Infection and Drug Resistance

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ORIGINAL RESEARCH

Polymorphisms in the K13-Propeller Gene in Artemisinin-Resistant *Plasmodium* in Mice

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Introduction: Artemisinin-based combination therapies (ACTs) act as first-line antimalarial drugs and play a crucial role in the successful control of *falciparum* malaria. However, the recent emergence of resistance of *Plasmodium falciparum* to ACTs in South East Asia is of particular concern. Hence, there is an urgent need to identify the genetic determinants of and understand the molecular mechanisms underpinning such resistance. Artemisinin resistance (AR) is primarily driven by the mutations in the *P. falciparum* K13 protein, which is widely recognized as the major molecular marker of AR. However, association of K13 mutations with in vivo AR has been ambiguous due to the absence of a tractable model.

Methods: In this study, we have successfully produced artemisinin- and piperaquine-resistant *P. berghei* K173 following drug administrations. Prolonged parasite clearance and early recrudescence were found following daily exposure to high doses of artemisinin and piperaquine. We have also sequenced the DNA of artemisinin-resistant strains and piperaquine-resistant strains of *P. berghei* K173 to explore the relationship between *PfK13* and AR.

Results: The resistance index of *P. berghei* K173 reached 12.4 after 30 artemisinin-resistant generations, but AR declined gradually after 30 generations. On the 50th generation, the resistance index of artemisinin-resistant strains was only 5.0 compared with the severe drug resistance of piperaquine-resistant strains (I_{90}=148.8). DNA sequencing of artemisinin-resistant strains showed that there were 9 meaningful mutations at *P. berghei* K13-propeller domain, but the above mutations did not include common clinical point mutations.

Conclusion: Our data show that artemisinin is less susceptible to severe resistance compared with other antimalarial drugs. In addition, mutation on *P. berghei* K13 has a multi-drug-resistant phenotype and may be used as a biomarker to monitor its resistance. More studies need to be conducted on the new mutations detected so as to understand their association, if any, with ACT resistance.

Keywords: malaria, *Plasmodium berghei*, *P. berghei* K13 gene, artemisinin resistance

Plain Language Summary

Resistance against artemisinin poses a great threat to global malaria control and elimination. So, there is a major need to figure out the mechanism of the resistance to artemisinin. Widely prevalent in the economically less developed regions, it is hard to collect live *Plasmodium* with resistance to artemisinin. Given this, we cultured artemisinin- and piperaquine-resistant strains in laboratory by the way of drug high-pressure cultivation, and then we analyzed the DNA sequence to detect the possible mechanism of resistance. Through this study, we found that artemisinin-resistant strains evolved more slowly than the piperaquine-resistant strains. More interestingly, the resistance index decreased as the number of breeding generations increased. Moreover, we found that there were 9 meaningful mutations in *P. berghei* K13 of artemisinin-resistant strains via DNA sequencing, which were different from the clinical mutant points. The points may be related to the multi-drug resistance phenotype of *P. berghei* K13 variants. Awaiting in-depth study, these meaningful mutations may be used as biomarkers to monitor drug resistance of artemisinin-resistant strains.

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Introduction

Since its isolation in the 1970s from the plant *Artemisia annua*, artemisinin (ART) and artemisinin derivatives have evolved as important components of artemisinin-based combination therapies (ACTs) worldwide.[1] ART and artemisinin derivatives are highly effective and act quickly against the blood stages of malaria parasites. The administration of ART and artemisinin derivatives can lead to up to 10,000-fold parasite reductions in the first replication cycle of the *Plasmodium*.[2] Recently, reported reductions in the malaria morbidity and mortality have been partly attributed to the usage of ACTs for malaria treatment and prevention.[3] Malaria parasites generally develop resistance to the new antimalarial drugs after 10 to 15 years of usage.[4] In 2008–2009, the New England Journal of Medicine published two different articles describing the clinical AR to *P. falciparum* strains found in Cambodia and Thailand border areas.[5,6] These two studies found that there was a markedly decreased sensitivity of *P. falciparum* to artesunate, parasite clearance time was significantly prolonged, and these changes were not due to pharmacokinetic or patient factors, but rather related to alterations in malarial parasite whole-genome.[7]

In 2014, Arief et al[8] collected and sequenced the whole genome of artemisinin-resistant strains from malaria-endemic areas in Africa and Cambodia. The genetic variation of *P. falciparum* (*Pf*3D7_1343700) kelch propeller domain (K13-propeller, amino acid residues 350 to 726) was found to be associated with AR. Phenotypically, AR is defined as a potential delay in the parasite clearance. These AR strains recrudesce more frequently than artemisinin-sensitive parasites after standard 3-day therapeutic courses with ACTs.[9] Genetic mutations of K13-propeller domain are associated with delayed parasite clearance in vivo and reduced susceptibility of the ring-stage parasites in vitro in ring-stage survival assays (RSA).[6,10] *PfK13* regional mutations have been often reported in most areas of South East Asia. The meta-analysis of the WWARN K13 Genotype-phenotype Study Group[11] reported that many isolates with the C580Y allele of *PfK13* were found at the western border of Thailand, Cambodia, Laos and Vietnam. Moreover, isolates with the Y493H and R539T mutant codons were more commonly found in Cambodia, and those with A578S were more common in Africa. It has been reported that upon being influenced by the drug pressure, life environment, mode of transmission, host body and other factors in the process of its evolution, *Plasmodium* K13 gene mutates in diverse ways, but the reasons behind this mutation are still unclear. Meanwhile, there are no sufficient evidences indicating that ART might be completely ineffective against artemisinin-resistant strains of *P. falciparum*, but the reduced sensitivity of *P. falciparum* to ART could potentially become a huge obstacle in complete eradication of malaria. Hence, it is important to study the mechanism(s) underlying these mutations in detail.

