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Transient temperature fluctuations severely decrease *P. falciparum* susceptibility to artemisinin *in vitro*

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A B S T R A C T

Clinical studies suggest that outcomes for hospitalised malaria patients can be improved by managed hypothermia during treatment. We examined the impact of short pulses of low temperature on ring-stage susceptibility of *Plasmodium falciparum* to artemisinin *in vitro*. The usually artemisinin-sensitive clone 3D7 exhibited substantially reduced ring-stage susceptibility to a 4-h pulse of 700 nM dihydro-artemisinin administered during a 5-h pulse of low temperature down to 17 °C. Parasite growth through the subsequent asexual cycle was not affected by the temperature pulse. Chloroquine and pyronaridine susceptibility, in a standard 48-h test, was not affected by brief exposures to low temperature. Fever-like temperature pulses up to 40 °C were also accompanied by enhanced ring-stage survival of 700 nM artemisinin pulses, but parasite growth was generally attenuated at this temperature. We discuss these findings in relation to the possible activation of parasite stress responses, including the unfolded protein response, by hypo- or hyper-thermic conditions. Physiological states may need to be considered in artemisinin-treated *P. falciparum* patients.

1. Introduction

Malaria remains a significant cause of disease and mortality worldwide. Elimination efforts are complicated by developing drug resistance to artemisinin combination therapies (ACT) by *Plasmodium falciparum* in Southeast Asia. Though the genetic basis of this resistance is clearly linked to mutations in *pfkelch13*, encoding a cytosolic protein of generally unknown function, understanding the biochemical and environmental effectors of parasite susceptibility to antimalarial drugs remains an active avenue of research (Imwong et al., 2017; Noedl et al., 2008; Ménard et al., 2016). The importance of this work is underscored by sporadic evidence of ACT treatment failure across sub-Saharan Africa, which seem to be occurring in the absence of *pfkelch13* mutations (Muwanguzi et al., 2016; Taylor et al., 2015). This is corroborated by several recent reports of treatment failure in travellers infected with *P. falciparum* after visiting the African continent (Sondén et al., 2017; Sutherland et al., 2017).

When left inadequately treated, *P. falciparum* infections may progress to severe disease such as cerebral malaria, which in turn may result in coma and death. Survivors frequently suffer measurable neurological deficit during convalescence (Mishra and Newton, 2009; Idro et al., 2016). A recent case report suggested the use of controlled hypothermia (32-34 °C) improved the resolution of neurological consequences of cerebral oedema and reduced cranial perfusion, as in other vascular medical emergencies like heart attack and stroke (Gad et al., 2018). Additionally, an *in vitro* model of mild hypothermia suggested that this treatment may also potentiate certain frontline antimalarials including artemisinin (Rehman et al., 2016).

Given a recent study which indicated that the cell stress response underlies ring-stage artemisinin resistance (Zhang et al., 2017), and evidence that temperature modulations induce a similar stress response in higher eukaryotes (Lee et al., 2016; Rzechorzek et al., 2015a; Liu et al., 2013; Kim et al., 2013; Xu et al., 2011), we sought to clarify the impact of temperature variation on *P. falciparum* susceptibility to artemisinin in the ring stage survival assay (RSA). Unlike the methods used in previous studies, the *in vitro* readouts from this assay correlate well with clinical treatment outcomes (Witkowski et al., 2013). Parasite susceptibility to artemisinin in the early ring stage, within the first 7 h post-invasion of a host erythrocyte, was investigated in an otherwise artemisinin- and chloroquine-sensitive parasite background under carefully calibrated exposures to both low and elevated temperatures designed to mimic both hypothermia and fever cycles.

2. Materials and methods

2.1. Parasite culture

Plasmodium falciparum culture was performed in AB^+ red blood cells obtained from the UK Blood Bank. 3D7 parasites were cultured in RPMI supplemented with Albumax II, L-glutamine, and gentamycin at 5%

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haematocrit under 5% CO_2 conditions at 37 °C. Parasite cultures were synchronised using 70% Percoll gradients to capture schizonts and 5% sorbitol to selectively isolate ring-stage parasites.

