Azmi *et al. Malaria Journal* (2024) 23:267
https://doi.org/10.1186/s12936-024-05071-1

Malaria Journal

RESEARCH

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Antimalarial drug sulfadoxine induces gametocytogenesis in *Plasmodium berghei*

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Abstract

Background The spread of antimalarial drug resistance parasites is a major obstacle in eliminating malaria in endemic areas. This increases the urgency for developing novel antimalarial drugs with improved profiles to eliminate both sensitive and resistant parasites in populations. The invention of the drug candidates needs a model for sensitive and resistant parasites on a laboratory scale.

Methods Repeated Incomplete Treatment (RIcT) method was followed in raising the rodent malaria parasite, *Plasmodium berghei*, resistant to sulfadoxine. *Plasmodium berghei* were exposed to an adequate therapeutic dose of sulfadoxine without finishing the treatment to let the parasite recover. Cycles of drug treatment and parasite recovery were repeated until phenotypic resistance appeared.

Results After undergoing 3–4 cycles, phenotypic resistance was not yet found in mice treated with sulfadoxine. Nevertheless, the molecular biology of *dhps* gene (the target of sulfadoxine) was analyzed at the end of the RIcT cycle. There was no mutations found in the gene target. Interestingly, the appearance of gametocytes at the end of every cycle of drug treatment and parasite recovery was observed. These gametocytes later on would no longer extend their life in the RBC stage, unless mosquitoes bite the infected host. This phenomenon is similar to the case in human malaria infections treated with sulfadoxine-pyrimethamine (SP).

Conclusions In this study, the antimalarial drug sulfadoxine induced gametocytogenesis in *P. berghei*, which could raise the risk factor for malaria transmission.

Keywords *Plasmodium berghei*, Sulfadoxine, Repeated incomplete treatment, Gametocyte, Resistant parasite

Background

The emergence of *Plasmodium* resistance to antimalarial drugs poses a tremendous hindrance to malaria control and treatment. This disease remains a major health issue that causes people's morbidity and mortality in malaria-endemic countries. Globally, there were 249 million cases of malaria in 2022 reported by the World Health Organization (WHO) [1].

Parasite resistance to sulfadoxine-pyrimethamine has emerged even before the drug was declared the first-line antimalarial drug [2]. The parasite resistance to other antimalarial drugs such as chloroquine, atovaquone, artemisinin, and mefloquine, was also emerging, disrupting malaria treatment control and prevention protocols.

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Thus, the use of artemisinin-based combinations was introduced by the WHO as the first-line antimalarial drug for malaria prevention and treatment.

The resistance of *Plasmodium falciparum* to sulfadoxine was harboured by mutations in dihydropteroate synthase (*dhps*) gene that encodes protein related to the folate biosynthesis pathway [3, 4]. Several mutations have been found related to parasite resistance to sulfadoxine, such as I431V, S436A/F, A437G, K540E/N, A581G, and A613S/T [5]. The A437G single-mutation in the *Pfdhps* gene is equivalent wih A394G mutation in *P. berghei*. The parasite resistance manifests as reduced susceptibility of the parasite to sulfadoxine.

Despite the spread of parasite resistance to the combination of sulfadoxine-pyrimethamine, the WHO still recommends it to prevent malaria as Intermittent Preventive Treatment (IPT) for pregnant women (IPTp) and for infants (below 5 years old) (IPTi), especially in sub-Saharan Africa, where malaria in pregnancies is at the highest risk [6–9]. Malaria infection in pregnant women represents a considerable risk for both the mother and fetus or infant. The infection can lead to severe malaria, fetal and maternal anaemia, cerebral malaria, abortion, low birth weight, and infant mortality [10, 11]. This risk can be reduced by preventing malaria during pregnancy using IPT [12, 13]. The combination of sulfadoxine-pyrimethamine is also used as a partner drug in some artemisinin-based combinations that are widely prescribed in the endemic regions in the Middle East and India [1].

To deal with the spread of *Plasmodium* resistance to sulfadoxine, novel antimalarial drug discovery and development is crucial. A malaria model which is sensitive and resistant to sulfadoxine could be used in laboratory experiments to mimic the field conditions in a natural population. *Plasmodium berghei* is a rodent malaria model which is commonly used in laboratory settings. The present study aimed to select *P. berghei* that is resistant to sulfadoxine within the host body (in vivo) by using a Repeated Incomplete Treatment (RIcT) method which later on could be used as a model for development of novel anti-malarial drug.