AR in *P. falciparum* has been associated with *PfK13* polymorphisms, and the K13-propeller domain serves as a molecular marker in the surveillance.[12] More than 200 nonsynonymous K13 single-nucleotide polymorphisms have been reported previously, including 11 candidate resistance mutations (P441L, G449A, C469F/Y, A481V, R515K, P527H, N537I/D, G538V, V568G, R622I, A675V, associated with delayed parasite clearance) and 10 validated mutations (F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P74L, C580Y, reduced in vitro sensitivity).[13] Although K13-propeller mutations are highly predictive of resistance, very little is known about the molecular mechanisms that render *P. falciparum* insensitive to artemisinins. Therefore, it is urgent to further study the mechanisms of resistance of *Plasmodium* parasites to ART. Meanwhile, ART resistance is well established in Cambodian provinces but which is uncommon and less prevalent. Moreover, *P. falciparum* cannot be tested in vivo in rodents, and data from rodents do conform to the patterns of many drug resistance that are observed in *P. falciparum* in nature.[14,15] Moreover, *P. berghei* and *P. falciparum* K13 structural homology are highly conserved (over 87% sequence identity overall) at DNA sequences. In order to further explore the correlation between K13 gene polymorphism and AR, we obtained artemisinin- and piperaquine-resistant strains in *P. berghei* through continuous drug high-pressure culture in the laboratory and conducted relevant DNA sequencing. Our results suggested that the mutations at *PbK13* can play a causal role in the development of the drug resistance in *P. berghei* and could serve as the potential target for monitoring and preventing AR in *Plasmodium*.

Materials and Methods

Ethical Consideration

The experimental protocol for in vivo studies was approved by the Ethics Committee of Science and Technology, Guangzhou University of Chinese Medicine, China (PZ14016). All the procedures related to animals were conducted

according to the Regulations of the Experimental Animal Administration, State Committee of Science and Technology of the People's Republic of China.

Parasite, Animal and Drugs

Parasite strains: We used *P. berghei* K173, which is a parental, drug-sensitive *P. berghei* line, developed by the National Institutes of Health (NIH) in 1997. *P. berghei* K173 was maintained in cryopreserved state in the liquid nitrogen and administered by intraperitoneal injections of infected blood into the mice. *P. berghei* kelch protein K13 (putative) was downloaded from the European Nucleotide Archive database (coding: CXJ03505).

Among Kunming mice (18–22g) used, half were male and half were female. They were supplied by the Laboratory Animal Center of Guangzhou University of Chinese Medicine or Southern Medical University, Guangdong, China. The license numbers for animal maintenance are SCXK (Yue) 2013–0034 and SCXK (Yue) 2016–0041. The animals were raised in an experimental room at 20–24°C and 70–80% relative humidity and fed on 60 Co irradiated forage and water ad libitum. The mice were infected with parasitized red blood cells (pRBCs) via intraperitoneal (i.p.) injection. Daily monitoring was carried out on infected mice, and infected bloods were transmitted to the receptor mouse.

The frozen tube containing malaria infected blood was removed from the liquid nitrogen and placed in warm water at 40°C until it has completely melted. The pRBCs were injected intraperitoneally into 4~10 mice while maintaining the amount of blood injected into each mouse as constant. Eight days later, parasitemia was assessed in Giemsa-stained thin smears from the tail-vein blood of infected mice. Mice with the highest infection rate were selected as the donor mice. The blood was diluted with physiological saline to contain 1×10^7 pRBCs per 0.2mL of blood. The mice were thereafter infected by intraperitoneal injection of blood containing about 1×10^7 pRBCs.

ART was obtained from Sichuan Tongrentai Pharmaceutical Co., Ltd. (Sichuan, China), Lot No.141101, 160401. Piperaquine (PQ) was obtained from Artepharm Co., Ltd. (Guangdong, China), Lot No. 110804. ART was prepared in a 1:1 mixture of DMSO and Tween 80 (Sigma) and diluted 10-fold in sterile distilled water immediately before administration. PQ was dissolved directly in distilled water. All drugs were prepared fresh before in vivo administration, and drug delivery was carried out by intragastric administration.

