

# In Vivo Curative and Protective Potential of Orally Administered 5-Aminolevulinic Acid plus Ferrous Ion against Malaria

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**5-Aminolevulinic acid (ALA) is a naturally occurring amino acid present in diverse organisms and a precursor of heme biosynthesis. ALA is commercially available as a component of cosmetics, dietary supplements, and pharmaceuticals for cancer diagnosis and therapy. Recent reports demonstrated that the combination of ALA and ferrous ion (Fe<sup>2+</sup>) inhibits the *in vitro* growth of the human malaria parasite *Plasmodium falciparum*. To further explore the potential application of ALA and ferrous ion as a combined antimalarial drug for treatment of human malaria, we conducted an *in vivo* efficacy evaluation. Female C57BL/6J mice were infected with the lethal strain of rodent malaria parasite *Plasmodium yoelii* 17XL and orally administered ALA plus sodium ferrous citrate (ALA/SFC) as a once-daily treatment. Parasitemia was monitored in the infected mice, and elimination of the parasites was confirmed using diagnostic PCR. Treatment of *P. yoelii* 17XL-infected mice with ALA/SFC provided curative efficacy in 60% of the mice treated with ALA/SFC at 600/300 mg/kg of body weight; no mice survived when treated with vehicle alone. Interestingly, the cured mice were protected from homologous rechallenge, even when reinfection was attempted more than 230 days after the initial recovery, indicating long-lasting resistance to reinfection with the same parasite. Moreover, parasite-specific antibodies against reported vaccine candidate antigens were found and persisted in the sera of the cured mice. These findings provide clear evidence that ALA/SFC is effective in an experimental animal model of malaria and may facilitate the development of a new class of antimalarial drug.**

Malaria is a devastating disease, affecting about 200 million and killing about 600,000 people annually, mostly children under 5 years of age in sub-Saharan Africa (1). The causative agents of malaria are protozoan parasites belonging to the *Plasmodium* genus; the most lethal species for humans is *Plasmodium falciparum*. Female *Anopheles* mosquitoes transmit these parasite species. The transmitted parasites invade the hepatocytes and mature into merozoites, which are released to infect red blood cells (RBCs) (2). In the RBCs, the parasites differentiate into the following stages: ring, trophozoite, and schizont. After that, the infected RBCs (iRBCs) burst and release merozoites, which invade uninfected RBCs.

So far, there is no effective vaccine against malaria, due to the parasites' complex life cycle and polymorphisms in their key antigens (3). Thus, it is pertinent to rely on chemotherapy for controlling malaria. However, resistance to currently available antimalarial drugs is widely reported, even to the most effective treatments, artemisinin-based combination therapies (ACTs) (4, 5). To prevent and control the disease, new antimalarial drugs with different therapeutic and structural characteristics are urgently required, including agents for malaria prophylaxis (6, 7), along with clear understanding of the mechanism of resistance to existing drugs (8, 9).

5-Aminolevulinic acid (ALA), which is ubiquitously found in plants, bacteria, fungi, and animals, is a precursor for the biosynthesis of tetrapyrroles such as chlorophyll, vitamin B<sub>12</sub>, and heme (10). In cancer cells, uptake of large amounts of ALA results in mitochondrial accumulation of protoporphyrin IX (PpIX), an intermediate of the heme biosynthesis pathway. PpIX also serves as a photosensitizer, a molecule that generates reactive oxygen spe-

cies (ROS) upon exposure to light, leading to death of cancer cells (11–13). As a result, ALA has been widely applied in medical fields: e.g., photodynamic diagnosis (PDD) and therapy (PDT) of various cancers (14–17). In addition, the combination of ALA and sodium ferrous citrate (ALA/SFC) has been shown to reduce the risk of type II diabetes development in a large-scale clinical trial of prediabetic volunteers (18, 19) and has been approved as a dietary supplement and a cosmetic in Middle Eastern and Asian countries.

Malaria parasites express heme biosynthesis enzymes in three compartments—mitochondrion, cytosol, and apicoplast, a plant-like but nonphotosynthetic plastid (see Fig. S1 in the supplemental material) (20–23)—and localization of the enzymes is different from the corresponding activities in mammalian cells (24, 25). The heme biosynthesis pathway is essential for the survival of ma-