2.2. Ring-stage survival assay (RSA^{4h})

Synchronised ring-stage cultures (0–2 h post-invasion) at 2% haematocrit and 0.5% parasitaemia were exposed to 700 nM dihydroartemisinin (DHA) or equivalent DMSO for 4 h from 3 to 7 h post-invasion. Infected cells were then washed five times in media before being returned to culture. Survival at 72 h post-invasion was measured using a flow cytometry protocol based on published methods (Yang et al., 2016). Briefly, infected cells were incubated with 8 vol of FACS-stain solution (SYBR Green (1:1000; Invitrogen), MitoTracker Deep Red (1:10,000; Invitrogen) in PBS) for 20 min at 37 °C. Parasite proliferation was determined by FACS (LSR-II flow cytometer, BD Biosciences), as previously described (Yang et al., 2016). Ring-stage survival was quantified as the ratio of $SYBR^+/MTDR^+$ DHA-treated to DMSO-treated events.

For temperature-variant RSA experiments, synchronised cultures were pre-incubated at the experimental temperature from hours 2–3 post-invasion before being treated with DHA or DMSO, with continued incubation at the experimental temperature during the drug pulse from hours 3–7 post-invasion. After this pulse, infected cells were washed with media pre-warmed to 37 °C and returned to culture at 37 °C until FACS was performed. RSA experiments were performed in technical duplicate, with each replicate enumerating at least 100,000 gate-stopping events. The mean of at least three biological replicates is presented with standard error. The temperature pulse was thus 5 h, and the drug pulse 4 h in duration, nested within the temperature pulse. Data were analysed in Graph Pad Prism 7. Significance was determined by analysis of variance (ANOVA), with correction for inter-experiment and intra-experiment variance. Pairwise comparisons between 37 °C and the lowest temperature tested, and between 37 °C and the highest temperature tested were statistically evaluated by the *t*-test of Student.

2.3. Chloroquine and pyronaridine 48 h susceptibility assays

Susceptibility to these two drugs was determined by incubating parasites in serial dilutions of chloroquine or pyronaridine at 2% haematocrit and 0.5% parasitaemia in 96-well plates. These plates were incubated at experimental temperatures during the same temperature window described above and returned to 37 °C incubation after this transient temperature modulation. After 48 h, parasite proliferation in these plates was quantitated using SYBR Green as previously described (van Schalkwyk et al., 2017). Each experiment was performed in technical duplicate. The mean EC_{50} of at least three biological replicates is presented with standard error.

3. Results

3.1. Transient temperature variation affects ring-stage artemisinin susceptibility in vitro

The dependence on environmental temperature of ring-stage susceptibility to artemisinin was examined by transiently exposing *pfk13* wild-type 3D7 parasites to a range of temperatures before and during the RSA^{4h}. Given previous data suggesting hypothermic conditions (32-34 °C) could enhance anti-plasmodial effects of artemisinin *in vitro*, estimated as EC_{50} in a full life-cycle assay (Rehman et al., 2016), we probed a range of low temperatures (17ºC-36 °C) alongside normothermic (37 °C) conditions in parallel. In contrast to these previous results, our data suggest that environmental temperature and ring-stage artemisinin susceptibility in the 3D7 reference parasite line are negatively correlated *in vitro* (Fig. 1A) (Gad et al., 2018). In the RSA, reduced susceptibility to artemisinin is expressed as enhanced survival following a pulse of 700 nM DHA, compared to known susceptible parasites. Surprisingly, 3D7 parasite survival in the RSA was enhanced by transient hypothermic incubations immediately post-invasion, exceeding 20% at temperature pulses below 25 °C, rising to almost 50% survival at 17 °C (Fig. 1A). By ANOVA, RSA survival was significantly associated with falling temperature, after correction for inter- and intra-experiment variation ($P < 0.001$; 63 d.f.). By comparison, parasites harbouring the R539T *pfkelch13* mutation conferring reduced susceptibility to artemisinin in Southeast Asia display survival rates of 28 ± 2.4% in our assay format under normothermic conditions (Fig. 1A), similar to published estimates (Yang et al., 2016). These transient exposures to hypothermic environments had no significant effect on parasite proliferation in the absence of drug (Fig. 1B) and did not affect susceptibility to chloroquine in a standard EC_{50} assay with 48-h drug exposure (Fig. 1C).

