A Basis for Rapid Clearance of Circulating Ring-Stage Malaria Parasites by the Spiroindolone KAE609

Rou Zhang,1 Rossarin Suwanarusk,2 Benoit Malleret,3,4 Brian M. Cooke,5 Francois Nosten,6,7 Yee-Ling Lau,4 Ming Dao,9 Chwee Teck Lim,7 Laurent Renia,3 Kevin Shyong Wei Tan,10,a and Bruce Russell1,a

Department of 1Microbiology, Yong Loo Lin School of Medicine, 2Biomedical Engineering, National University of Singapore, and 3Singapore Immunology Network (SIgN), Agency for Science Technology and Research (A*STAR), Biopolis; 4Department of Microbiology, Monash University, Melbourne, Victoria, Australia; 5Shoklo Malaria Research Unit, Mae Sot, Tak, and 6Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; 7Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, University of Oxford, United Kingdom; 8Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur; and 9Department of Materials Science and Engineering, Massachusetts Institute of Technology, Cambridge

Recent clinical trials revealed a surprisingly rapid clearance of red blood cells (RBCs) infected with malaria parasites by the spiroindolone KAE609. Here, we show that ring-stage parasite–infected RBCs exposed to KAE609 become spherical and rigid, probably through osmotic dysregulation consequent to the disruption of the parasite’s sodium efflux pump (adenosine triphosphate 4). We also show that this peculiar drug effect is likely to cause accelerated splenic clearance of the rheologically impaired Plasmodium vivax– and Plasmodium falciparum–infected RBCs.

Keywords. Plasmodium vivax; Plasmodium falciparum; malaria; red blood cell; spiroindolones; KAE609.

The emergence of Plasmodium parasites with increased tolerance to artemisinins, the only remaining globally effective antimalarial drug, poses a serious threat to malaria control. The recently discovered spiroindolone class of compounds show promise as effective antimalarials [1]. In human phase II clinical trials, the most advanced of these, KAE609 (cipargamin; formerly NITD609; Novartis Institute for Tropical Diseases), proved to be highly effective in treating adults with uncomplicated Plasmodium vivax or Plasmodium falciparum malaria [2]. Of particular interest was the unexpectedly rapid clearance of red blood cells (RBCs) infected with early blood-stage (ring-stage) parasites, with median half-lives for parasite clearance of <1 hour (Figure 1A) [2], which compares favorably with a geometric mean of 2.6 hours measured for artesunate treatment of uncomplicated falciparum malaria in 2001 on the Thai-Myanmar border [3].

Given that KAE609 and artemisinins have distinct mechanisms of action, we have explored and elucidated the nature of the rapid clearance. We hypothesized that KAE609, known to be a potent adenosine triphosphatase 4 (ATPase4; malaria parasite sodium efflux pump adenosine triphosphate 4 [ATP4]) inhibitor [4], is likely to alter the biochemical and rheological properties of parasite-infected RBCs by dysregulating the parasite’s ionic homeostasis.

METHODS
Reexamination of Slides From a Clinical Study
We obtained approval to reexamine slides from the 4 patients with malaria (the entire group of patients enrolled in the clinics associated with the Shoklo Malaria Research Unit from a phase II, open-label study of KAE609 [2]). Although the original study reports parasitemia, it does not provide any data on the stage of the parasites. Because the ring-stage parasitemia is central to this study, it was important to stage the parasites carefully. We used 2 expert microscopists to examine and quantify the number of parasites by stage per 1000 white blood cells in the thick film and per 8000 RBCs in the thin film (Figure 1A).

The original study and the collection of clinical isolates (P. vivax) used in this study was approved by the ethics committees of the Faculty of Tropical Medicine, Mahidol University, the Thai Ministry of Public Health, the Institute for the Development of Human Research Protection, and the Oxford Tropical Research Ethics Committee (OxTREC).

Cells and Reagents
We used the P. falciparum Dd2 strain, a drug-resistant Dd2609R line derived from it (a gift from the Novartis Institute of Tropical Diseases), and P. vivax from 10 clinical isolates (>80% ring stage) collected from individuals attending health clinics of the Shoklo Malaria Research Unit in Thailand (collected in 5-mL lithium-heparinized tubes and sent to the laboratory within 6 hours), under the ethical guidelines OxTREC 58-09 and 04-10. JC-1 and Hoechst 33 342 were purchased from Life Technologies, ionomycin was purchased from Invitrogen, and the Annexin V:FITC Apoptosis Detection kit was purchased from BD Biosciences.

Short-Term Treatment of Infected RBCs With KAE609 and Artesunate
Stage-synchronous P. vivax– or P. falciparum–infected RBCs (>80% ring stage <8 hours after invasion) were maintained in 200 µL of McCoy 5A medium (supplemented with 2.4 g/L
D-glucose and 20% heat-inactivated human AB serum), at a 2% hematocrit. This blood medium mixture was treated with artesunate at 100 ng/mL and KAE609 at 100 ng/mL, and then incubated in of McCoy medium for 2 hours in a gas mix incubator (5% carbon dioxide, 5% oxygen, and 90% nitrogen) at 37.5°C before assessment of its morphological and biomechanical changes. An additional control group was prepared to assess the properties of untreated infected RBCs [5]. The sphericity of uninfected reticulocytes and normocytes from control and treated isolates was also determined.

**Micropipette Aspiration and RBC Sphericity Measurement**

The sphericity of infected RBCs was measured using the method of Waugh et al [6]. Cell suspensions were viewed under a
×100 oil immersion objective (Olympus IX73). A borosilicate micropipette with an inner diameter of 1.5 ± 0.2 µm was used to aspirate the infected RBCs at a negative pressure of 588.6 Pa (6 cm H2O). Cell deformation was recorded using a Dual CCD Digital Camera DP80 (Olympus) and analyzed using cellSens Dimension software (version 1.9).

Microfluidics
Polydimethylsiloxane microfluidic flow chambers with 2-µm openings were used for ring-stage–infected RBCs. Channels were prefilled and incubated with 1x phosphate-buffered saline with 1% bovine serum albumin for 1 hour to prevent RBC adhesion to polydimethylsiloxane before experiments. After incubation with KAE609, 1 µL of packed RBCs (containing 5% P. falciparum–infected RBCs or <1% P. vivax–infected RBCs) was injected into the microfluidic channels. Cells were pushed through the channel at a constant pressure gradient of 0.1 Pa/µm.

JC1 Cell Viability Assay
We used methods derived from Ch'ng et al [7] and Pasini et al [8]. Briefly, after incubation with KAE609, P. falciparum–infected RBCs were resuspended in Roswell Park Memorial Institute 1640 medium, and P. vivax–infected RBCs were resuspended in McCoy's medium. JC1 (6 µmol/L) and Hoechst 33 342 (1 µg/mL) were added. The cell suspension was incubated at 37°C for 30 minutes, and then washed with 1x phosphate-buffered saline. Fluorescence images were captured under a ×100 oil immersion objective (Olympus IX73) with additional ×2 tube magnification. Images were analyzed using cellSens – Dimensions software.

Annexin V Apoptosis Assay
KAE609-treated infected RBCs were resuspended in 200 µL of ×1 annexin V binding buffer containing 10 µL of annexin V–fluorescein isothiocyanate and 1 µg/mL Hoechst 33 342. Cells were incubated at room temperature for 15 minutes in the dark and then washed with 1x binding buffer. Samples were analyzed using an LSR II flow cytometer (Becton Dickinson). FlowJo software (version 10.0.7) (Tree Star) was used for flow cytometry analyses. Phosphatidylserine expression was also confirmed using fluorescence microscopy (Olympus IX73). A phosphatidylserine-positive control group was prepared by incubating uninfected RBCs in Ringer solution (125 mmol/L sodium chloride, 5 mmol/L potassium chloride, 1 mmol/L magnesium sulfate, 32 mmol/L HEPES, 5 mmol/L D-((+)glucose, and 1 mmol/L calcium chloride, pH adjusted to 7.4) with 1 µmol/L ionomycin for 30 minutes at 37°C, followed by annexin V–fluorescein isothiocyanate staining.

