COMMENTARY

Timing is everything for artemisinin action

David J. Sullivan, Jr.[1]

W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205

In Cambodia, the same region where chloroquine resistance developed in the 1950s, a significant delay in the *Plasmodium falciparum* parasite clearance rates to artemisinins has been persistently observed over the past 5 y, raising the specter of artemisinin "resistance" (1). The parasites in this region have imprints of genetic selection associated with the delay in parasite clearance rates measured by serial patient blood films (2–4). However, the traditional continuous inhibition concentration 50% (IC_{50}) drug assays measured directly with parasites from patient's blood indicate no significant difference in IC_{50}s (1, 5). In PNAS, Klonis et al. (6) expand upon our knowledge of intraerythrocytic action of artemisinins, in a tour de force of *P. falciparum* in vitro culture work, by exposing three laboratory isolates to short 1-, 2-, 4-, or 6-h pulses of artemisinins every hour throughout the 48-h life cycle to determine pulse drug IC_{50}. An important unique finding is that an early ring stage that is less than 8-h-old is hypersensitive to artemisinins, and that intrinsic differences preexist among decades-old laboratory *P. falciparum* isolates not thought to be exposed to the artemisinin drugs before isolation.

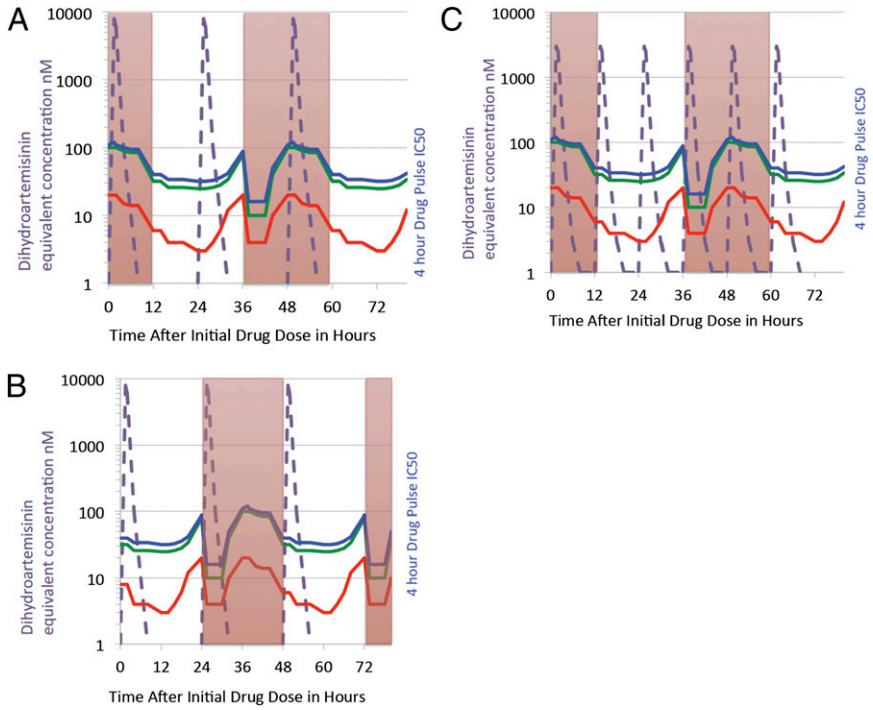

Fig. 1. Artemisinin killing varies by stage of erythrocyte life cycle. During the 48-h erythrocyte cycle, *P. falciparum* spends the initial 24 h as a ring stage (red shaded columns) before progressing to a heme crystal-containing trophozoite/schizont stage (white columns) for the last 24 h of the cycle. By careful short 1- to 6-h drug pulsing starting at every hour of the 48-h life cycle, Klonis et al. (6) demonstrate a unique initial hypersensitivity nadir at low nanomolar drug for rings up to 8 h, followed by a 16-h period of relative insensitivity to pulsed artemisinins with IC_{50} 10-times as high. At the onset of the hemoglobin digestion period, the IC_{50} to a 4-h drug pulse decreases from the ring zenith to a range of 5–20 nM for the trophozoite stages. During the end of the 48-h cycle, when hemoglobin digestion stops, the IC_{50} rises at late schizont stage. Interestingly the work compared three laboratory isolates to note an intrinsic large four- to eightfold variation in the 4-h drug pulse IC_{50}, with the isolate 7G8 (red line) being the most sensitive compared with isolates D10 or 3D7 (green line). The model described in the article takes into account the timing of short half-life artemisinins, which may result in an altered delayed parasite clearance rates in *P. falciparum* with artemisinin treatment, which has been described in Palin, Cambodia. The delay in clearance rate could be accounted by the intrinsic difference in pulse drug IC_{50} throughout the life cycle or by timing of the 24-h artesunate dosing, such that in the worst case (depicted in *A*) the artesunate dihydroartemisinin equivalent short-pharmacokinetic profile (dotted purple line) is initiated with parasite biomass predominately at 12-h rings, showing that most of artemisinin (two of three drug doses) drug exposure coincides with ring stages (red boxes), which require higher drug inhibitory concentrations to kill. (*B*) If by chance the drug dosing was to coincide with a parasite biomass predominately at early trophozoite stages, then all three 24-h doses of the drug produce high drug peaks coincident with the more susceptible trophozoite stages or hypersensitive early-ring stage. (*C*) An alternative way to maximize both time and peak drug concentrations above inhibition concentrations is to dose every 12 h, as is done with artemether-lumefantrine. Continuous in vitro drug inhibition assays have shown little difference in IC_{50} because of influence of the hypersensitive early-ring stage and trophozoite stages.