To compare our findings of reduced artemisinin susceptibility in the hypothermic RSA with another drug thought to display some ring-stage activity (Chen et al., 1987), we generated EC_{50} estimates for

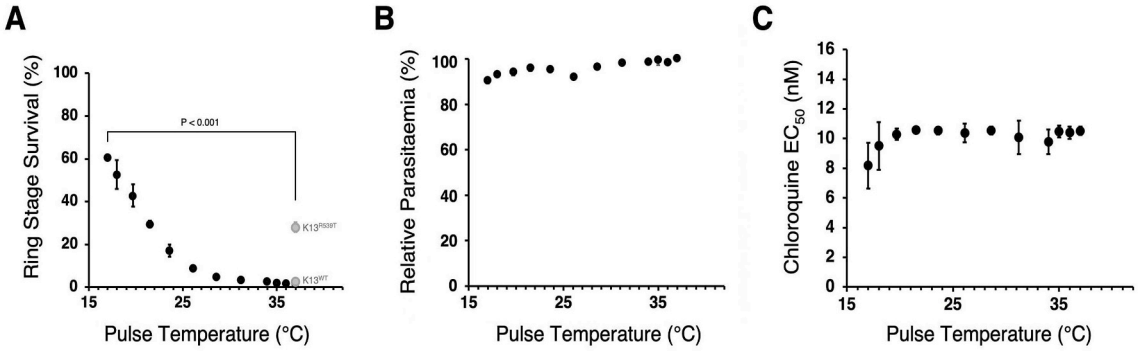

Fig. 1. Effect of transient hypothermia on ring-stage artemisinin susceptibility of *P. falciparum* (3D7). A. Ring-stage survival (proportion of control) of DHA pulse-treated 3D7 parasites (black circles) transiently incubated at hypothermic and normothermic temperatures. Open circles represent RSA survival estimates, in our hands, for previously described Cambodian parasite lines Cam 3.11 (reduced DHA susceptibility) and Cam 3.11^{REV} (fully DHA susceptible) (Straimer et al., 2014), with corresponding K13 genotypes in superscript. P value was estimated by two-sided *t*-test between 17 °C (n = 4) and 37 °C (n = 6) (mean difference 58.7% survival; 95% CI 57.7–59.8% survival; 8 d.f.).**B.** Effects of temperature modulation on 3D7 parasite proliferation in the absence of drug. Relative drug-free parasitaemia of temperature-pulsed parasite cultures was measured 72 h post-invasion as a proportion of the parasitaemia of parallel cultures incubated at 37 °C for the duration of the experiment. Each data point in **A** and **B** represents the mean of at least four biological replicates at each temperature, each performed in technical duplicate, with standard error. Each technical replicate enumerates at least 100,000 gate-stopping events by flow cytometry.**C.** Effects of transient (3–7 h post-invasion) incubation at hypothermic temperature on parasite susceptibility to a 48-h exposure to chloroquine.
Mean of at least three biological replicates is shown, each performed in technical duplicate; standard error is shown.

Table 1
Impact of a 4-h pulse of low temperature[a] on pyronaridine EC_{50} estimates[b] for 3D7 parasites, with and without a drug wash-out.