Methods

Inoculation of mice with *P. berghei*

Naïve BALB/c mice were obtained and maintained in the pathogen-free animal house facility of the Eijkman Research Center for Molecular Biology, National Research and Innovation Agency. *Plasmodium berghei* strain Leiden was obtained from the parasite collection of the Eijkman Research Center for Molecular Biology, National Research and Innovation Agency. The parasites were maintained by serial blood passages in 8- to 12-weeks-old BALB/c mice from the stock. Parasites (10^6 infected red blood cells) were inoculated into 8- to 12-week-old naïve BALB/c mice through intraperitoneal injection. In this study, 10 mice were used for the experiments. The parasitaemia level was monitored daily by preparing peripheral blood smears from tail vein bleeds. The thin blood smears were fixed in absolute methanol and then stained with 10% Giemsa. The parasitaemia level was determined under 100 × light microscopy for at least 2500 red blood cells.

Preparation of drug solution

Sulfadoxine was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Sulfadoxine was dissolved in dimethyl sulfoxide (DMSO) and stored as stock solution at − 20 ℃ and diluted to the required concentration with phosphate-buffered saline (PBS) before being administrated.

In vivo selection of *Plasmodium berghei* resistant to sulfadoxine by repeated incomplete treatment (RIcT)

Selection of *P. berghei* resistant to sulfadoxine was carried out within the host (in vivo) by conducting repeated incomplete treatment (RIcT) procedures (adequate dose of drug, but incomplete treatment). The infected mice with 2–5% parasitaemia level were treated with 25 mg/kg body weight dose of sulfadoxine. The treatment was interrupted when the parasitaemia level dropped to ≤ 0.5% and allowed the parasite to recover. The next treatment was done when the parasitaemia level reached ≥ 2% with the same dose of sulfadoxine and interrupted again after the parasitaemia level dropped to ≤ 0.5%. Drug treatment and interruption were done alternately like a cycle until the drug did not affect the increase of parasitaemia level of the infected mice (which indicates phenotypic resistance of the parasite). Blood collection was carried out at the start of every new cycle after parasite recovery for further analysis (DNA extraction and cryopreservation). Approximately 100 µl of blood was collected from the peripheral blood of the experimental mice into 1.5 ml heparinized Eppendorf tube and stored at − 20 ℃.

DNA extraction and PCR

Approximately 50 µL of blood was collected at the start of every cycle in RIcT for DNA extraction. *P. berghei* DNA was isolated with saponin-chelex method. The blood was washed with phosphate-buffered saline (PBS) pH 7.2 and lysed with saponin (Sigma-Aldrich, St. Louis, MO, USA) with centrifugation. Minerals and other contaminants were bound with chelex-100 (Sigma-Aldrich, St. Louis, MO, USA) by incubating the samples in boiling water. After centrifugation, the isolated DNA was preserved at − 20 ℃ and used for PCR reaction.

Fragments of *dhps* gene were amplified by employing a specific primer pair: Pbdhpss-F 5′-CCGAATATTGCC GTACACAGAATG-3′ and Pbdhpss-R 5′-CAGCGC AAGTTTGTGCCAAAG-3′. The primers were designed specifically for targeting the *P. berghei dhps* gene in the area where mutations mostly occur in sulfadoxine-resistant parasites with a product size of 697 bp.

Sequencing of *dhps* gene

The PCR products were purified using a QIAquick® PCR purification kit. Sequencing of the purified DNA was done using ABI 3500XL and aligned using BioEdit program to find possible mutations in the *dhps* gene.

Results

Sulfadoxine RIcT on *P. berghei* infected mice

The results of cycles of incomplete treatment of *P. berghei*-infected mice with a therapeutic dose of sulfadoxine are represented by a mouse shown in Fig. 1.

In general, after undergoing 3–4 cycles of drug treatment and parasite recovery, the RIcT cycles could not be continued due to low parasitaemia level with the last parasitaemia level of 0.1% or dissapearance of parasites. The average days of treatment in cycle 1 was 3 days, while the average parasite recovery time in cycle 1 was 7 days. In cycle 2, the average days of treatment was 3 days and the average time for parasite recovery was 9 days (Table 1).

All the experiment mice underwent drug treatment in cycle 3 with an average time of 3 days. However, only 2 mice finished the parasite recovery time with an average of 13 days. The remaining mice could not finish the cycle due to low parasitaemia levels. The two mice that finished cycle 3 underwent drug treatment in cycle 4. After 2 days of treatment in cycle 4, the parasitaemia level decreased (Table 1). Following all the experiments above, none of the mice showed phenotypic resistance after 3–4 cycles of drug treatment and parasite recovery until 245 days of observation (leading to termination of RIcT cycle).