In an earlier report by Klonis et al. (7), as well as a report by ter Kuile et al. (8), the timing of artemisinin action had been demonstrated to coincide with the onset of hemoglobin ingestion during the late-ring or early trophozoite stages. This experimental window of artemisinin duration of action during the 48-h *P. falciparum* erythrocyte life cycle is longer than quinoline drugs, such as quinine, mefloquine, or lumefantrine, which inhibit heme crystallization (8). Many malariologists believe artemisinins work after iron activation to induce carbon-centered radical damage of nearby proteins for low 1- to 10-nM inhibition of *Plasmodium* trophozoite stages, which catabolize hemoglobin (9). The artemisinins also inhibit *Babesia* (10), *Toxoplasma* (11), and human cancer cells (12), albeit at 10- to 100-times higher drug concentrations. Furthermore, these microbes (cells) do not catabolize hemoglobin but either have enough bioavailable iron or heme to activate the artemisinin's endoperoxides for radical damage, or the artemisinin molecules at the higher drug concentrations interact with specific proteins or calcium channels to kill (13, 14).

Reminiscent of bacterial antibiotic resistance discovered in an ancient isolated section of Carlsbad cave with no exposure to modern human antibiotics for millions of years (15), Klonis et al. show that older laboratory isolates of malaria have almost 10-fold different response rates to drug pulses of artemisinins during the 48-h erythrocyte cycle (6). This work correlates well with the

Author contributions: D.J.S. wrote the paper.

The author declares no conflict of interest.

See companion article on page 5157.

[1]E-mail: dsulliva@jhsph.edu.

very recent 2013 observation of altered ring-stage sensitivity to 6-h pulse artemisinins in laboratory isolates, as well as isolates from Palin, Cambodia (5), where most parasites from this region have a four- to fivefold more pulse drug IC_{50} for ring stage compared with the trophozoite stage. A possible troubling conclusion from this work is that the delayed artemisinin clearance phenotype may be more widespread than previously thought if an isolate from Africa-3D7 or Papua New Guinea-D10 have elevated pulse drug IC_{50} for artemisinins, even though the pulse drug IC_{50} is less than the Palin, Cambodia isolates.

The work by Klonis et al. provides experimental evidence and a mathematical model to account for a slower clearance rate, based on the altered higher drug concentrations needed for pulse-dose short half-life artemisinins to inhibit ring stages (6). The timing of the 24-h artesunate drugs, used in many of the studies to describe delayed parasite clearance, may make a difference based on the percentage of parasites that are more susceptible to drugs. Fig. 1 depicts the model output with the high peak but short time intervals above 10-nM blood concentrations of either artesunate or artemether—dosed every 24 or 12 h—converted to the active metabolite dihydroartemisinin equivalents. As one can see from the shape of the pulse drug IC_{50} curves for two laboratory isolates, D10 and 7G8, as well as modeled ring-stage isolates from Palin just published as an additional work (5), the initiation of artemisinin dosing on a predominate single patient population of rings at 12 h of age (Fig. 1*A*) will result in two doses of the artemisinin coinciding with intrinsically higher pulse drug IC_{50} for ring-stage parasites. Even though only ring-stage parasites are visible on microscopy, clinicians are not able to discern if the total *P. falciparum* biomass at the time of microscopy is either 80% ring and 20% trophozoite stage or 20% ring and 80% trophozoites. If the same dosing regimen is initiated by chance on a predominately parasite population of 26-h trophozoites (Fig. 1*B*), then the dosing regimen will result in three doses coinciding with the lower-pulse drug IC_{50} for trophozoite stages. