parasite inoculation [24,180]. Mean survival time is also determined during Peter's suppressive test, which estimates how long the animals survive post-treatment [184]. Parasitemia and percentage suppression are determined from the experiments as a measure of chemo-suppression [180].

5.3. Curative Test

Rane's test is the most widely used to assess the curative efficacy of new chemical entities in vivo [180,185]. During this experiment, treatment of the animals inoculated with the rodent Plasmodium strain begins from day 3 post-infection and lasts for four days. Curative treatment is usually

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administered orally, intraperitoneally, and through other avenues [185]. Curative tests conducted for lead molecules are particularly essential when treating cerebral malaria [185,186].

Routes of administration of drug treatments include oral, intraperitoneal, intravenous, and subcutaneous. Other routes of administration aside from oral dosage are explored when test compounds are not quickly soluble [103].

5.4. Parasite Viability

In vivo parasite viability estimation post-treatment has been considered a more informative measure of drug efficacy than just the number of parasites or strength of a reporter signal [187]. Analogous to the concept of colony-forming units in virology, this method uses limiting dilution and in vitro regrowth to determine the number of viable parasites after drug-wash out. While this is very laborious and time-consuming, it aids in measuring the lead compound's cidal effect and the maximal concentration to obtain the optimal cidal effect [187]. Parasite viability as a read-out can correct for underestimation of a drug's efficacy e.g. with artesunate, which appeared to leave some parasites in circulation after treatment [187] . Nevertheless, these were not actually viable and over-estimation of efficacy was observed with a *P. falciparum* field isolate that appeared to respond to piperaquine but actually would not be cleared by it [188].

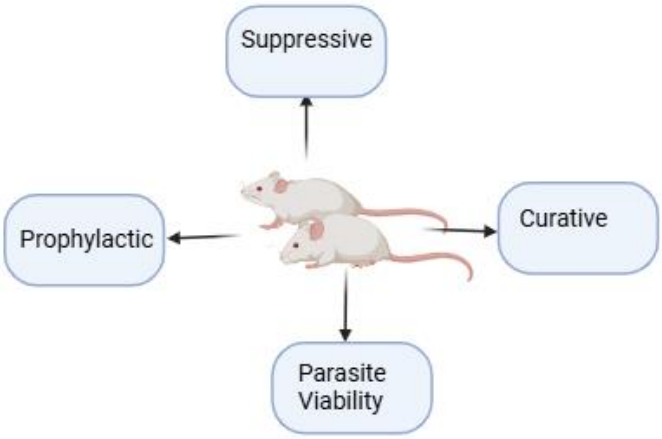

Figure 5. In vivo Efficacy Parameters in Antimalarial Drug Discovery.

Murine models are currently being explored in other areas of malaria research apart from drug discovery, emphasizing their indispensable utility.

Table 2. Current Areas in Malaria Research Utilizing Murine Models Apart from Drug Discovery.

Murine Model	Area of Malaria Research	Reference
Theiler's Original (TO)	Drug resistance mechanisms	[24]
Humanized	Liver and Blood Stage Malaria Pathogenesis	[24,189]
C57BL/6	Kinetics of the Infection and Disease Progression in mice compared to humans	[190]
C57BL/6	Malaria-induced kidney impairment	[191]
C57Bl/6	Vaccine Development	[192,193]
CBA	Lipidome Profile Assessment in Cerebral Malaria	[194]
ICR based	Understanding metabolic responses from immune perturbations through liver transcriptomics	[195]

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C57BL/6	Alterations in Cardician of parasite development and the resulting effect in cerebral malaria	[196]
C57BL/6	Understanding malaria infection during pregnancy and the consequential birth effects	[197,198]
BALB/c	Vaccine Development against Malaria Relapse	[199]
C57BL/6	Malaria Pathogenesis and Associated Lung Impairment	[200–202]
BPH/2 (transgenic)	The possibility of association between Malaria and Hypertension	[203]
BALB/c/C57BL/6	Understanding the difference in metabolic responses between Uncomplicated and severe malaria	[204]
C57BL/6	Immunity and Malaria infection	[205]
BALB/c/C57BL/6	Environmental temperature and its association with malaria disease progression	[206]
C57BL/6	Neonatal Immune response in malaria pathogenesis	[207,208]

6. Conclusion

Selecting appropriate lead molecules during the antimalarial drug discovery program is essential to prevent drug failures during clinical trials. Therefore, the availability of murine resources in in vivo validation experiments of in vitro identified lead compounds provides a rich basis for assessing these new chemical entities for their safety, toxicity, dosage-regimen and efficacy in preclinical trials. This informs the Phase I clinical trials and can reduce the risk of failures during clinical trials.

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Abbreviations

AAG	Albumin and alpha-1-acid glycoprotein
BALB/c	Bagg Albino
BBB	Blood-brain barrier
GLP	Good Laboratory Practice
hERG	human ether-ago-go related gene
HPLC-ESI-MS/MS	high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry
ICR	Institute of Cancer Research
PPB	Plasma protein binding
PK	Pharmacokinetic
RDT	- Repeat dose toxicology

TO	Theiler's Original
Vd	Volume of distribution
MDPI	Multidisciplinary Digital Publishing Institute
DOAJ	Directory of open access journals
TLA	Three letter acronym
LD	Linear dichroism

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