Table 1 Treatment and recovery cycle of RIcT in *P. berghei*

Mouse's code	Duration of treatment—recovery (Days)				Cycles*
	1	2	3	4	
PbLSDXr1	3 – 5	2 – 11	3 – ●	–	3
PbLSDXr2	3 – 6	3 – 11	4 – ●	–	3
PbLSDXr3	3 – 7	2 – 11	3 – ●	–	3
PbLSDXr4	3 – 8	2 – 9	3 – ●	–	3
PbLSDXr5	2 – 6	2 – 10	3 – ●	–	3
PbLSDXr6	4 – 6	3 – 10	3 – ●	–	3
PbLSDXr7	3 – 7	2 – 5	3 – 12	2 – ●	4
PbLSDXr8	3 – 6	2 – 7	2 – ●	–	3
PbLSDXr9	3 – 6	2 – 8	2 – 14	2 – ●	4
PbLSDXr10	3 – 6	2 – 8	3 – ●	–	3

* One cycle consists of days of treatment (to bring parasitaemia level down to below 0.5%)

– days of recovery/treatment interruption (to bring parasitaemia level up to more than 2%)

● = termination of treatment cycles due to low parasitaemia level

RIcT of sulfadoxine-treated parasites in new mice

Four *P. berghei* isolates from mice PbLSDXr4, PbLSDXr5, PbLSDXr6, and PbLSDXr10 were transferred into each one of naïve BALB/c mice to observe whether the

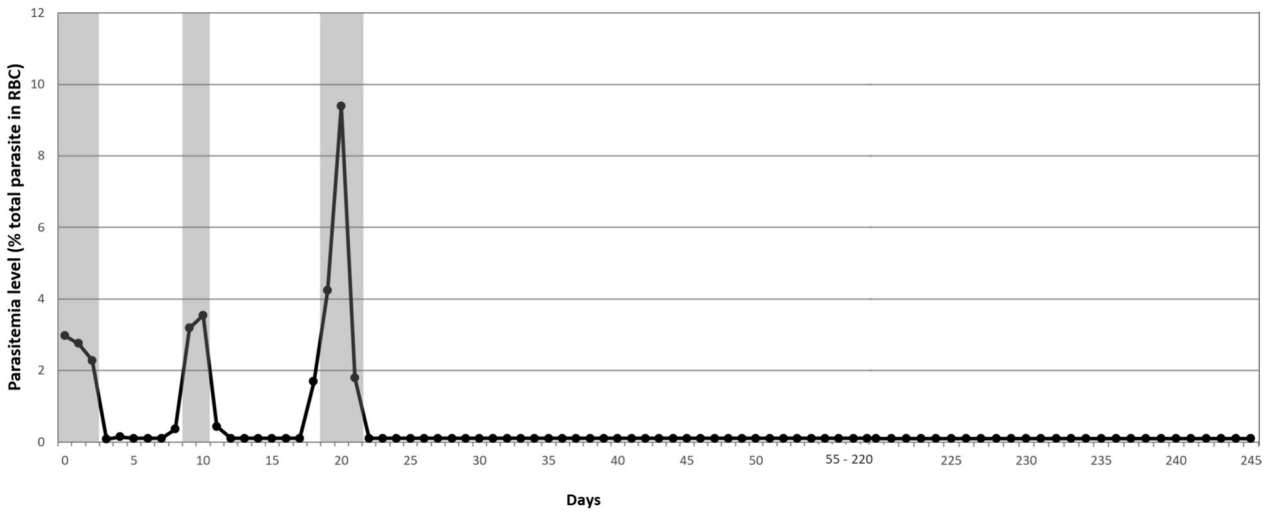

Fig. 1 Model of cycles of sulfadoxine RIcT (PbLSDXr10) of *P. berghei* in a mouse. Shaded areas indicated the treatment periods. The whole mice model are represented in Table 1 which summarize the duration of treatment and recovery times observed during the treatment of *P. berghei*-infected mouse with sulfadoxine

immune system of the parasitized mouse is responsible for the presence of parasites. These mice underwent the same RIcT regimen as the previous group. The results of the RIcT system in this group are described in Fig. 2.

The average time needed for drug treatment in cycle 1 was 2 days with an average recovery time of 7 days. On the other hand, the average time of drug treatment in cycle 2 was 3 days with an average recovery time of 15 days. PbLSDXr4.1 and PbLSDXr10.3 finished cycle 3 with an average drug treatment time spent 2 days followed by 59 days of parasite recovery. After 2 days of drug treatment in cycle 4, the parasitaemia level did not increase and the RIcT regimen was interrupted (Table 2). None of the four sub-clone mice in this regimen showed phenotypic resistance after 3–4 cycles of drug treatment and parasite recovery.