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alaria parasites and therefore recognized as a potential drug target in *Plasmodium* (26, 27). Smith and Kain applied ALA-PDT to malaria parasites and demonstrated complete inhibition of the *in vitro* (blood culture) growth of *P. falciparum* by 0.2 mM ALA in combination with exposure to white light (28). This treatment is, however, not applicable for clinical treatment of malaria patients because the PDT application was developed for elimination of malaria parasites from blood prior to transfusion. After extensive screening of the protocols for a clinical use of ALA, we recently found that ALA efficiently inhibits the growth of *P. falciparum* in the presence of ferrous ion (Fe<sup>2+</sup>) without light irradiation, suggesting ALA as a potential antimalarial treatment in humans (23). Furthermore, an ALA/SFC dietary supplement was found to be beneficial for human malaria cases in the Solomon Islands: oral ALA phosphate and SFC ingestion (50 and 57.36 mg/day, respectively) decreased fever and improved typical symptoms in malaria patients, including children, in a day and led to fast recovery (29). These findings suggest that ALA/SFC could be a promising drug combination for malaria chemotherapy. It is thus necessary to demonstrate the efficacy of ALA/SFC in treating malaria in an animal model as a next step to the design of a large-scale clinical trial in human malaria patients. Here, we performed a preclinical drug evaluation of orally administered ALA/SFC for the treatment of mice infected with the lethal rodent malaria parasite *Plasmodium yoelii* 17XL (Py17XL). Furthermore, the cured mice were tested for resistance to reinfection to determine the immunological effect on *Plasmodium*-infected mice treated with ALA/SFC.

## MATERIALS AND METHODS

**Animals.** Female inbred C57BL/6J mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). Mice were obtained as 4- to 5-week-old animals (17 ± 1 g each, mean ± standard deviation [SD]) for passage and primary infection and as 17-week-old animals (23 ± 2 g each) for controls in the reinfection study. Mice were maintained in the animal facilities at the Faculty of Medicine, The University of Tokyo, where the animals were provided with *ad libitum* access to a standard commercial pellet diet, CE-2 (Clea Japan, Inc., Tokyo, Japan), and tap water. All animal experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Graduate School of Medicine, The University of Tokyo, and were performed in accordance with the *Guidelines for the Care and Use of Laboratory Animals* of the Faculty of Medicine, The University of Tokyo.

**Drugs.** 5-Aminolevulinic acid hydrochloride (ALA-HCl) was obtained from Cosmo Oil Co., Ltd. (Tokyo, Japan). SFC, which is used as a food additive in Japan, was a kind gift from the Komatsuya Corp. (Osaka, Japan). Sterile 0.5% (wt/vol) methyl cellulose (MC) and concentrated hydrochloric acid (HCl) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ALA-HCl was dissolved in 0.5% (wt/vol) MC. This ALA solution was used for dosing the ALA-only group and for combination administration: SFC was formulated with the ALA solution. For administration of SFC only, SFC was dissolved in 0.5% (wt/vol) MC containing 0.3 M HCl (final concentration). All drugs were freshly prepared on the day of use.

**Parasite and infection.** Lethal strains of rodent malaria parasites Py17XL and *Plasmodium berghei* NK65 (PbNK65) and the human malaria parasite *P. falciparum* 3D7 (Pf3D7) maintained in our laboratory were used. Infections of mice were performed as described previously with minor modification (30). Briefly, the stocks of the rodent malaria parasites were stored at -80°C. Freshly thawed parasites were passaged twice through naive C57BL/6J mice to confirm infectivity, and 1 × 10<sup>4</sup> iRBCs were injected intraperitoneally into each mouse used in this study. The human malaria parasite culture was performed as previously described (23). The human malaria parasite experiment was approved by the Re-

TABLE 1 Effect of treatments with ALA, SFC, or both on survival rate in mice infected with *Plasmodium yoelii* 17XL

Group	Dose (mg/kg) of:		Survival rate <sup>b</sup> (no. living/infected <sup>c</sup> )
	ALA-HCl	SFC	
A <sup>a</sup>			0/10 <sup>d</sup>
B		300	0/5
C	600		2/5
D	100	300	0/5
E	300	300	5/10 <sup>d</sup>
F	600	300	3/5

<sup>a</sup> MC was administered at 0.5% (wt/vol) as a vehicle.

<sup>b</sup> Survival rates were determined on day 30 postinfection.

<sup>c</sup> All mice used were infected.

<sup>d</sup> Data were combined from two independent experiments.

search Ethics Committee of the Graduate School of Medicine, The University of Tokyo. Parasitemia, defined as the infection ratio of malaria parasite, was calculated by counting 500 to 10,000 RBCs in thin blood films that were prepared on glass slides, fixed with methanol, and stained with Giemsa solution.

**Survival analysis.** The overall schedule of this study is shown in Fig. S4 in the supplemental material. Prewedged mice were randomly divided into six groups (A to F) of five mice per group. Treatments were started from 1 day postinfection (dpi) by once-daily oral administration of the various drug combinations. Treatment was continued until the infected mice were cured, for about 5 weeks (the determination of mouse cure is described below). Group A (the challenge control) was administered an equivalent volume of the sterile vehicle, 0.5% (wt/vol) MC. Groups B and C were administered 300 mg/kg SFC only or 600 mg/kg ALA-HCl only, respectively. Groups D, E, and F were administered the ALA-HCl/SFC combination at doses of 100/300, 300/300 and 600/300 mg/kg, respectively (Table 1; see Fig. S4B). Two independent experiments were conducted for groups A and E (*n* = 10).