Procedures for Exerting Drug-Selection Pressure

The "serial technique" (ST) was used to select resistant line[16]. Eight Kunming mice were selected as the parent generation and were randomly divided into 2 different groups with 4 mice in each group. The day of *P. berghei* K173 infection was denoted as D0. The mice were treated by a single dose of ART or PQ on D2 after *P. berghei* K173 inoculation (The doses used for the drug-selection pressure have been shown in Table 1). From D3 to D7, the parasitemia of each infected mouse was assessed and mice with the highest parasitemia were selected as potential donors of pRBCs for the next generations. Peter's 4-day suppressive test was performed at every five generations to determine the resistance index. The drug dosage was increased every 5 generations depending on the parasite response.

Peter's 4-Day Suppressive Test

Mice were infected intraperitoneally with 1×10^7 pRBCs, and drug was administered at 2h, 24h, 48h, and 72h, respectively, postinfection.[17] Parasitemias were assessed by the microscopic examination of Giemsa-stained slides from tail vein blood collected on day 4 post-infection, in order to manually determine the levels of infection.

Assessment of Resistance

Blood was obtained by trimming the tip of the tail and smeared on a microscope slide (thin blood smear) and then fixed with absolute methanol for 10s. After fixation, the slides were dried and stained with 10% Giemsa stain for 15 min, rinsed with running water, and dried at room temperature. The parasite-infected red blood cells were quantified using a light microscopy (CX41RF, OLYMPUS, Corporation, TOKYO, Japan). The blood films which did not visualize protozoa in 50 microscope fields were considered as negative, and the infection rate was found to be 0. The Peters 4-day suppression test was used to measure the resistance index once every five generations.[17] Then, the "index of resistance", I_{90} (defined as the ratio of the ED_{90} of the resistant line to that of the sensitive parent line), was calculated. The degree of

Table 1 Selection of Artemisinin and Piperaquine Resistance in *P. berghei K173* Using the Serial Technique

Passage NO.	ART Group			PQ Group		
	Drug-Selection (mg/kg)	ED_{90}	I_{90}	Drug-Selection (mg/kg)	ED_{90}	I_{90}
5	188.85	208.9	3.4	6.57	11.8	3.2
10	271.49	293.1	4.7	11.75	35.5	9.7
15	293.10	478.6	7.7	35.48	39.8	10.9
20	478.67	359.6	5.8	39.80	56.2	15.4
26	574.20	596.4	9.7	47.63	72.5	19.9
30	689.04	759.9	12.4	57.31	106.8	29.3
35	803.60	252.3	4.1	69.84	124.5	34.1
40	803.60	280.4	4.6	69.84	538.1	147.4
45	883.96	334.1	5.4	76.82	400.3	109.7
50	883.96	305.3	5.0	76.82	543.2	148.8

Notes: The data has been presented as doses for drug-selection pressure in different procedures, effective doses that can reduce parasitemia by 90% (ED_{90}) and 90% index of resistance (I_{90}, defined as the ratio of the ED_{90} of the resistant line to that of the parent strain).

resistance was grouped into four levels by the values of I_{90} as previously reported:[18–20] (1) $I_{90} \leqq 1.0$, sensitive, (2) I_{90} =1.01–10.0, slightly resistance, (3) I_{90}=10.01–100.0, moderately resistant and (4) I_{90}>100.0, strongly resistant. The formula for I_{90} used was as follows: $I_{90} = \frac{ED_{90}\ resistant\ -\ line}{ED_{90}\ parent\ -\ line} \times 100\%$.

Blood Sample Collection

In the process of resistance cultivation, after each generation of resistance cultivation, the blood samples were respectively harvested by heparinized capillary tubes from the mice orbitals after the mice were narcotized using diethyl ether. About 700 μL of the blood samples were obtained from each mouse in the anticoagulant collection vessels of Ethylenediamine Tetraacetic Acid (EDTA). The resistance cultivation algebra was then labeled. The plasma was kept at −80°C before use.

DNA Extraction

Genomic DNA of parent strains (sensitive *P. berghei* K173) and drug-resistant strains (ART- and PQ-resistant strains of the 30th and 50th generation) was extracted from 100 μL of each whole blood sample using DNA blood kit according to the manufacturer's protocol (Takara Bio Inc. Nojihigashi 7-4-38, Kusatsu, Shiga 525–0058, Japan, Lot No.: AK1901). The extracted DNA was eluted in 60 μL of TE buffer (10 mM Tris–HCl, 0.1 M EDTA, pH 8.0) and stored at −20°C until further use. The quality of genomic DNA was detected using 1.0% agarose gel electrophoresis. The gel was stained with Goldview nucleic acid stain used as an ethidium bromide substitute (Sangon Bio Inc., Shanghai, China).