Table 2 Treatment and recovery cycle of RIcT in sulfadoxine-treated *P. berghei* (following the previous cycle set of RIcT)

Mouse's codes	Duration of treatment—recovery (Days)				Cycles*
	1	2	3	4	
PbLSDXr4.1	2 – 7	2 – 18	2 – 65	2 – ●	4
PbLSDXr5.3	1 – ●	–	–	–	1
PbLSDXr6.2	2 – 6	4 – ●	–	–	2
PbLSDXr10.3	2 – 7	2 – 12	1 – 53	3 – ●	4

* One cycle consists of days of treatment (to bring parasitaemia level down to below 0.5%)

– days of recovery/treatment interruption (to bring parasitaemia level up to more than 2%)

● = termination of treatment cycles due to low parasitaemia level

Molecular analysis of *dhps* gene

The resulted sequences of *dhps* gene were aligned with wild-type *P. berghei* sequence from NCBI (NC_036172.2:c1032585-1030129 *Plasmodium berghei* ANKA genome assembly, chromosome: 14) and wild-type *P. berghei* sequence from this study (Fig. 3). The sequence length was 697 bp and there was no mutations found as indicated by the sequence alignment results.

Gametocyte production rates

During recovery of the parasites on the last day of all cycles of RIcT regimen, low parasitaemia levels were accompanied by the appearance of gametocytes in all of the experiment mice. The gametocyte production rates were counted at all cycles of the RIcT Regimen. The results showed that in 7 experimental mice, the average increase of gametocyte production was in line with the parasitaemia level during drug treatment from Day 1 until Day 3 in the last cycle. The percentage increased from Day 1 to Day 2 and decreased on Day 3. On Day 4, when the drug treatment was interrupted, the gametocytes still remain (Table 3). Similar results were shown by the other 3 mice (PbLSDXr7, PbLSDXr8, PbLSDXr9), the gametocytes still remains on Day 1 and Day 2 of drug treatment. The rate of gametocytes still present on Day 3, when drug treatment was interrupted. The gametocytaemia was compared in the sulfadoxine-treated mouse and untreated mouse (Table 3).

Figure 4 shows the appearance of male and female gametocytes at the end of the cycle of the RIcT regimen when the drug treatment was released.

Discussion

In this study, the RIcT experiment was carried out to raise a new rodent malaria mutant parasite model, *P. berghei*, which is resistant to sulfadoxine. The RIcT regimens were undertaken by exposing *P. berghei* to

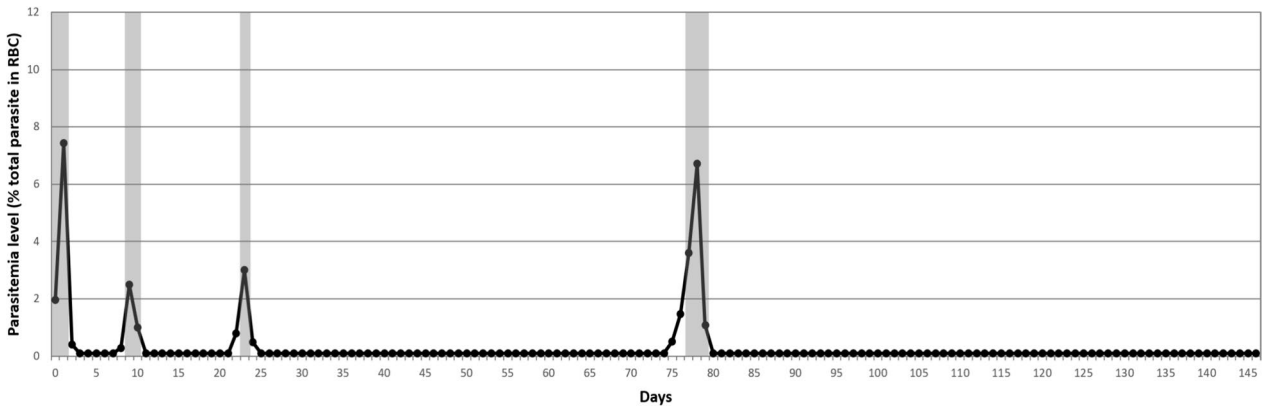

Fig. 2 Model of cycles of sulfadoxine RIcT (PbLSDXr10.3) of *P. berghei* in a mouse. Shaded areas indicated the treatment periods. The whole mice model are represented in Table 2 which summarize the duration of treatment and recovery times observed during the treatment of *P. berghei*-infected mouse with sulfadoxine