Note that our mouse chow contained 30 mg of iron per 100 g of feed (Clea Japan; [http://www.clea-japan.com/en/diets/diet\\_a/a\\_03.html](http://www.clea-japan.com/en/diets/diet_a/a_03.html)); a C57BL/6J mouse was expected to consume feed at about 3 g/day (data provided by the feed supplier, Clea Japan, Inc.). Thus, a mouse in our study was expected to ingest about 1 mg of total iron per day from the diet, equivalent to a total iron dose of about 60 mg/kg body weight.

Following the initiation of dosing, parasitemia was monitored every 2 to 4 days for the first month and then once per week thereafter. Survival rates were evaluated by counting the number of living mice every day. In addition, the body temperature and weight of each mouse were measured before infection and once per week after infection. Surviving mice were considered cured if the parasite DNA was not detected by PCR. The follow-up duration of this study was decided as follows. When few parasites (e.g., 3 parasites per mouse) are intravenously injected to mice, the parasitemia is, typically, between 0.05 and 0.1% in 30 to 50% of the mice at 8 dpi (31). Therefore, we considered that there was no relapse if the result of diagnostic PCR had been negative 1 week after the day at which the mice had been cured. We defined this as the endpoint of primary infection.

To investigate reinfection of the mice cured by ALA/SFC, the 10 mice were rechallenged with the same parasite strain at 6 and 33 weeks postrecovery from the primary infection, when the mice were 17 weeks old (*n* = 4) and 44 weeks old (*n* = 6), respectively. As a challenge control group for this experiment, five 17-week-old naive mice were infected in parallel. Reinfected (and control) mice were assessed by diagnostic PCR, quantification of parasitemia, and survival rate over the course of the month following reinfection.

**Diagnostic PCR.** Heparinized blood was collected from snipped tails of infected or cured mice. Total DNA was extracted from the blood using the DNeasy blood and tissue kit (Qiagen K.K. Japan, Tokyo, Japan). A hot-start PCR was carried out to amplify a 426-bp segment of the Py17XL

mitochondrial cytochrome *b* gene (*cob*). The nucleotide sequences of the primer pair were 5'-CTA ATC CTT TAG GGT ATG ATA CAG CA-3' and 5'-CTT GTG GTA ATT GAC ATC CTA TCC A-3'. After the initial denaturation at 95°C for 2 min, the reaction mixture was subjected to 30 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 15 s, followed by a final extension at 72°C for 3 min. The PCR products were electrophoresed on 1.2% agarose gels. DNA isolated from mice infected with Py17XL (without subsequent treatment) was used as the positive control in all of the PCR amplifications.

**Western blot.** Infected blood was collected from Py17XL- and PbNK65-infected mice by retro-orbital puncture or tail vein sampling; *P. falciparum* iRBCs were obtained directly from Pf3D7 blood culture. The parasite lysates were prepared as described previously with minor modification (30) and served as parasite antigens. Three micrograms of each lysate was electrophoresed on SuperSep Ace (5 to 20% gradient, Wako Pure Chemical Industries, Ltd.) and transferred onto Immobilon-P PVDF membranes (Merck Millipore Corp., Tokyo, Japan) following the manufacturer's protocol.

Blood samples were collected from normal mice and infected mice 30 days after primary infection and from 44-week-old cured mice 1 day before and 5 and 14 days after reinfection. Sera were prepared from the collected blood as described in the manufacturer's protocol with minor modification. Serum from individual animals was pooled and was used as the primary antibody in Western blotting for detection of parasite antigens on the membranes.

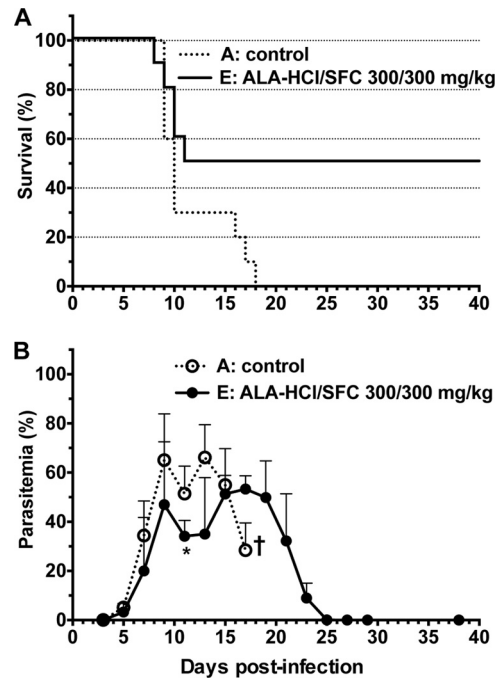
Bound primary antibody was detected using horseradish peroxidase (HRP)-conjugated anti-mouse IgG (GE Healthcare Japan Corp., Tokyo, Japan) as the secondary antibody, in combination with the ECL (enhanced chemiluminescence) Prime Western blotting detection system (GE Healthcare Japan Corp.), which was used in accordance with the manufacturer's protocol. Developed membranes were visualized using a LAS-4000 mini (GE Healthcare Japan Corp.).