PCR Amplification and Sequencing of *P. berghei* K13-Propeller

Kelch13 fragments of *P. berghei* sensitive and resistant strains were amplified and were identified by agarose gel electrophoresis. PCR amplification products were sent to Shenzhen BGI Technology Co., Ltd. (Guangdong, China) for sequencing of *P. berghei* K13 fragments. The sequencing was completed by using three reactions. DNASTAR and DNAMAN9.0 were used to compare the above sequences with ENA CXJ03505. The primers were designed for *P. berghei* K13, 2126752 to 2128968 base pairs on chromosome 13 (GenBank: LT160033.1, locus_tag="PBK173_000418100"). PCR primer:[21] Upstream primer sequence: 5'-AGTCAAACAGTATCTCTAACT-3', downstream primer sequence: 5'-ACGGAATGTCCAAATCTTG-3'. The PCR amplification reaction of components was thereafter combined in a master mix composed of TAKARA Master Mixture (5U/μL), 25μL; PCR Forward Primer (100μM), 2μL; PCR Reverse Primer (100μM), 2μL; Template DNA (20~50ng/μL), 2μL and RNAse Free dH_2O used to reach a total reaction volume of 50μL. The cycling program was set at 1 cycle of

predenaturation at 95°C for 5 min, denaturation at 94°C for 1 min, annealing process at 51°C for 30s, and extension at 72°C for 1.5 min, followed by 2~4 cycle for 30 times, and finally repair extension at 62°C for 10 min.

Quantification and Statistical Analyses

All the data were statistically analyzed using SPSS (IBM SPSS Statistics 20.0). Statistical analysis was performed using one-way analysis of variance (ANOVA). A significance level of $P<0.05$ was required for all tests and considered as statistically significant.

Results

P. berghei Did Not Develop Severe Resistance to ART

Artemisinin- or piperaquine-resistance *P. berghei* K173 strain was obtained by a serial passaging of the sensitive malaria parasites under incremental ART or PQ dosages for 50 generations in our laboratory. The resistance index of PQ was determined to be 148.8 ($I_{90}>100$, Table 1) in our laboratory, which increased rapidly and indicated severity of infection. Interestingly, the resistance index of ART increased from the 5th generation to 30th, which indicated a moderate resistance in the 30th generation ($I_{90}=12.4$, $10\leq I_{90}<100$, Table 1). However, in subsequent 31–50 generations, although the dosage of artemisinin was increased by up to 53.95% (Table 1), the resistance index of ART decreased significantly to less than 10, showing mild resistance ($I_{90}=5.0$, $I_{90}<10$, Table 1). From 21 to 50 generations of resistant culture, the difference between artemisinin- and piperaquine-resistant strain I_{90} was statistically significant ($P<0.05$, Figure 1). In conclusion, *P. berghei* K173 strain did not effectively develop severe resistance to artemisinin under continuous drug pressure, which may be related to the special mechanism of artemisinin. Artemisinin has been used clinically for several decades. Although resistance has been reported (prolonged protozoa clearance time), there has not been a large-scale spread of severe resistance, thereby proving that potential application of artemisinin is more advantageous and irreplaceable than other antimalarial drugs.

Variation at the K13 Locus of *P. falciparum* or *P. berghei* Can Be Used as a Possible Biomarker to Predict the Resistance Development

According to the primers, a total of 2179 bp fragments from position 20 to position 2198 were amplified. After sequencing, the splicing sequences were compared in NCBI-BLAST (Table 2), which confirmed that the measured sequence was the K173 KELCH13 fragment of *P. berghei*. We next downloaded the K173 KELCH13 gene sequence CXJ03505 from the European Nucleotide Archive (ENA). DNAMAN9.0 was used to compare the gene sequences with K173 sensitive strains, ART-resistant strains of the 30th generation (A30), ART-resistant strains of the 50th generation (A50), PQ-resistant strains of the 30th generation (P30) and PQ-resistant strains of the 50th generation (P50), and the changed locations were marked blue (Supplementary Figure 1). It could be seen that the nucleotide sequence of *P. berghei* resistant strains (A30, A50, P30 and P50) has many identical changes compared with CXJ03505 (Table 3), while the similarity between the sensitive strains (*P. berghei* K173) and CXJ03505 was up to 99.95%.

It was found that compared with the sensitive strains, resistant strains had 9 missense mutations, such as A128T, A189G, A311G, G361A, A368G, G398A, A464G, A531T and C1643T. The corresponding amino acid changes were Y43F, I63M, N104S, A121T, N123S, S133N, N155S, E177D and S548L, and there were 29 silent mutations. However, *PfK13* mutation sites, such as C458Y, R539T, Y493H, M476I, which have been closely related to artemisinin-resistant strains of *P. falciparum* in South East Asia, were not detected. It was observed that the variation of *P. berghei* K13 genes under the drug pressure was diverse due to the influence of environment, transmission route, host and other factors. The *P. berghei* K13 variation detected in this study needs to be further verified.

Mutations at *P. berghei* K13 occurred in both artemisinin-resistant and piperaquine-resistant strains of *P. berghei*. In A30 and A50 strains, although the I_{90} resistance decreased from moderate to mild, there were no observed differences between them. We conclude that the detection of *P. berghei* K13 variants can be used as the biomarkers to predict the development of malaria resistance but could not be employed to quantify the severity of resistance.