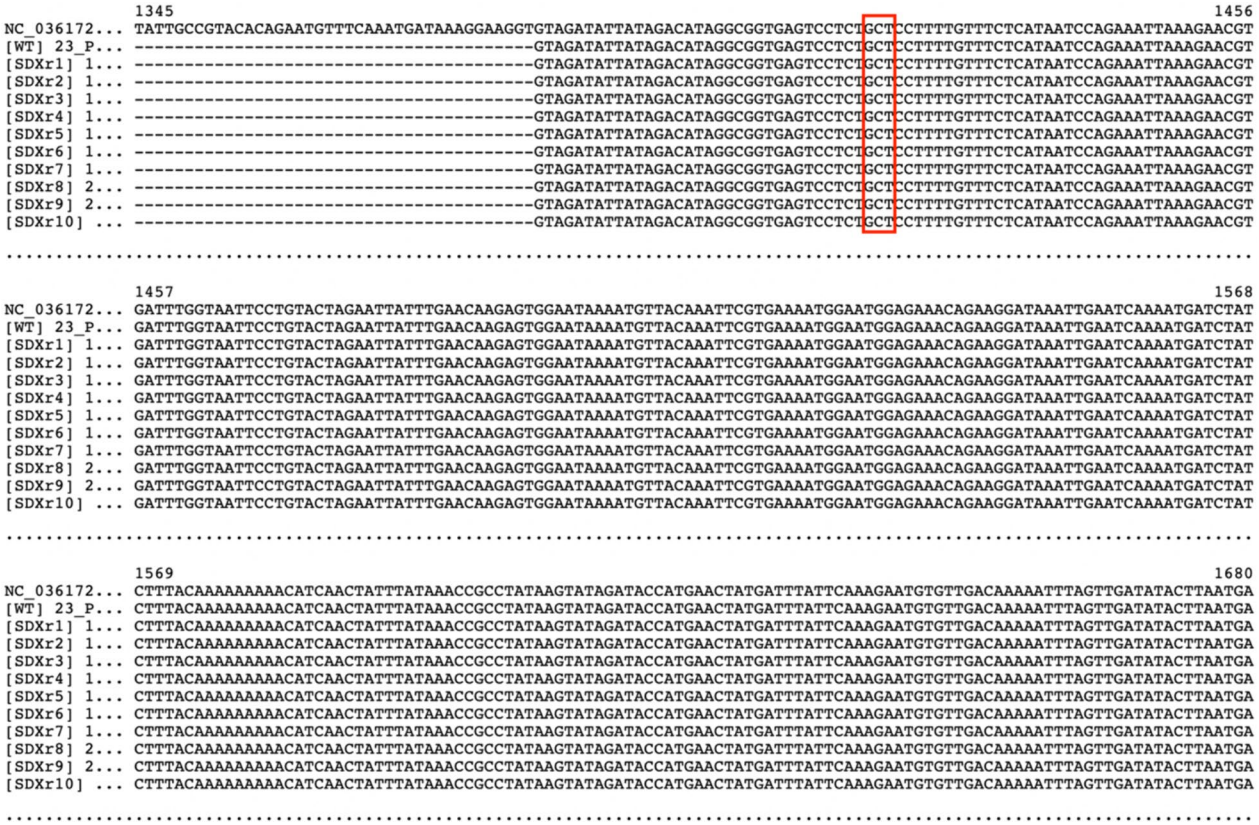

Fig. 3 Alignment of *P. berghei dhps* gene sequences from NCBI, *P. berghei* wild-type, and 10 isolates of *P. berghei* from RIcT experiment in this study

an adequate therapeutic dosage of sulfadoxine (25 mg/kg BW) without finishing the treatment to let parasites recover. This method mimics repeated treatment failures that happened naturally in populations. Out of 10 mice used in this experiment, 8 underwent 3 drug treatment and parasite recovery cycles, while the other 2 underwent 4 cycles. However, after experiencing 3 to 4 cycles of repeated drug treatment and parasite recovery, phenotypic resistance to sulfadoxine was not yet observed. Moreover, the parasitaemia level did not increase in the parasite recovery step after 245 days of observation leading to termination of RIcT cycles. The different lengths of cycles might be due to the role of mice immunity to the parasites since the selection was done within the host body [14, 15]. Thus, in this repeated sulfadoxine treatment and parasite recovery regimen, mice's immune systems potentially affect the number of cycles and periods of the drug treatment and parasite recovery.