**Statistical analysis.** Parasitemia was expressed as the mean + SD, and values were compared using the Student *t* test. The survival curves of mice were statistically analyzed using a log rank test following analysis by the Kaplan-Meier method. In both tests, differences were considered significant only when the *P* value was less than 0.05. All tests were performed using the GraphPad Prism 6.0 statistical program.

## RESULTS

### ALA/SFC-cured mice infected with the lethal malaria parasite.

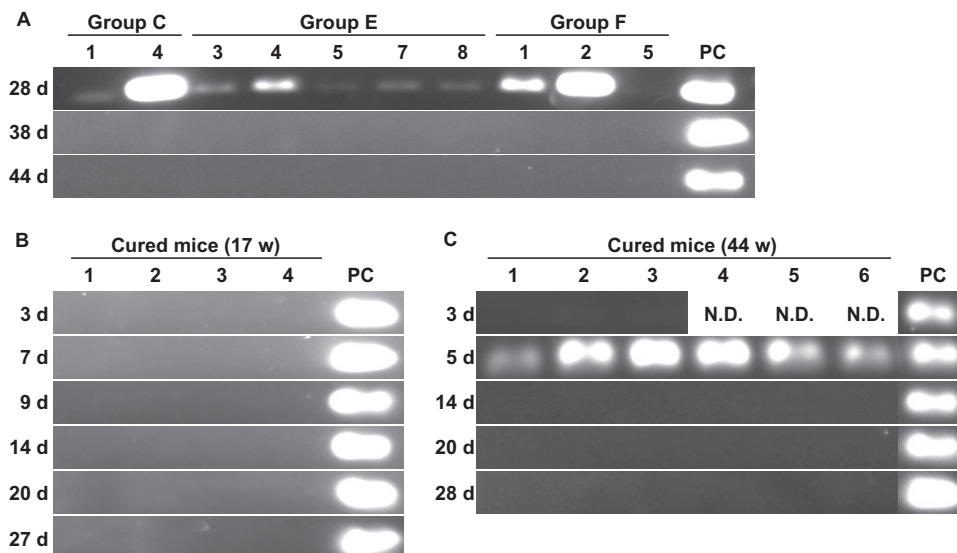
Following the demonstration that ALA/SFC inhibits the *in vitro* growth of *P. falciparum* (23), we conducted animal experiments using C57BL/6J mice and the rodent malaria parasite Py17XL (lethal strain) to demonstrate the antimalarial effects of ALA/SFC *in vivo*. Oral administration was adopted for this study, because our aim is to evaluate the potential use of ALA/SFC as a real clinical treatment in areas where malaria is endemic. Indeed, ALA and SFC previously have been approved as oral drugs for PDD of glioma (32) and for treatment of anemia (33), respectively. For the present study, treatments were administered until the infected mice were completely cured, for about 5 weeks. The survival rates obtained at 30 dpi are presented in Table 1. All infected mice administered 0.5% MC (group A, control), 300 mg/kg SFC only (group B), or 100 mg/kg ALA·HCl with 300 mg/kg SFC (group D) died. In contrast, 40% of the mice treated with 600 mg/kg ALA·HCl alone (group C) survived despite infection with the lethal parasite. Furthermore, infected mice were cured by 300 or 600 mg/kg ALA·HCl in combination with 300 mg/kg SFC (group E, 50%; group F, 60%). The survival curves and parasitemia profiles for groups A (control) and E (ALA/SFC 300/300 mg/kg) are provided in Fig. 1. Fifty percent (5 of 10) of the treated mice were rescued, a survival rate that was significantly higher than that of



**FIG 1** Effects of ALA/SFC treatments on mice infected with *Plasmodium yoelii* 17XL. Female C57BL/6J mice ( $n = 10/\text{group}$ ) were inoculated intraperitoneally with  $1 \times 10^4$  *P. yoelii* 17XL-infected RBCs and treated once daily from 1 day postinfection (dpi) with ALA or SFC alone or in combination. The data provided for group E (ALA-HCl/SFC at 300/300 mg/kg) are representative of the treated groups in which mice were cured. Data were combined from two independent experiments. (A) Survival curves of treated animals. Results are presented as the survival percentage. The log rank test was carried out after analysis by the Kaplan-Meier method. The statistical analysis indicated a significant difference ( $P < 0.05$ ) between the two groups. (B) Course of infection of treated animals. The parasitemia of each group is plotted as the mean + SD. (Data for individual animals are provided in Fig. S2 in the supplemental material.) Values at each time point were compared between groups by Student's *t* test; the difference was significant at 11 dpi (\*,  $P < 0.05$ ). † indicates that all of the remaining group A mice died.

the vehicle-treated animals ( $P < 0.05$ ) (Fig. 1A). The parasitemia profile indicated that ALA/SFC affected the parasite growth *in vivo* (11 dpi,  $P < 0.05$ ), although no statistical difference was found between the two groups until 9 dpi (Fig. 1B). The parasitemia of surviving mice declined gradually from 20 dpi, and eventually no parasite could be detected by microscopic analysis of RBCs in surviving mice at 1 month after infection (Fig. 1B). Likewise, the cured mice in other groups besides group E displayed similar profiles (see Fig. S2 in the supplemental material). In addition, while the body temperature and weight of infected mice decreased during the 2 weeks following infection, these values gradually recovered thereafter (see Fig. S3 in the supplemental material).