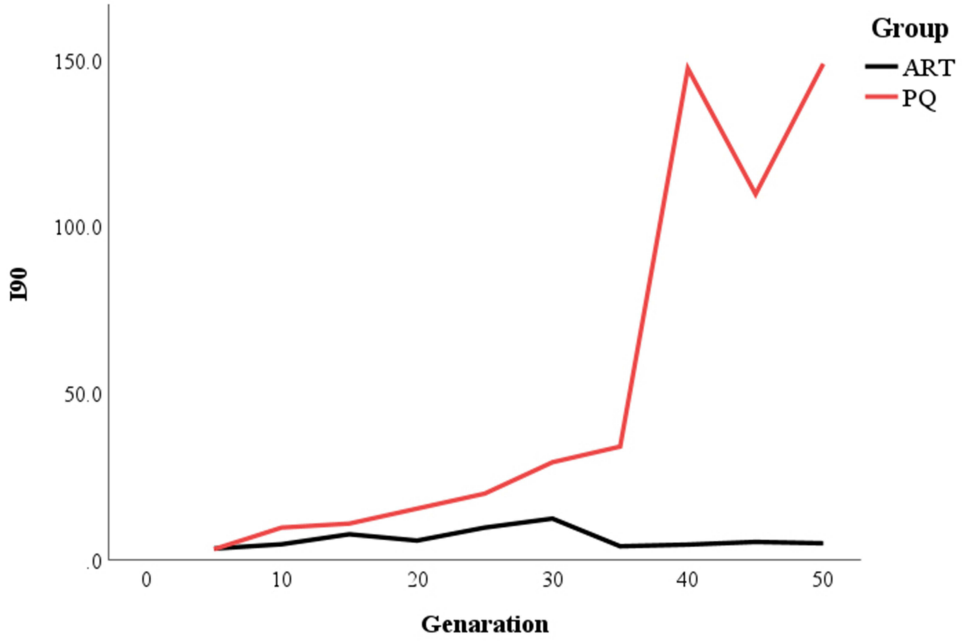

Figure 1 Resistance index line chart (ART Vs PQ:*P*=0.035). After 50 generations of culture, the dosage of artemisinin-resistant culture was observed to increase from 188.85 mg/kg to 883.96 mg/kg, but the resistance index peaked at the 30th generation (I_{90}=12.4) and began to decrease thereafter, and remained at about 5 in 30–50 generations. The dose of piperaquine resistance was increased from 6.57 mg/kg to 76.82 mg/kg, and the resistance index was increased from 3.2 in the fifth generation to 148.8 in the 50th generation.

Resistant Strains in *P. berghei* Display Multidrug Resistance Phenotypes

We complied both artemisinin- and piperaquine-resistance *P. berghe*i, and the *P. berghei* K13 fragment of the two drug-resistant strains showed almost identical mutations. This finding clearly indicated that *Plasmodium* resistant strains have a multidrug resistance phenotype, making *Plasmodium* resistant to either artemisinin or artemisinin combination. Moreover, sequencing results also revealed that the 1643th nucleotide change is C (cytosine) into T (thymine), which can cause the 548th in the amino acid sequence generated by the S (ser), the change of L (leucine) (Supplementary Figure 2), the specific variation may be in *P. berghei* K13 protein six propellers area, or directly related to artemisinin resistance. The multidrug resistance phenotype of *Plasmodium* directly leads to the inevitable resistance of all the antimalarial drugs after long-term chronic use. It has been established that different drugs have different antimalarial mechanisms, so the time and severity of the resistance are also different. Artemisinin is activated mainly by the degradation products of hemoglobin in *Plasmodium*, thus producing Reactive Oxygen Species (ROS) to kill *Plasmodium*. Although there is evidence that *PfK13* can significantly attenuate the sensitivity of *Plasmodium* to artemisinin by functionally reducing its endocytosis of hemoglobin[22] (Figure 2),

Table 2 Comparison of Each Sequence with *K13* Gene Sequence of *P. berghei*

NO.	Max Score	Total Score	Query Cover	Per. Ident
K173	3751	3751	100%	100.00
A30	3530	3530	99%	98.08
A50	3530	3530	100%	98.03
P30	3530	3526	99%	98.03
P50	3530	3530	100%	98.03

Abbreviations: K173, the sensitive strains in *P. berghei* K17; A30, Artemisinin-resistant strains of the 30th generation in *P. berghei* K173; A50, Artemisinin-resistant strains of the 50th generation in *P. berghei* K173; P30, Piperaquine-resistant strains of the 30th generation in *P. berghei* K173; P50, Piperaquine-resistant strains of the 50th generation in *P. berghei* K173.