Sequencing PfATP4 and PvATP4
To determine allelic differences in PF3D7_1211900 (PfATP4) and PVX_084625 (PvATP4), the gene was subjected to polymerase chain reaction (PCR) amplification with Phusion DNA polymerase (Thermo Scientific) from genomic DNA, using primers and master mix, as detailed in Supplementary Figure 1. PCR products were purified with the QIAquick PCR purification kit according to the manufacturer’s instructions and sequenced with AITbiotech sequencing. Sequences were aligned and analyzed using Lasergene SeqMan Pro software (version 9.1.0) (DNASTAR).

Statistical Analysis
Nonparametric analysis of data in Figure 1A (Mann–Whitney) and Figure 1D (Kruskal–Wallis with Dunn’s post-hoc tests) was performed using GraphPad Prism 6 software.

RESULTS
To examine the effect of KAE609 on the rheological properties of infected RBCs, we obtained ring-stage parasites collected from individuals infected with P. falciparum (n = 10) or from in vitro cultures of P. falciparum (line Dd2 and the KAE609-resistant counterpart, Dd2<sup>609R</sup>), which were then exposed for 2 hours to KAE609 or to artesunate at a concentration equivalent to that commonly observed therapeutically (100 ng/mL) [2]. The resulting alterations in sphericity and rigidity were then measured by micropipette aspiration of subsets of single RBCs (infected and uninfected). The sphericity of control RBCs (normocytes and reticulocytes) was not significantly affected by exposure to either drug (Figure 1B) [9]. Parasite–infected RBCs were also unaffected by exposure to artesunate. By contrast, the drug-sensitive P. vivax–infected and P. falciparum Dd2–infected RBCs showed a marked increase in sphericity after exposure to KAE609, a phenomenon not observed for the KAE609-resistant Dd2<sup>609R</sup> parasites (Figure 1B and 1C). Amplification and sequencing confirmed that Dd2<sup>609R</sup> harbors 2 of the 3 key pfATP4 mutations associated with resistance [4], and all of the P. vivax isolates used in this study carried the wild-type genotype at the orthologous ATPase loci (Supplementary Figure 1).

The increase in sphericity is associated with a decrease in the deformability (increase in rigidity) of the RBCs (infected or uninfected), as evidenced by a shorter tongue of the RBC that is aspirated in the micropipette at a constant negative pressure of 588.6 Pa (6 cm H2O) (Figure 1C). We then ascertained whether the demonstrable increase in the sphericity and rigidity of the parasite-infected RBCs subsequent to exposure to KAE609 were sufficient to result in their retention (and subsequent clearance) in the spleen. Thus, control and KAE609-treated infected RBCs were perfused through an in vitro microfluidic device that models the sinusoids in the spleen [10]. The proportion of KAE609-treated infected RBCs that remained trapped in the 2-µm restrictions of the flow channels was significantly higher than that observed for both the untreated RBC controls and the drug-exposed KAE609-resistant Dd2<sup>609R</sup> P. falciparum clone (Figure 1D).

DISCUSSION
It should be noted that RBCs infected with young, ring-stage parasites, during their first 6 hours of intraerythrocytic
development, do not alter measurably the rheological properties of normal infected RBCs [11]. Thus, infected RBCs containing ring-stage parasites would not be expected to be retained by the mechanical barriers imposed by the splenic sinusoids (approximately 2 µm in diameter). On the other hand, using an elegant and unusual system of perfused ex vivo human spleens, Safeukui et al [9] demonstrated that at least half of the RBCs whose sphericity index exceeds 0.90 are retained in the splenic sinusoids. Given our observations here that exposure to KAE609 increased the sphericity of approximately 24%–31% of ring-stage-infected RBCs beyond a sphericity index of 0.9 (Figure 1B), making them grossly poorly deformable, implies that a significant proportion would be unable to traverse the splenic sinusoids and then be trapped and destroyed by splenic phagocytes. This phenomenon is unlikely to occur with infected RBCs exposed to high levels of artesunate because this drug did not alter RBC sphericity (Figure 1B).