Diagnostic PCR was performed to confirm complete parasite clearance from peripheral blood. Specifically, PCR amplification of a fragment of the plasmodial mitochondrial *cob* gene was used as an indicator of parasite presence. Although the parasite was not detected by microscopy in 7/10 of surviving mice at 28 dpi (see Fig. S2 in the supplemental material), this amplicon was detected in 9/10 of surviving mice (Fig. 2A). This result corroborates the higher sensitivity of diagnostic PCR compared to microscopy. Neither the parasite (by microscopy) nor its DNA (by PCR) was detectable in any of the surviving mice after 38 dpi (Fig. 2A; see Fig. S2).



**FIG 2** Diagnostic PCR of surviving mice at primary infection and reinfection. A segment (426 bp) of the *P. yoelii* 17XL mitochondrial *cob* gene was amplified from mouse blood by PCR; products were electrophoresed on 1.2% agarose gels. (A) Products obtained at 28, 38, and 44 days after primary infection. Data were combined from two independent experiments. Although the parasite was not observed by microscopy in 7/10 surviving mice on day 28 after infection (see Fig. S2 in the supplemental material), the parasite DNA was detected in 9/10 surviving mice. (B) Results from 3, 7, 9, 14, 20, and 27 days after reinfection of cured mice at 17 weeks old. (C) Results from 3, 5, 14, 20, and 28 days after reinfection of cured mice at 44 weeks old. DNA isolated from mice infected with *P. yoelii* 17XL and untreated was used as the positive control (PC). N.D., not determined.

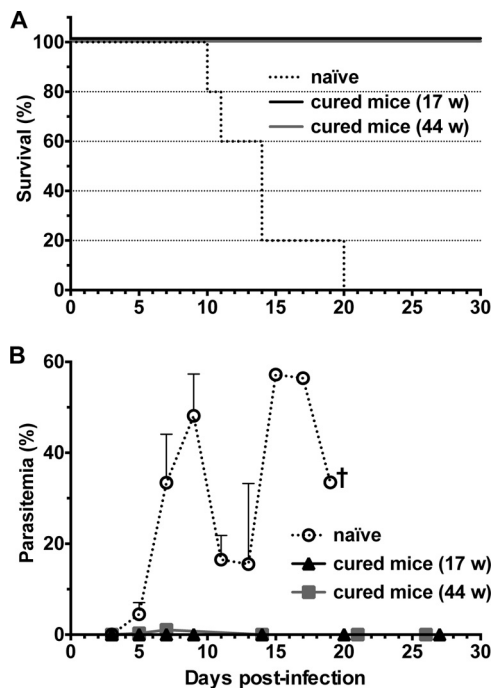
#### Cured mice exhibited resistance to homologous reinfection.

To verify whether cured mice possessed resistance against reinfection, four 17-week-old cured mice were re inoculated (6 weeks after recovery from the primary infection) with the same dose of Py17XL iRBCs as the primary infection. Whereas all the five control (naive) mice developed parasitemia and died of the infection, all of the rechallenged mice survived (Fig. 3A), and no parasite was detected in blood smears prepared from the mice (Fig. 3B). These results were consistent with diagnostic PCR conducted on rechallenged mice on multiple days (from 3 to 27 dpi) after reinfection (Fig. 2B). Thus, cured mice exhibited resistance to reinfection after primary successful treatment with ALA/SFC.

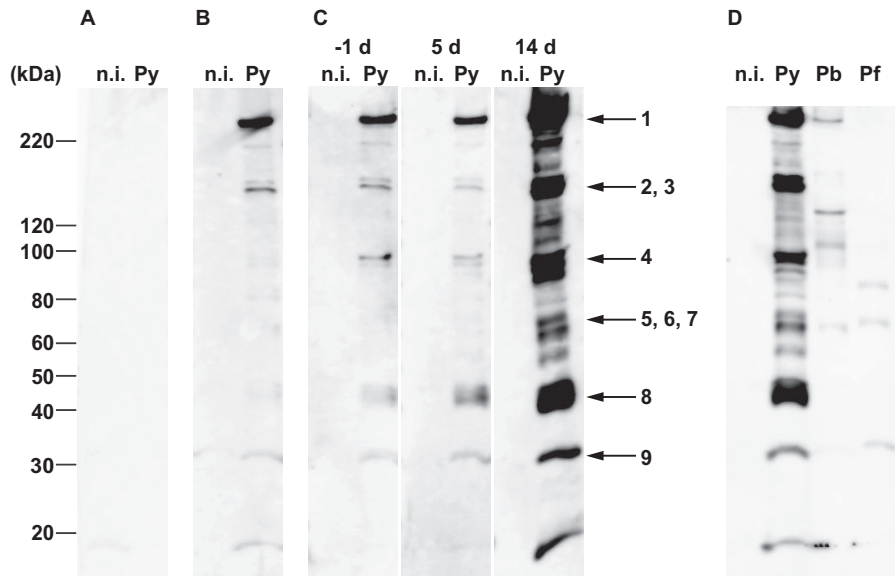
To ascertain if the resistance was sustained, the other 6 cured mice were rechallenged 33 weeks after being cured of the primary infection, at 44 weeks of age. Parasitemia was drastically suppressed upon reinfection of these animals, with values of 1% or less at 7 days after reinfection (Fig. 3B). All (6/6) of these previously cured mice survived this second round of exposure (Fig. 3A). Diagnostic PCR detected parasite DNA in all 6 animals at 5 days after rechallenge, but not thereafter (through 28 days postinfection [Fig. 2C]), confirming parasite clearance. These data demonstrated that long-lasting resistance was established in the mice cured with ALA/SFC.