Table 3 Potential Changes of *P. berghei K13* Caused by Resistance Breeding

Variation Type	Nucleotide Changes and Positions	Amino Acid Changes and Positions
Missense mutation	A128T	Y43F
	A189G	I63M
	A311G	N104S
	G361A	A121T
	A368G	N123S
	G398A	S133N
	A464G	N155S
	A531T	E177D
	C1643T	S548L
Silent mutation	G192A	T64T
	C258T	A86A
	A474G	A158A
	T522C	C174C
	A558G	T186T
	C615A	G205G
	A684G	T228T
	C787T	C263C
	A789G	T228T
	T897C	A299A
	A984C	A299A
	T1059A	A328A
	A1110C	A370A
	T1128A	A376A
	A1275G	A425A
	T1347A	A449A
	C1366T	T456T
	G1497A	G499G
	G1650A	G550G
	C1677T	A559A
	C1701T	A569A
	C1755T	A585A
	C1767A	C589C
	C1809G	A603A
	C1878T	A626A
	C1983A	T661T
	G1998A	C666C
	A2031C	A677A
	T2064C	A688A

hemoglobin is still the main raw material required for the maintenance of growth and development of *Plasmodium*, and artemisinin can act through modulating multiple binding location or pathways. Activated artemisinins can effectively form adducts with a variety of biological macromolecules, including haem, translationally controlled tumor protein (TCTP), *P. falciparum* phosphatidylinositol-3-kinase (*PfPI3K*), inhibiting a calcium ATPase (*PfATP6*) and other high-molecular-weight proteins. Moreover, Bing Zhou and his team[23,24] have reported that artemisinin can also cause mitochondrial depolarization, and mitochondria can serve as potential targets of both artemisinin and activators of artemisinin. This may be an important reason why malaria parasites have not yet been developed widespread and severe resistance to artemisinin.

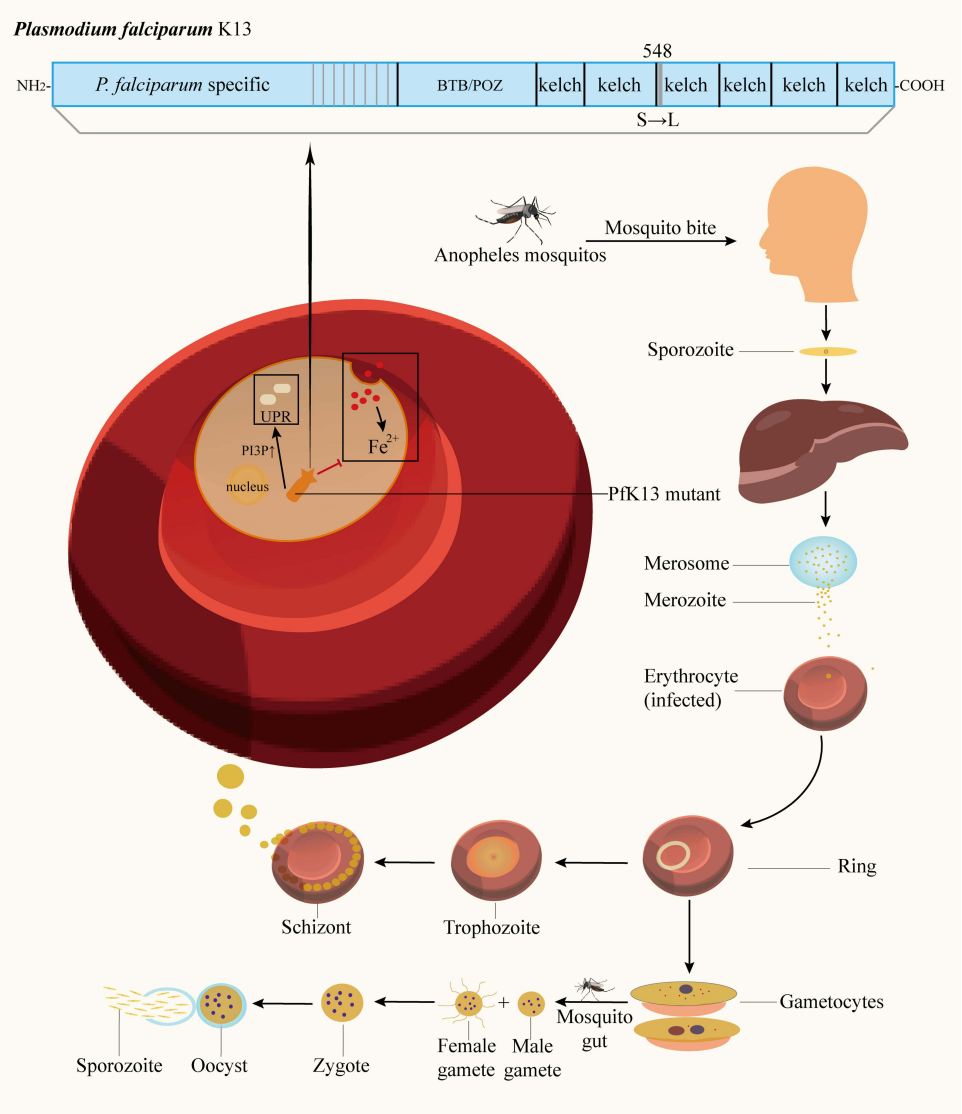

Figure 2 The growth cycle of *Plasmodium* and the mechanism of resistance induced by *PfK13* mutation. *PfK13* mutations can reduce sensitivity to artemisinin by inducing the unfolded protein response (UPR) in the resistant parasite and reducing endocytosis of hemoglobin.