This rapid perturbation by KAE609 of the rheological properties of parasite-infected RBCs could be due to ATP4-mediated osmotic dysregulation and/or to the induction of parasite cell death and eryptosis. JC1 (a mitochondrial probe that permits visual discrimination of live and dead parasites) and annexin V (as a measure of phosphatidylserine exposure) were used to assess parasite and RBC viability [7, 12]. Whereas Plasmodium vivax- and P. falciparum Dd2-infected RBCs exposed to 100 ng/mL KAE609 remained viable for a period of 2–4 hours (data not shown), those exposed to 1000 ng/mL of the drug showed clear signs of mitochondrial stress and structural disruption.

Figure 2. To investigate whether KAE609 treatment is lethal for parasites, we used JC1 (a mitochondrial probe that allows visualization of live [red staining] and/or dead [absence of red staining] parasites) as a marker of parasite viability. With KAE609 at 100 ng/mL, we saw no evidence of cell death (data not shown). A, B. However, after 2 hours of KAE609 treatment at 1000 ng/mL (approximately maximum concentration 2–4 hours after oral administration of 30 mg), Plasmodium vivax and Dd2 parasites showed clear signs of death with the loss and dispersal of JC1 monomer and aggregate staining. C. As expected, the Plasmodium falciparum Dd2R609 showed no sign of cell death. Abbreviation: DIC, differential interference contrast.
and often displayed ruptured parasitophorous vacuoles, with parasite contents spilling into the RBC cytoplasm (Figure 2A and 2B). The KAE609-resistant Dd2609R line remained unaffected by exposure at the higher concentration (Figure 2C). None of the treatments led to abnormal exposure of phosphatidylserine on the RBC surface (Supplementary Figure 2A and 2B), although KAE609-treated parasites did show other signs of eryptosis, including cell shrinkage and membrane blebbing after prolonged exposure to 1000 ng/mL KAE609.

To some extent, the phenomenon we document here, namely, alteration of the physical properties of infected RBCs, is not unique to KAE609. Indeed, 2 other preclinical lead compounds that affect the parasites’ ATP4 enzyme, a pyrazoleamide, PA21A092 [13], and a dihydroisoquinolone, (+)-SJ733 [14], were reported to induce swelling of infected RBCs, with a suggestion that this could be due to the induction of eryptosis/ senescence for (+)-SJ733. Nonetheless, the drug-induced rheological alterations caused by (+)-SJ733 (observations on PA21A092 in this context were limited) differed from those we have made with KAE609 in that they were made using infected RBCs with maturing trophozoites in which significant biomechanical changes became evident 7–24 hours after compound exposure [14]. Moreover, the overall parasite clearance rates observed in the humanized mouse model seemed slower than those measured for KAE609 in the clinical trial [2].

Ultimately, whereas antimalarial drugs, including KAE609, directly kill the parasite by inhibiting its key metabolic pathways or through oxidative damage, KAE609 is the first clinically tested drug that exerts part of its antimalarial activity through the biochemical disruption of the infected RBC. This dual killing mechanism seems to be unique to the spiroindolone class of drugs and probably contributes in large part to the remarkably fast parasite clearance observed, which exceeds that observed for artesunate, a drug that does not affect the rheological properties of circulating RBCs infected with ring-stage parasites (the only form usually found in the peripheral blood of patients with nonsevere falciparum malaria). Given the consequences of the altered deformability of infected RBCs on their normal function and survival in vivo [5, 10, 11, 15, 16], this feature reinforces the potential role of KAE609 as an important contributor to malaria control. The fact that it promotes clearance of RBCs infected with very young asexual ring-stage parasites, that are least sensitive to artesinins, makes it a suitable drug partner in the fight to contain and possibly reverse the emergence of artesunate-tolerant parasites. Should additional studies indicate that it similarly promotes clearance of mature gametocytes, that would further raise its value as a transmission-blocking agent, a much sought-after characteristic in future drugs to be deployed for malaria elimination.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copYetted and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

Acknowledgments. We thank Ms Li Hua Tan for technical support of this study. We also thank the staff and patients of the Shoklo Malaria Research Unit, Mae Sot, Thailand, and we thank Drs Bryan Yeung and Thierry Diagana for helpful discussions and the supply of the KAE609 and P. falciparum clones.