Four isolates from the experiment mice group were passed into each naïve BALB/c mouse after the parasitaemia level was < 0.5% at the end of cycle 3, to continue the regimen in a new host, reducing the chance of mice immunity effect. However, in this regimen, there was also no significant difference regarding the time they spent during drug treatment. Meanwhile, the parasite recovery time increased within the cycles and took a longer recovery period in cycle 3 with an average days spent 59 days compared to the previous cycle (15 days). The RIcT regimen in the second mice group was also terminated after 3 to 4 cycles due to low parasitaemia levels. Phenotypic resistance was also not observed before terminating the regimen. To confirm the phenotypic observation, a molecular biology analysis of *dhps* gene was carried out, the target of sulfadoxine, from the parasites collected at the end of cycle 3 and 4 of the first group of experiment mice. Still, the results did not show any mutations, meaning resistant parasites were not yet raised in this experiment.

In a previous study, Syafuddin et al. [16] raised atovaquone-resistant *P. berghei* by exposing the parasites to increased sub-therapeutic doses of atovaquone every day for 7 days. Though the resistant parasite model was obtained after 5 months of experiment with an in vivo Serial Technique method, this method is costly and requires more mice than RIcT which is more effective and reproducible [14]. In another experiment, Siregar et al. [17] raised *P. berghei* with stable resistance to atovaquone by interrupted treatment with a therapeutic

Table 3 Comparison of % gametocyte in total RBC and % asexual stage in total RBC at the last cycle model of RIcT

Isolates	Day 1 %gametocyte [%asexual stage]	Day 2 %gametocyte [%asexual stage]	Day 3 %gametocyte [%asexual stage]	Day 4 %gametocyte [%asexual stage]
PbLSDXr1	0.18% [**8.01%**]	1.24% [**9.38%**]	0.69% [**1.50%**]	0.13% [**0.04%**]
PbLSDXr2	0.56% [**7.06 %**]	1.27% [**5.95%**]	0.50% [**2.67%**]	0.26% [**0.13%**]
PbLSDXr3	0.32% [**5.32%**]	0.69% [**4.43%**]	0.21% [**1.11%**]	0.09% [**0.09%**]
PbLSDXr4	0.12% [**4.47%**]	0.44% [**3.52%**]	0.04% [**0.79%**]	0.14% [**0.24%**]
PbLSDXr5	0.40% [**2.85%**]	0.45% [**4.50%**]	0.05% [**0.54%**]	0.36% [**0.04%**]
PbLSDXr6	0.27% [**2.38%**]	0.27% [**3.63%**]	0.55% [**2.01%**]	0.18% [**0.13%**]
PbLSDXr7	0.57% [**2.51%**]	0.20% [**1.64%**]	0.09% [**0.04%**]	-
PbLSDXr8	0.11% [**2.25%**]	0.84% [**2.37%**]	0.61% [**0.30%**]	-
PbLSDXr9	0.11% [**1.25%**]	0.04% [**0.63%**]	0.09% [**0.04%**]	-
PbLSDXr10	0.35% [**4.23%**]	0.74% [**9.39%**]	0.28% [**1.79%**]	0.14% [**0.04%**]
PbL untreated	0.00% [**1.07%**]	0.00% [**3.30%**]	0.00% [**5.70%**]	0.00% [**6.31%**]

* Shaded column: Drug treatment; non-shaded column: Drug release; unbold percentage: %gametocytes; bold percentage: %asexual stage

dosage of atovaquone to the infected mice (RIcT). Phenotypic resistance of the parasites against atovaquone was observed after 2.5 cycles of drug treatment and parasite recovery.

Additionally, Nuralitha et al. [14] used the RIcT method to select *P. berghei* with stable resistance to pyrimethamine. Phenotypic resistance of the parasites against pyrimethamine was observed after 5 cycles of repeated incomplete treatment which requires 37 days of experiment. The number of drug treatment and parasite recovery cycles differs from atovaquone since the two drugs have different profiles in their pharmacokinetics and pharmacodynamics. Though atovaquone's half-life is 5.9 days, which is longer than pyrimethamine (3.5 days), its mechanism as antimalarial targets at different sites [18, 19]. The parasite's resistance to antimalarial drugs is usually identified by mutations in the gene that encodes a protein targeted by the drug. Mutation in the *cytb* gene is associated with the parasite's resistance to atovaquone, while mutation in *dhfr* gene is related to parasite's resistance to pyrimethamine. This is in line with the number of RIcT cycles needed to raise resistant *P. berghei* against atovaquone and pyrimethamine since the mutation in mitochondrial DNA is faster than mutation in nuclear DNA [14, 17]. Sulfadoxine targets dihydropteroate synthase, an enzyme needed in the *Plasmodium* folic acid pathway. This is aligned with the long cycles of pyrimethamine treatment and could be the reason why raising *P. berghei* resistant to sulfadoxine through RIcT is less successful.

Compared to pyrimethamine RIcT which needed 5 cycles until phenotypic resistance was observed, the sulfadoxine RIcT in this experiment did not show any increase in parasitaemia level after undergoing 3 to 4 cycles. The long parasite recovery period, especially in the last cycle, was in line with the half-life of sulfadoxine which stays up to 9 weeks in the plasma [20–22]. Thus, the cut-off for stopping the sulfadoxine treatment

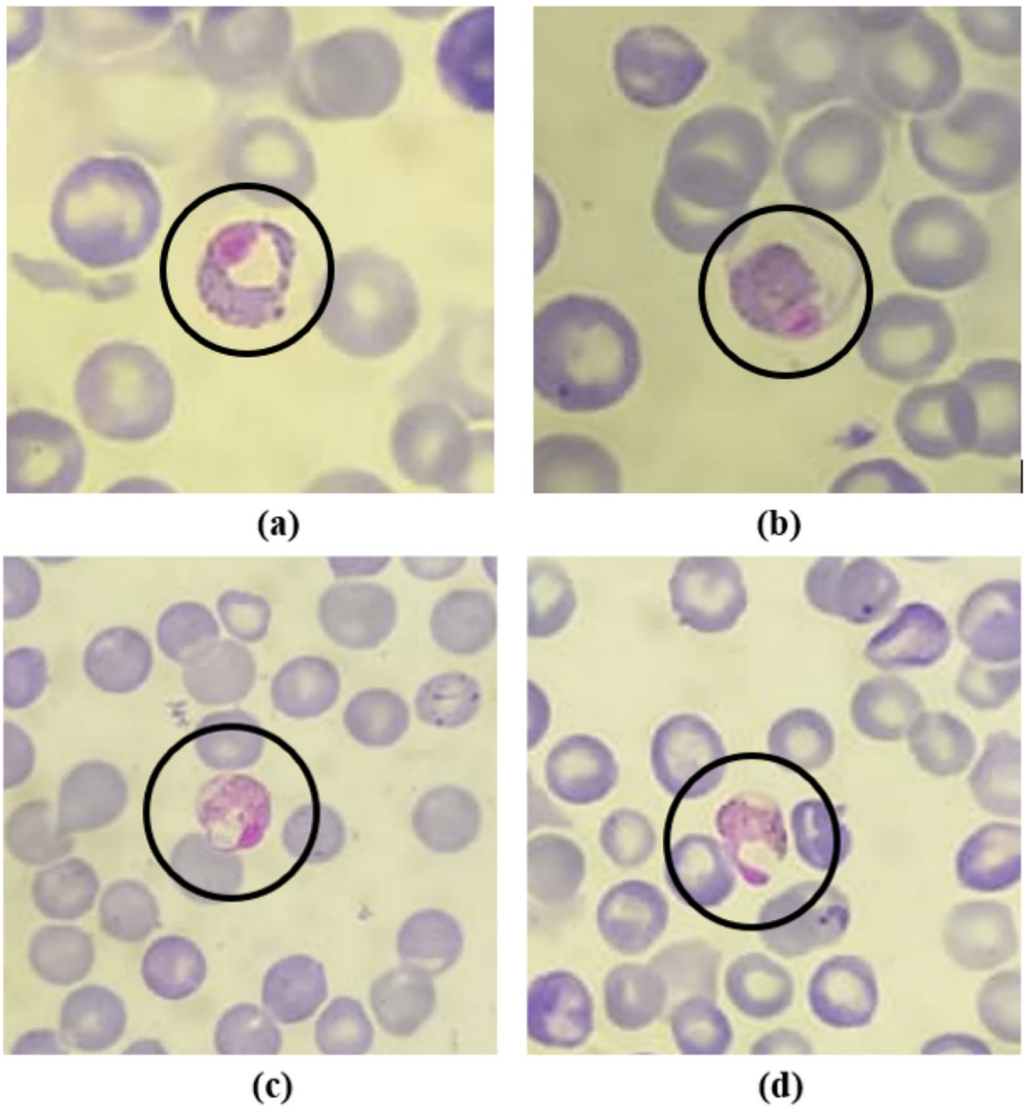

Fig. 4 Gametocyte stages on the first day of drug release of RIcT cycle 3 and 4. **a** female gametocyte in PbLSDXr6 cycle 4; **b** female gametocyte in PbLSDXr6 cycle 4; **c** male gametocyte in PbLSDXr1 cycle 4; **d** male gametocyte in PbLSDXr8 cycle 3

at < 0.5% parasitaemia levels might be too low since sulfadoxine is still effective in killing the parasite up to 9 weeks during parasite recovery phase. Thus, this can lead to the loss of parasites before reaching the phenotypic resistance.

On the other hand, there was an interesting finding in sulfadoxine RIcT, in which gametocytaemia was observed at the last cycle. In *Plasmodium* spp. life cycle, a small percentage of merozoites differentiates into gametocytes [23, 24. Gametogenesis in *P. berghei* is followed by an abundant increase of stress and folding proteins [25]. Thus, the increased level of gametogenesis of *P. berghei,* which leads to gametocytaemia, is in line with the effect of sulfadoxine exposure to the parasite. However, since sulfadoxine is not gametocidal, the drug only kills the asexual forms of the parasites in peripheral blood and increases gametocytaemia [26].

In RIcT atovaquone undertaken by Siregar et al. [27], the selected stable resistant parasites were transmitted to mosquitoes to observe the parasite development in the mosquito gut. The study then showed that despite the resistance mutations rescuing the parasite from atovaquone, it turned out that it was lethal to the parasite's development in the mosquito gut [27]. In the RIcT sulfadoxine case, the number of gametocyte was abundant compared to the asexual stage. However, the maturation of gametocyte takes place in the midgut of mosquitoes, therefore the gametocyte stage

will eventually die during the erythrocytic cycle. This phenomenon is quite intriguing for further research in understanding *Plasmodium* resistance to sulfadoxine.

Yamauchi et al. [28] have experimented with raising *P. berghei* resistant to sulfadoxine (PbDHPS-A394G) through genetic modification (in vitro). However, in this study, there was no significant difference in parasite growth and gametocyte production between PbDHPS-A394G and wild-type clones. A similarity in the increase of parasitaemia level between sulfadoxine-resistant parasite and wild-type parasite indicated that parasites resistance to sulfadoxine did not affect its growth in mice. Moreover, the development of sulfadoxine-resistant parasites into oocysts inside mosquito gut was not significantly different from the susceptible parasites [28]. Unlike the issue in atovaquone-resistant parasites, where the development of the oocyst is defective, the unaffected fitness of sulfadoxine-resistant parasites could explain the persistence of this resistant parasite in natural populations.

The findings of gametocytaemia were also discovered in human malaria cases treated with sulfadoxine-pyrimethamine (SP). Even though parasites were cleared and there was a beneficial effect on the patients, gametocytaemia was observed in pregnant women treated with SP continuously in IPTp [26, 29]. The production of gametocytes during *Plasmodium* infection is initially very low and continuous parasite culture may induce reduction of gametocytogenesis or even its loss [30]. Thus, the increased rate of gametocyte production in parasite infection is not typical. The high density of gametocytes will raise the possibility of parasite transmission into mosquitoes. It will be more dangerous if the gametocyte already has mutations or resistance before transmission. Thus, finding the sulfadoxine-resistant parasites model will enhance the development of novel antimalarial drugs with different targets to kill sensitive and resistant parasites.

Conclusions

Sulfadoxine RIcT method in the present study failed to raise parasite resistant to sulfadoxine. After undergoing 3 to 4 cycles of drug treatment and parasite recovery, there is no parasitaemia increase observed. Molecular analysis in *dhps* gene that induces *Plasmodium* resistance to sulfadoxine, showed no mutations occurred. Interestingly, after continuous exposure to the incomplete treatment of sulfadoxine, gametocytaemia levels rose after discontinuation of the drug in the last cycle of RIcT. The high level of gametocytes in the erythrocytic cycle could explain why the transmission of sulfadoxine-resistant parasites is massive, since there is no fitness effect of a mutation to the resistant parasites.

Abbreviations

RIcT	Repeated incomplete treatment
IPT	Intermittent preventive treatment
IPTp	Intermittent preventive treatment for pregnant women
IPTi	Intermittent preventive treatment for infants
dhps	Dihydropteroate synthase
DMSO	Dimethyl-sulfoxide
PBS	Phosphate-buffered saline
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-024-05071-1.

Additional file 1.

Acknowledgements

We thank John Acton for his assistance at the manuscript stage.

Author contributions

WAA and JES concept and design the experiments. WAA and AFM performed the experiments and collected the data. AS participated in the experiment. WAA and JES performed data analysis, data interpretation and wrote the manuscript. WAA wrote the original draft and drawing figures. WAA, AFM, AS, YD, IMA, and JES reviewed and revised the manuscript. All authors read and approved the final manuscript.

Funding

This work was carried out under the auspices of the Health Research Organization, National Research and Innovation Agency 2023.

Availability of data and materials

All data and material of the experiments are located at Cibinong Science Center, National Research and Innovation Agency, Indonesia.

Declarations

Ethics approval and consent to participate

Ethical clearance approval from the Animal Care and Use Ethics Committee National Research and Innovation Agency Ref No.:033/KE.02/SK/02/2023.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 3 June 2024 Accepted: 7 August 2024
Published online: 02 September 2024

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