Median Pyronaridine EC_{50} (IQR)	Constant 37 °C (n = 5, 5)	18–19 °C 2–7 h post-invasion, then 37 °C (n = 5, 4)[c]	P value[d]
48 h continual drug exposure	5.15 nM (1.21–7.87 nM)	3.91 nM (1.17–13.16 nM)	0.50
3–7 h post-invasion, washed out	14.03 nM (1.92–15.51 nM)	32.64 nM (20.95–37.01 nM)	0.065

h: hour.
[a] Ambient room temperatures used for the 5 experiments performed were 18, 18, 19, 18 and 19 °C, respectively.
[b] A full dose-response curve was generated for each treatment, in five experiments each in technical duplicate.
[c] Meaningful data were obtained for only 4 of 5 experiments in the temperature-pulsed, drug pulsed treatment.
[d] Paired sign test, one-sided (H_o: 18 °C increases EC_{50}.).

pyronaridine acting upon 3D7 parasite cultures synchronised as described for the RSA experiments, with and without incubation at low temperature during early ring-stage development, and with and without a drug wash-out at 7 h post-invasion (Table 1). Exposure to low temperature during early ring-stage development had no impact on parasites exposed to pyronaridine for a full life-cycle, and a minor impact when drug was washed out at 7 h; this was not statistically significant (Table 1).

In clinical infections, fevers are triggered by schizont egress, and therefore, fever conditions overlap with the window of ring-stage development when reduced susceptibility to artemisinin is known to occur. Given these striking results under hypothermia, we wondered whether hyperthermic environments mimicking fever conditions had a similar inhibitory effect on ring-stage susceptibility to artemisinin. To this end, we examined a range of hyperthermic conditions from 38 to 41 °C. As seen for the hypothermia experiments, a statistically significant increase in RSA survival was observed (Fig. 2A; ANOVA $P < 0.001$, 14 d.f.), though conditions exceeding 39 °C strongly inhibited parasite proliferation in the absence of drug (Fig. 2B), as previously reported (Rehman et al., 2016; Chen et al., 1987). Ring-stage survival was not estimated for parasites treated with a 41 °C pulse because this killed nearly all mock-treated cells. Parasite susceptibility to chloroquine in the standard 48-h exposure assay was unaffected by the transient raised temperature (Fig. 2C).

4. Discussion

Fever cycles, important symptomatic manifestations of malaria infection, coincide with significant chemical and immunological changes to the host environment. However, the impacts of these transient temperature fluctuations on parasite development and antimalarial susceptibility are relatively unclear. The results of this study suggest that ring-stage parasite susceptibility to artemisinin derivatives may be exquisitely sensitive to environmental temperature even in the absence of *pfkelch13* mutations. Parasite susceptibility to DHA is slightly reduced under transient fever conditions, approaching 10% survival in the RSA. In contrast, parasite ring-stage susceptibility to DHA is dramatically reduced in a temperature-dependent manner with transient pulses below 30 °C, while not directly affecting parasite survival across the full life cycle *per se*. We see no impact of transient hypothermia on susceptibility to the unrelated comparator drug chloroquine, assessed with the standard 48-h readout for aminoquinolines, and a minimal EC_{50} increase for pyronaridine when drug was washed out at 7 h post-invasion. This increase was non-significant, and the drug remained highly potent (median EC_{50} 32.6 nM). Future studies could explore the effects of transient hypothermia or other cell stresses on ring-stage activity of other antimalarial drug classes.

It is unclear whether *in vitro* RSA outcomes are reliable predictors of clinical outcomes. However, these results show that hypothermic conditions, under exploration for the co-treatment of severe malaria patients, moderately attenuate artemisinin ring-stage cytotoxicity *in vitro*. As this study specifically examined the effects of transient decreases in environmental temperature, it is likely that the anti-plasmodial effects of continuous hypothermia override the brief contribution of these conditions to reducing ring-stage artemisinin susceptibility (Rehman et al., 2016). As reported previously (Rehman et al., 2016; Long et al., 2001), in our hands hyperthermia exerts strong anti-plasmodial effects on ring-stage cultures, even under transient fever conditions.