#### Cured mice developed long-lasting and specific antibodies.

The possible mechanism of this resistance may due to specific humoral immunity. To confirm this hypothesis, Western blot analyses were performed using parasite lysates and sera collected from naive animals, primarily infected mice, and reinfected mice at various time points. Normal serum did not recognize any proteins in either uninfected or Py17XL-infected RBCs (Fig. 4A). In contrast, sera from infected and reinfected mice recognized many proteins in a lysate of Py17XL iRBCs (Fig. 4B and C). More importantly, there was little difference in cross-reactivity of sera be-



**FIG 3** Resistance of cured mice to homologous rechallenge. Naive and 17- and 44-week-old cured C57BL/6J mice were intraperitoneally inoculated with  $1 \times 10^4$  *P. yoelii* 17XL-infected RBCs. Naive ( $n = 5$ ), 17-week-old ( $n = 4$ ), and 44-week-old ( $n = 6$ ) animals were used in each group. (A) Survival curves of the three groups. Results are presented as the percentage of survival. The log rank test was carried out after analysis by the Kaplan-Meier method. The statistical analysis indicated a significant difference ( $P < 0.001$ ) among the three groups. (B) Course of reinfection with *P. yoelii* 17XL. Parasitemia in each group is plotted as the mean + SD. Dotted line, naive mice; black solid line, 17-week-old cured mice; gray solid line, 44-week-old cured mice. † indicates that all of the remaining naive mice died.



**FIG 4** Detection of parasite-specific antibodies in antisera from infected and reinfected mice. Lysates of the indicated *Plasmodium* species (or negative controls), which were prepared from iRBCs as described previously with minor modification (30), were separated on 5 to 20% gradient gels and blotted onto membranes. Blots were probed with pooled sera from mice that were uninfected, infected, or cured and then homologously reinfected. Bound antibodies were detected by HRP-coupled secondary antibody (anti-mouse IgG-HRP). (A to C) Immunoblot analysis against blank and *P. yoelii* 17XL iRBC lysate using pooled sera taken from uninfected mice (A), infected mice (30 dpi [B]), or 44-week-old cured mice (C) after reinfection (–1 day, left lane, 5 days, middle lane, and 14 days, right lane). (D) Immunoblot analysis against different species of parasite lysates using pooled sera taken from 44-week-old cured mice at 14 days after reinfection. Aliquots of 3  $\mu$ g of each lysate were loaded on each lane. The dilution ratio of all sera used was 1/250, except for 44-week-old cured mice 14 days after reinfection (1/1,000). The arrows indicate the identified antigens (with the gene ID registered in PlasmoDB at <http://plasmodb.org/plasmo/> shown in parentheses or brackets). (For details of separation and identification, see Methods and Fig. S5 in the supplemental material.) The arrows are numbered as follows: 1, merozoite surface protein 1 (MSP1) precursor (PY05748); 2 and 3, fragment protein of MSP1 precursor (PY05748); 4, MSP1-83 (PY05748); 5, heat shock protein 70 (HSP70 [PY06158]); 6 and 7, heat shock protein Hsp70 homologue Pfhs70 (PY06981); 8, MSP7-like protein (MSRP2 [PY17X\_1354000]); 9, early transcribed membrane protein (ETRAMP [PY17X\_0203000]) or uncharacterized protein (PY00763). n.i., uninfected RBC lysate; Py, *P. yoelii* 17XL iRBC lysate; Pb, *P. berghei* NK65 iRBC lysate; Pf, *P. falciparum* 3D7 iRBC lysate.

tween 30 days after primary infection and 1 day before reinfection of the 44-week-old mice, indicating specific antibodies were retained for more than 230 days without booster immunization (Fig. 4B and C). Lysates of iRBCs infected with either of three species of malaria parasites were blotted and compared to check the species specificity of the antibodies (Fig. 4D). As expected, fewer bands with weaker intensities were detected in the lysates of Pf3D7 and PbNK65 iRBCs than in a lysate of Py17XL iRBCs, indicating that Py17XL-specific antibodies were developed in the cured mice.