The problem is the opportunity as the multi-resistant phenotype of *Plasmodium* can act as both the cause of multi-resistant *Plasmodium* and the potential target for the future treatment of *Plasmodium* resistance.

Discussion

Our study showed that compared with PQ, ART was less prone to severe drug resistance. We found that *P. berghei* K13 gene mutation could effectively predict the development of drug resistance in *Plasmodium*. Furthermore, we also found that *P. berghei* K13 gene mutation could predict the emergence of resistance to *Plasmodium*. During the breeding of *P. berghei* 1–50 generations in the laboratory, we noticed that ART did not show severe resistance, and its I_{90} was maintained at around 5 after 30 generations. The subsequent DNA sequencing of the parent strains (*P. berghei* K173) and drug-resistant strains (A30, A50, P30 and P50) almost displayed the same mutations, while the parent strains did not exhibit the same mutations. Overall, combined with the existing literature, our study provided biological support for the hypothesis that *P. berghei* K13 site mutation can lead to *Plasmodium* resistance.

We successfully cultivated the *P. berghei* K173′s resistant strains against ART and PQ in our laboratory. Artemisinin-resistant strains hardly showed severe resistance and their resistance was unstable. One study found that artemisinin-

resistant *P. falciparum* isolates displayed a loss of health, the drug-resistant strains could not undergo the transition from ring forms to trophozoites as well as schizonts, and fewer offspring were found in red blood cells during development cycle.[25] In case of an insufficient supply of exogenous amino acids, the loss of adaptability of the resistant strains will increase significantly. The drug-resistant parasites are immature, so the anti-artemisinin phenotype is often unstable. Repeated *Plasmodium* resistance was first reported by Elfawal et al in their study on artemisinin-resistant strains of *P. yoelii*.[26] Their study also found that ART was less prone to severe drug resistance.[24] Moreover, when drug dosage was increased, the antimalarial effect of ART could be temporarily restored.[27] The above findings indicated that in areas where artemisinin-resistant strains were endemic, increasing the amount of artemisinin in ACTs or prolonging the treatment course of ACTs can be applied as an important strategy to improve the clinical efficacy of ACTs but not as a long-term solution.

The *P. berghei* K13 gene mutation of the artemisinin-resistant strain cultivated in our laboratory was different from the *PfK13* mutation point of the artemisinin-resistant strain of *P. falciparum* detected in South East Asia. This may be due to the difference of the life cycle of the two malaria parasites and the difference of the host bodies. In the existing literature, *PfK13* mutation of artemisinin-resistant strains in South East Asia mostly occurred in C580Y, E25Q, Y493H, R539T;[28] while in Africa,[29] *PfK13* mutation primarily occurred in A578S, T549C, and G553A. One study in Uganda[30] monitored *P. falciparu*m resistance in 3 different local villages and found that the K13 mutations were closely associated with artemisinin resistance of *Plasmodium* in the target areas were not common and did not increase over time. Another study conducted in Equatorial Guinea (Ngonamanga and Miyobo)[31] sequenced the K13-propeller domain of *P. falciparum* in two different villages in 2005 and 2013, respectively, and found that the mutations in the K13 region in 2005 and 2013 were not consistent. It can be observed that the mutation of *PfK13* was inherently polymorphic. Many studies have confirmed that the artemisinin resistance of the malaria parasite could be directly related to the mutation of the K13 gene, thus regular detection of *PfK13* gene sequence mutations in malaria-endemic areas can potentially serve as an effective means to predict the occurrence of malaria parasite resistance, and adjust malaria treatment plans in a timely manner. Although there are no reports of large-scale transmission of resistant strains of *P. falciparum* in Asia and Africa recently, there are evidences to suggest that *Anopheles gambiae* can be used as the main vector for the transmission of the resistant strains of *Plasmodium* in Asia and Africa.[32] Therefore, monitoring the drug susceptibility of the malaria parasite strains and controlling the population movement in malaria-endemic areas could be two important means to prevent and control the spread of malaria-resistant strains on a global scale.