Unravelling the mechanisms behind these observations is beyond the scope of this study. The thermodynamics of artemisinin activation and cellular development are unlikely to completely explain these results. The oxidative opening of the critical endoperoxide bridge in the artemisinin molecule is likely a temperature-dependent process. Under hypothermic conditions, the rate of this opening may be slower and therefore could contribute to the reduced susceptibility of cells under hypothermia. However, under this explanation hyperthermia should be

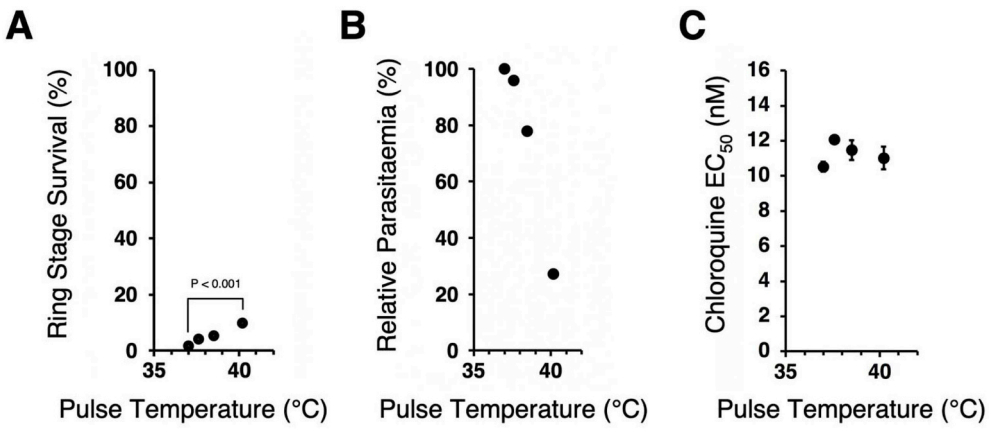

Fig. 2. Effect of transient hyperthermia on ring-stage artemisinin susceptibility of *P. falciparum* (3D7). A. Ring-stage survival (proportion of control) of DHA pulse-treated parasites transiently incubated at normothermic and hyperthermic temperatures. The data represent 15 RSA survival estimates, with 3 technical replicates for each timepoint above 37 °C. P value from two-sided *t*-test between 40.2 °C (n = 3) and 37 °C (n = 6) (mean difference 7.73% survival; 95% CI 6.56–8.90% survival; 7 d.f.).**B.** Effects of temperature modulation on parasite proliferation in the absence of drug. Relative drug-free parasitaemia measured 72 h post-invasion as a percentage of the parasitaemia of replicates incubated at 37 °C. Each data point in **A** and **B** represents the mean of at least three biological replicates at each temperature, each performed in technical duplicate, with standard error. Each technical replicate enumerates at least 100,000 gate-stopping events by flow cytometry.**C.** Effects of transient (3–7 h post-invasion) incubation at hyperthermic conditions on parasite susceptibility to a 48-h exposure to chloroquine.
Mean of at least three biological replicates is shown with standard error.

correlated with enhanced susceptibility, yet we observe the opposite. Likewise, if hypothermic conditions slow parasite development, this might be expected to elicit an extension of the ring stage, known to be hypersensitive to artemisinin in wild-type parasites (Klonis et al., 2013). Additionally, since hyperthermia exerts anti-plasmodial effects, one might expect increasing temperature to decrease the fractional inhibitory concentration of artemisinin required to sterilise the culture. However, we again observe the opposite.

Previous results have shown that the cell stress response underlies reduced ring-stage artemisinin susceptibility in *P. falciparum* (Zhang et al., 2017). Hyperthermia and hypothermia are known to trigger this mechanism in mammalian cells by destabilising proteins and slowing protein folding and translation, respectively (Lee et al., 2016; Kim et al., 2013; Xu et al., 2011; Rzechorzek et al., 2015b). Therefore, it is possible that a similar cell stress response is triggered in malaria parasites by these environmental modulations. This requires further investigation.