To identify the parasite-specific antibodies, the parasite lysate was subjected to SDS-PAGE and 2-dimensional gel electrophoresis (2-DE) followed by silver staining or Western blotting (see Fig. S5 in the supplemental materials). To determine antiserum-specific antigens, nine bands and spots were selected and further analyzed by nano liquid chromatography tandem mass spectrometry (LC-MS/MS). The peptides were identified as follows: merozoite surface protein 1 (MSP1), its related protein, MSP7-like protein (MSRP2), heat shock protein 70 (HSP70), and its homologue, Pfhs70, as well as early transcribed membrane protein or uncharacterized protein (Fig. 4; see Fig. S5 in the supplemental material). MSP1 is a well known vaccine candidate developed in clinical trials (34, 35). HSPs play important roles in host-parasite interaction (36). Anti-HSP70 antibody is markedly elevated in sera from both malaria patients and *P. yoelii*-infected mice (37), and Pfhs70 has been proposed as a malaria vaccine candidate (38). Hence, vaccine candidates and molecules involved in immunity against malaria parasites were identified specifically

in the cured mice, suggesting that these antibodies are important to protect mice from reinfection.

## DISCUSSION

We have been trying to discover and develop drugs for various parasites (23, 39–41). In the present study, we demonstrated that oral administration of the combination ALA/SFC rescued more than 50% of mice from the lethal malaria parasite Py17XL. Although all infected mice temporarily exhibited decreases in body weight and hypothermia, these parameters recovered in the treated and cured mice. Abnormally low body temperature is a symptom of *Plasmodium*-infected mice, in contrast to the case in human malaria (42, 43). These results support the therapeutic efficacy of ALA. In the cured mice, no relapse was observed after clearance of the parasite, because the absence of cryptic infection was confirmed by a PCR assay for the presence of plasmodial DNA. It is noteworthy that “orally” active chemotherapy with ALA/SFC against malaria is favorable because such a route is preferable for administration of antimalarial drugs in areas where malaria is endemic (44). Furthermore, in our study the ALA/SFC combination was administered to mice for over 30 days (until the mice were cured) without any clinical signs; this absence of adverse effects is suggestive of the safety of ALA/SFC for long-term treatment. Indeed, ALA/SFC already has been proven to be safe in humans, as shown in a phase I clinical trial completed with Caucasian and Japanese populations and by its historical use in various commercial applications. Based on the results of the safety

study with adult volunteers (phase I), a phase II clinical trial using ALA/SFC for mitochondrial diseases has commenced, targeting children younger than 2 years old in Japan (<https://dbcentre3.jmacct.med.or.jp/JMACTR/App/JMACTRS06/JMACTRS06.aspx?seqno=4904>), a similar age group that is of greater concerns in regions of malaria endemicity such as Africa. Although an extremely large iron dosage can cause hepatotoxicity as well as gastrointestinal disturbances, a maximum safety level for a repeated dose is indicated as 1,000 mg/kg/day SFC in rats in the generally recognized as safe (GRAS) notice of FDA in the United States, which was then approved as a food ingredient (45). In addition, the safety of ALA/SFC as a dietary supplement has been reviewed (46); the safety features of ALA/SFC have also been shown in a study with type II diabetes patients under the therapy with antidiabetes drugs (47). From the viewpoint of these high levels of safety of ALA/SFC, it is expected that ALA/SFC-based combination therapies with conventional drugs (e.g., ALA/SFC with artemisinin) can be available to reduce the dosage of conventional ones. Recently, concerns about neuroauditory toxicity (i.e., hearing impairment) associated with ACTs have been reported even though ACTs are widely viewed as safe drugs (48). This is particularly a serious problem for children because hearing impairment will have severe adverse effects on speech, behavior, linguistic understanding, and language acquisition, contributing to global disability and mortality; however, another combination strategy using ALA/SFC with conventional antimalarial drugs would be beneficial to children who are malaria patients. We are planning to conduct the additional strategy in our future study. Thus, the administration of ALA in combination with ferrous ion could be a promising antimalarial drug candidate.

ALA/SFC administration did not rescue all of the infected mice in our model of infection with the lethal malaria parasite Py17XL. In fact, we have conducted two independent *in vivo* experiments and observed that up to 60% of infected mice were cured in each study. This limit of efficacy may be attributable to several possible reasons. It may be important that ALA/SFC has different activities against parasites at distinct life cycle stages. Notably, growth inhibition of *P. falciparum* by ALA/SFC is more effective in synchronized culture than in nonsynchronized culture (29). The most severe effect was observed when ALA/SFC was added to parasites at the ring stage (K. Komatsuya, S. Suzuki, and K. Kita, unpublished data). In this context, note that *P. yoelii* has a shorter life cycle than *P. falciparum* (<12 h versus <24 h at the ring stage, respectively), and that the parasite stage in the present study was presumably random, given that the test was performed using a nonsynchronized *in vivo* assay. Consequently, this stage-specific activity may have permitted non-ring-form parasites to escape growth inhibition by ALA/SFC. However, ALA/SFC was actually effective on the human malaria parasite: oral ingestion of ALA/SFC was enough to improve malaria symptoms of children in the Solomon Islands (29) as well as the complete growth inhibition of the parasite in *in vitro* culture (23). Hence, it is expected that treatment with ALA/SFC is more effective on human malaria cases than in this mouse model. For application of ALA/SFC to human malaria in areas of endemicity, a low-cost drug supply is essential. As a large-scale production of ALA by fermentation technology has been established, further cost reduction satisfying this requirement is becoming feasible (49, 50).

The mice treated with 600 mg/kg ALA·HCl alone (group C) were cured even without SFC, although we demonstrated that

ferrous ion is essential for *in vitro* inhibition of the parasite growth in the presence of 200  $\mu$ M ALA (23). It has been reported that 2 mM ALA alone can inhibit parasite growth *in vitro* (28), suggesting that high concentrations of ALA alone may inhibit parasite growth even *in vivo*. Moreover, the mice used in our study were expected to ingest about 60 mg/kg total iron per day from the laboratory diet (see Materials and Methods), while 300 mg/kg SFC (as used in the present study) is equivalent to about 30 mg/kg ferrous ion. Thus, it is likely that our infected mice ingested sufficient ferrous ion from their diet, although the iron regulatory hormone hepcidin is expressed in *Plasmodium*-infected hosts and reduces dietary iron absorption from the small intestine (51, 52). We speculate that the endogenous ferrous ion content of the diet was sufficient to permit group C mice (treated with ALA alone) to recover from plasmodial infection even in the absence of explicit SFC supplementation. Since it was indicated that addition of ALA/SFC to *in vitro* malaria parasite culture caused the accumulation of heme intermediates and production of ROS in malaria parasites (23), similar mechanisms may play a role in the mouse model.

One of the important findings of the current study is that the ALA/SFC treatment resulted in resistance to reinfection. Recently, maslinic acid, a triterpene isolated from olive pomace, was shown to arrest the *in vitro* growth of *P. falciparum* (53). Related to that *in vitro* growth inhibition, intraperitoneal administration of maslinic acid cured almost all of the mice infected with Py17XL, and the cured mice possessed resistance to homologous rechallenge (30) as observed in the present study. However, the ALA/SFC treatment has an advantage over maslinic acid, given that the ALA/SFC combination is orally administered and provides long-lasting acquired immunity. Neither maslinic acid nor ALA/SFC provides drastic suppression of parasite growth *in vivo*. However, subcurative treatments by lower doses of drugs to maintain chronic infection have been reported to enable mice to acquire better protective immunity than radical treatments (54), indicating the importance of the moderate growth suppression by these drugs until acquisition of the immunity. Thus, the mild effects of both treatments would increase the production of antimalarial antibodies. RTS,S/AS01 is the only malaria vaccine evaluated in a phase III clinical trial, and its trial has most recently been finished: the vaccine averted about 36% and 26% of the malaria cases in child and infant participants, respectively (55). Since these figures seem to be insufficient for vaccines, additional immunization strategies could be needed along with vaccine immunizations. Chemoprevention, such as chemoprophylaxis, intermittent preventive treatment, and controlled human malaria infection, is expected to be beneficial to development of naturally acquired immunity (56–58). Considering the safety and the protective activity of ALA/SFC, the combination treatment may be applicable for such a chemoprevention approach. It is further supported by the following two facts: (i) the antibodies induced in the mice during ALA/SFC treatment cross-reacted with effective antigens, such as MPS1, and molecules related to protective immunity; (ii) in our preliminary study, continuous ingestion of ALA phosphate/SFC (25/28.68 mg/day) prevented workers in an area of intense malaria transmission from malaria infection for a month (29). Subcurative drug administration results in slower spread of drug-resistant parasite than curative administration in a mouse model infecting drug-sensitive, drug-resistant, or both parasites, although it is generally accepted that subcurative treatment accelerates drug resistance evolution (59). Thus, our result clearly demonstrated a

merit of subcurative chemotherapy. In future work, we will explore how ALA/SFC modulates host immunity to provide chemoprevention of malaria infection.

**Conclusion.** ALA/SFC is commercially available as a supplement, and its safety has been verified as a phase I clinical study has been successfully completed. We previously demonstrated that exposure to ALA/SFC in the absence of light inhibits the *in vitro* growth of *P. falciparum* (23). In the present study, we demonstrated by *in vivo* experiments that oral administration of ALA/SFC provided curative efficacy on *Plasmodium*-infected mice, including long-lasting protection from reinfection. These findings indicate that ALA/SFC is a promising antimalarial drug candidate for humans. We are planning to test ALA/SFC as an antimalarial agent in phase II clinical trials for the next step.

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