Mutations in *PfK13* can induce it to express a multi-drug resistance phenotype. The experiment of Paloque L at al.[33] showed that *PfK13* mutation can cause cross-resistance between artemisinin and endoperoxide-based antimalarials. The highly homologous variation on artemisinin- and piperaquine-resistant strain *P. berghei* K13 in this experiment also illustrated this point. Mbengue A et al[34] proved that in the resistant clinical strains, increased *PfPI3K* expression was related to the C580Y mutation of *P. falciparum* Kelch13. It is currently known that *PfK13* mutations can increase phosphatidylinositol 3-phosphate (PI3P) tubules/vesicles, and elevated PI3P can functionally induce the resistant parasite unfolded protein response (UPR) of *P. falciparum*, thereby neutralizing the *Plasmodium* protein affected by the toxicity of artemisinin and thereby reduce the sensitivity of resistant strains to artemisinin.[35] In addition, *PfK13* mutation can also induce *Plasmodium* to reduce the endocytosis of the hemoglobin,[27,36] thereby reducing the activation of artemisinin. It should be noted that in the previous studies on *Plasmodium* resistance genes, some other locus mutations have also been confirmed that can lead to resistance. Pholwat S et al[37] used TaqMan array cards to test the samples from 18 different laboratory parasite strains and 87 clinically resistant strains. It was found that except for the samples from Malawi, most of the clinical samples showed mutations in *pfcrt*. All the samples showed *pfdhfr* and *pfdhps* mutations. The number and types of *pfmdr1* and *PfK13* mutations varied based on the country of origin, and *pfmdr* means multi-drug resistance. The Nag S team[38] performed high-throughput sequencing using Illumina NGS (Miseq) platform on the samples of 457 patients from the Bandim and Belém Health Care Centers in Guinea-Bissau. The results showed that various polymorphisms can be found in *pfcrt*, *pfmdr1, pfdhfr, pfdhps*, and *PfK13. Plasmodium* resistance has been found to be often accompanied by multi-site mutations. Therefore, multi-resistant phenotype can also arise because of the synergistic effects of multi-site mutations.

The study may be useful to explore the drug resistance mechanism of *P. falciparum*, but which also has several limitations. Firstly, in vivo malaria models have their own constraints. We used mouse tail vein blood with high malaria infection rate to dilute and vaccinate the healthy mice. This method is different from the clinical transmission of malaria caused through the bite of female Anopheles mosquitoes. Therefore, mice malaria models cannot completely include all the distinct characteristics of human malaria, which might be one of the reasons why the variation of resistant strains found in this study was significantly different from the clinical ones. Secondly, the sample size for DNA sequencing in this study was limited. We have reported 9 different missense mutations in the resistant strains of *Plasmodium*, which need to be verified by further experiments.

In the future, we will cultivate *Pf*3D7 and *P. berghei* K173 strains in vitro, with successional drug-selection pressure forming AR strains, followed by sequencing. At the same time, gene editing technology will use to knock out specific mutation sites in the various susceptible strains, and thus it can be verified that whether this strategy can be effectively used to prolong the clearance time and early relapse of *Plasmodium*.

Conclusions

In response to the artemisinin challenge, the slow clearance phenotype of *P. falciparum* parasite populations can at least involve one non-synonymous change in the *P. falciparum*-specific single mutations in the propeller region of the *PfK13* gene. In order to further define the other genomic changes in the parasite populations that can support *PfK13* mutants to potentially overcome the fitness deficits, genomic studies needed to validate whether these different propeller mutations can compromise the parasite fitness.

Our study demonstrated that 6 missense mutations and 29 silent mutations of *P. berghei* K13 propeller mutants could be considered molecular markers associated with prolonged parasite clearance. With its complexity of the phenotype, mutations in the propeller region of the *PfK13* gene were served as valuable markers for the surveillance of artemisinin responsiveness diminishing in the parasite populations. At the same time, the *P. berghei* K13 fragment of artemisinin- and piperaquine-resistant strains showed almost similar mutations in the study. This finding is sufficient to indicate that *Plasmodium* drug-resistant strains have a multidrug resistance phenotype, thereby attributing *Plasmodium* resistance to either artemisinin or artemisinin combination. Meanwhile, these *P. berghei* K13 mutant parasites from artemisinin-resistance *P. berghei* rodent model of malaria also provide evidence of their important role in mediating artemisinin resistance in vivo, which further supports observations related to in vitro artemisinin resistance. These obtained findings should be considered as a preliminary study and might provide a new insight into the drug resistance mechanisms against ARTs.

Abbreviations

ACTs, Artemisinin-based combination therapies; AR, Artemisinin resistance; RSA, Ring stage survival assays; NIH, National Institutes of Health; pRBCs, parasitized red blood cells; ART, Artemisinin; PQ, Piperaquine; DMSO, Dimethyl sulfoxide; ST, Serial technique; ED_{90}, 90% effective dose; EDTA, Ethylenediamine Tetraacetic Acid; A30, Artemisinin-resistant strains of the 30th generation; A50, Artemisinin-resistant strains of the 50th generation; P30, Piperaquine-resistant strains of the 30th generation; P50, Piperaquine-resistant strains of the 50th generation; ROS, Reactive Oxygen Species; TCTP, Translationally controlled tumor protein; *Pf*PI3K, *Plasmodium falciparum* phosphatidylinositol-3-kinase; *Pf*ATP6, *Plasmodium falciparum* calcium ATPase 6; *PfK13, P. falciparum Kelch 13; PbK13, P. berghei Kelch 13*; UPR, Unfolded protein response; ANOVA, One-way analysis of variance.

Data Sharing Statement

Data in tables used to support the findings of this study are included within the article. The sequences have been deposited in Supplementary Figures 1 and 2.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically

reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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