To date, attempts to understand the cellular mechanism of artemisinin resistance have started from the vantage point afforded by *pfkelch13* mutations. However, the results from this and previous studies suggest that the role of K13 is underpinned by fundamental mechanisms that may be mobilised independently to cause resistance (Zhang et al., 2017; Mok et al., 2015; Bhattacharjee et al., 2018). These results highlight a new approach to studying the ring-stage artemisinin resistance phenotype and underscore the potential importance of the physiologic environment upon the outcome of antimalarial assays, particularly with short-lived drugs like artemisinin. Lastly, our findings should be considered when planning future studies to evaluate the impacts of mild hypothermia on the treatment of severe malaria patients with artemisinin derivatives, as these effects may be parasite-stage dependent.

Acknowledegements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2018.12.003.

References

Bhattacharjee, S., Coppens, I., Mbengue, A., Suresh, N., Ghorbal, M., Slouka, Z., Safeukui, I., Tang, H.Y., Speicher, D.W., Stahelin, R.V., Mohandas, N., Haldar, K., 2018. Remodeling of the malaria parasite and host human red cell by vesicle amplification that induces artemisinin resistance. Blood 131, 1234–1247.

Chen, K.Y., Ha, S.H., Zhan, C.Q., Shao, B.R., 1987. Sensitivities of different stages of erythrocytic *Plasmodium falciparum* to pyronaridine. Zhongguo Yao Li Xue Bao 8, 348–351 (in Chinese).

Gad, A., Ali, S., Zahoor, T., Azarov, N., 2018. Case report: a case of severe cerebral malaria managed with therapeutic hypothermia and other modalities for brain edema. Am. J. Trop. Med. Hyg. 98, 1120–1122.

Idro, R., Kakooza-Mwesige, A., Asea, B., Ssebyala, K., Bangirana, P., Opoka, R.O., Lubowa, S.K., Semrud-Clikeman, M., John, C.C., Nalugya, J., 2016. Cerebral malaria is associated with long-term mental health disorders: a cross sectional survey of a long-term cohort. Malar. J. 15, 184.

Imwong, M., Hien, T.T., Thuy-Nhien, N.T., Dondorp, A.M., White, N.J., 2017. Spread of a single multidrug resistant malaria parasite lineage (PfPailin) to Vietnam. Lancet Infect. Dis. 17, 1022–1023.

Kim, J.H., Park, S.J., Kim, T.S., Park, H.J., Park, J., Kim, B.K., Kim, G.R., Kim, J.M., Huang, S.M., Chae, J.I., Park, C.K., Lee, D.S., 2013. Testicular hyperthermia induces unfolded protein response signaling activation in spermatocyte. Biochem. Biophys. Res. Commun. 434, 861–866.

Klonis, N., Xie, S.C., McCaw, J.M., Crespo-Ortiz, M.P., Zaloumis, S.G., Simpson, J.A., Tilley, L., 2013. Altered temporal response of malaria parasites determines differential sensitivity to artemisinin. Proc. Natl. Acad. Sci. Unit. States Am. 110, 5157–5162.

Lee, J.K., Wang, B., Reyes, M., Armstrong, J.S., Kulikowicz, E., Santos, P.T., Lee, J.H., Koehler, R.C., Martin, L.J., 2016. Hypothermia and rewarming activate a macroglial unfolded protein response independent of hypoxic-ischemic brain injury in neonatal piglets. Dev. Neurosci. 38, 277–294 (S).

Liu, X., Wang, M., Chen, H., Guo, Y., Ma, F., Shi, F., Bi, Y., Li, Y., 2013. Hypothermia protects the brain from transient global ischemia/reperfusion by attenuating endoplasmic reticulum response-induced apoptosis through CHOP. PLoS One 8, e53431.

Long, H.Y., Lell, B., Dietz, K., Kremsner, P.G., 2001. *Plasmodium falciparum*: in vitro growth inhibition by febrile temperatures. Parasitol. Res. 87, 553–555.