Author contributions. R. Z., R. S., B. M., F. N., K. S. W. T., and B. R. performed the experiment; B. M. C., L. Y. L., M. D., C. T. L., and L. R. contributed to the scientific discussion, facilities, and manuscript editing; and K. S. W. T. and B. R. who are joint directors and corresponding authors of this manuscript, conceived the project and wrote the manuscript.

Financial support. This work was supported by the Singapore Ministry of Education (tier 2 grant MOE2013-T2-1-145), the University of Malaya (UM-MOHE High-Impact Research grant UM.C/625/1/HIR/MOHE/CHAN/14/3 to L. Y. L.), the Singapore-MIT Alliance for Research and Technology (SMART) Centre (M. D.), the Singapore Immunology Network (L. R.), and the Horizontal Programme on Infectious Diseases under the Agency for Science, Technology and Research (A*STAR; L. R.). Shoklo Malaria Research Unit is part of the Mahidol Oxford University Research Unit, supported by the Wellcome Trust of Great Britain.

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Rottmann M, McNamara C, Yeung BK, et al. Spiroindolones, a potent compound class for the treatment of malaria. Science 2010; 329:1175–80.
2. White NJ, Pukrittayakamee S, Phyo AP, et al. Spiroindolone KAE609 for falciparum and vivax malaria. N Engl J Med 2014; 371:1403–10.
3. Phyo AP, Nikhoma S, Stepniewska K, et al. Emergence of artesinisin-resistant malaria on the western border of Thailand: a longitudinal study. Lancet 2012; 379:1960–6.
4. Spillman NJ, Allen RJ, McNamara CW, et al. Na+ regulation in the malaria parasite Plasmodium falciparum involves the cation ATPase PfATP4 and is a target of the spiroindolone antimalarials. Cell Host Microbe 2013; 13:227–37.
5. Suwanarusk R, Cooke BM, Dondorp AM, et al. The deformability of red blood cells paralyzed by Plasmodium falciparum and P. vivax. J Infect Dis 2004; 189:190–4.
6. Waugh RE, Narla M, Jackson CW, Mueller TJ, Suzuki T, Dale GL. Rheologic properties of senescent erythrocytes: loss of surface area and volume with red blood cell age. Blood 1992; 79:1351–8.
7. Ch’ng JH, Kotturi SR, Chong AG, Lear MJ, Tan KS. A programmed cell death pathway in the malaria parasite Plasmodium falciparum has general features of mammalian apoptosis but is mediated by clan CA cysteine proteases. Cell Death Dis 2010; 1:e26.
8. Pasini EM, van den Ierssel D, Vial JJ, Kocken CH. A novel live-dead staining methodology to study malaria parasite viability. Malar J 2013; 12:190.
9. Safekuku I, Buffet PA, Deplaine G, et al. Quantitative assessment of sensing and sequestration of spherocytic erythrocytes by the human spleen. Blood 2012; 120:424–30.
10. Handayani S, Chiu DT, Tjttra E, et al. High deformability of Plasmodium vivax-infected red blood cells under microfluidic conditions. J Infect Dis 2009; 199:445–50.
11. Cooke BM, Stuart J, Nash GB. The cellular and molecular rheology of malaria. Biochemistry 2014; 53:99–119.
12. Lang F, Qdadi SM. Mechanisms and significance of eryptosis, the suicidal death of erythrocytes. Blood Purif 2012; 33:125–30.
13. Vaidya AB, Morrissey JM, Zhang Z, et al. Pyrazoleamide compounds are potent antimalarials that target Na+ homeostasis in intraerythrocytic Plasmodium falciparum. Nat Commun 2014; 5:5521.
14. Jimenez-Diaz MB, Ebert D, Salinas Y, et al. (+)-SF733, a clinical candidate for malaria that acts through ATP4 to induce rapid host-mediated clearance of Plasmodium. Nat Protoc Nat Acad Sci U S A 2014; 11:ES455–62.
15. Angaran M, Zhang R, Law SK, et al. Host cell deformability is linked to transmission in the human malaria parasite Plasmodium falciparum. Cell Microbiol 2012; 14:983–93.
16. Mallett B, Li A, Zhang R, et al. Plasmodium vivax: restricted tropism and rapid remodeling of CD71-positive reticulocytes. Blood 2015; 125:1314–24.