Ménard, D., Khim, N., Beghain, J., Adegnika, A.A., Amodu, O., Barnadas, C., Berry, A., Boum, Y., Bustos, M.D.G., Cao, J., Caridad, J., Espino, F.E., Chen, J.H., Collet, L., Cui, L., Das Thakur, G., Dieye, A., Djallé, D.D., Dorkeno, o M.A., Eboumbou Moukoko, C., Fall, B., Fandeur, T., Ferreira-da-Cruz, M.F., Fola, A.A., Fuehrer, H.-P., Hassan, A.M., Herrera, S., Hongvanthong, B., Houzé, S., Hua, T.L., Huy, R., Jahirul Karim, M., Jiang, L., Kano, S., Khan, W., Khanthavong, M., Kremsner, P.G., Lacerda, M., Laminou, I.M., Leang, R., Leelawong, M., Lin, K., Mazarati, J.B., Mei, L., Menard, S., Morlais, I., Muhindo Mavoko, H., Musset, L., Na-Bangchang, K., Nambozi, M., Niaré, K., Noedl, H., Ouedraogo, J.-B., Pillai, D.R., Pradines, B., Quang Phuc, B., Rahim Awab, G., Ramharter, M., Randrianarivelojosia, M., Sattabongkot, J., Shafiul Alam, M., Sheikh Omar, A., Silué, K.D., Sirima, S.B., Sutherland, C.J., Syafruddin, D., Tahar, R., Toure, O.A., Tshibangu, P., Vigan-Womas, I., Warsame, M., Wini, L., Zakeri, S., Kim, S., Eam, R., Berne, L., Khean, C., Chy, S., Ken, M., Loch, K., Canier, L., Duru, V., Legrand, E., Barale, J.C., Stokes, B., Straimer, J., Witkowski, B., Fidock, D.A., Rogier, C., Ringwald, P., Ariey, F., Mercereau-Puijalon, O., 2016. A worldwide map of *Plasmodium falciparum* K13-propeller polymorphisms. 2016. N. Engl. J. Med. 374, 2453–2464.

Mishra, S.K., Newton, C.R.J.C., 2009. Diagnosis and management of the neurological complications of falciparum malaria. Nat. Rev. Neurol. 5, 189–198.

Mok, S., Ashley, E.A., Ferreira, P.E., Zhu, L., Lin, Z., Yeo, T., Chotivanich, K., Imwong, M., Pukrittayakamee, S., Dhorda, M., Nguon, C., Lim, P., Amaratunga, C., Suon, S., Hien, T.T., Htut, Y., Faiz, M.A., Onyamboko, M.A., Mayxay, M., Newton, P.N., Tripura, R., Woodrow, C.J., Miotto, O., Kwiatkowski, D.P., Nosten, F., Day, N.P., Preiser, P.R., White, N.J., Dondorp, A.M., Fairhurst, R.M., Bozdech, Z., 2015. Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance. Science 347, 431–435.

Muwanguzi, J., Henriques, G., Sawa, P., Bousema, T., Sutherland, C.J., Beshir, K.B., 2016. Lack of K13 mutations in *Plasmodium falciparum* persisting after artemisinin combination therapy treatment of Kenyan children. Malar. J. 15, 36.

Noedl, H., Se, Y., Schaecher, K., Smith, B.L., Socheat, D., Fukuda, M.M., 2008. Artemisinin resistance in Cambodia 1 (ARC1) study consortium. Evidence of artemisinin-resistant malaria in Western Cambodia. N. Engl. J. Med. 359, 2619–2620.

Rehman, K., Sauerzopf, U., Veletzky, L., Lötsch, F., Groger, M., Ramharter, M., 2016. Effect of mild medical hypothermia on in vitro growth of *Plasmodium falciparum* and the activity of anti-malarial drugs. Malar. J. 15, 162.

Rzechorzek, N.M., Connick, P., Patani, R., Selvaraj, B.T., Chandran, S., 2015a. Hypothermic preconditioning of human cortical neurons requires proteostatic priming. EBioMedicine 2, 528–535.

Rzechorzek, N.M., Connick, P., Patani, R., Selvaraj, B.T., Chandran, S., 2015b. Hypothermic preconditioning of human cortical neurons requires proteostatic priming. EBioMedicine 2, 528–535.

Sondén, K., Wyss, K., Jovel, I., Vieira da Silva, A., Pohanka, A., Asghar, M., Homann, M.V., Gustafsson, L.L., Hellgren, U., Färnert, A., 2017. High rate of treatment failures in nonimmune travelers treated with artemether-lumefantrine for uncomplicated *Plasmodium falciparum* malaria in Sweden: retrospective comparative analysis of effectiveness and case series. Clin. Infect. Dis. 64, 199–206.

Straimer, J., Gnädig, N.F., Witkowski, B., Amaratunga, C., Duru, V., Ramadani, A.P., Dacheux, M., Khim, N., Zhang, L., Lam, S., Gregory, P.D., Urnov, F.D., Mercereau-Puijalon, O., Benoit-Vical, F., Fairhurst, R.M., Menard, D., Fidock, D.A., 2014. K13-propellor mutations confer artemisinin resistance in Plasmodium falciparum clinical isolates. Science 347, 428–431.

Sutherland, C.J., Lansdell, P., Sanders, M., Muwanguzi, J., van Schalkwyk, D.A., Kaur, H., Nolder, D., Tucker, J., Bennett, H.M., Otto, T.D., Berriman, M., Patel, T.A., Lynn, R., Gkrania-Klotsas, E., Chiodini, P.L., 2017. pfk13-independent treatment failure in four imported cases of *Plasmodium falciparum* malaria treated with artemether-lumefantrine in the United Kingdom. Antimicrob. Agents Chemother. 61 AAC.02382-16.

Taylor, S.M., Parobek, C.M., DeConti, D.K., Kayentao, K., Coulibaly, S.O., Greenwood, B.M., Tagbor, H., Williams, J., Bojang, K., Njie, F., Desai, M., Kariuki, S., Gutman, J., Mathanga, D.P., Mårtensson, A., Ngasala, B., Conrad, M.D., Rosenthal, P.J., Tshefu, A.K., Moormann, A.M., Vulule, J.M., Doumbo, O.K., Ter Kuile, F.O., Meshnick, S.R., Bailey, J.A., Juliano, J.J., 2015. Absence of putative artemisinin resistance mutations among *Plasmodium falciparum* in sub-Saharan Africa: a molecular epidemiologic study. J. Infect. Dis. 211, 680–688.

van Schalkwyk, D.A., Moon, R.W., Blasco, B., Sutherland, C.J., 2017. Comparison of the susceptibility of *Plasmodium knowlesi* and *Plasmodium falciparum* to antimalarial agents. J. Antimicrob. Chemother. 72, 3051–3058.

Witkowski, B., Amaratunga, C., Khim, N., Sreng, S., Chim, P., Kim, S., Lim, P., Mao, S., Sopha, C., Sam, B., Anderson, J.M., Duong, S., Chuor, C.M., Taylor, W.R., Suon, S., Mercereau-Puijalon, O., Fairhurst, R.M., Menard, D., 2013. Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: in-vitro and ex-vivo drug-response studies. Lancet Infect. Dis. 13, 1043–1049.

Xu, X., Gupta, S., Hu, W., McGrath, B.C., Cavener, D.R., 2011. Hyperthermia induces the ER stress pathway. PLoS One 6.

Yang, T., Xie, S.C., Cao, P., Giannangelo, C., McCaw, J., Creek, D.J., Charman, S.A., Klonis, N., Tilley, L., 2016. Comparison of the exposure time dependence of the activities of synthetic ozonide antimalarials and dihydroartemisinin against K13 wild-type and mutant *Plasmodium falciparum* strains. Antimicrob. Agents Chemother. 60, 4501–4510.

Zhang, M., Gallego-Delgado, J., Fernandez-Arias, C., Waters, N.C., Rodriguez, A., Tsuji, M., Wek, R.C., Nussenzweig, V., Sullivan Jr., W.J., 2017. Inhibiting the *Plasmodium* eIF2α kinase PK4 prevents artemisinin-induced latency. Cell Host Microbe 22, 766–776.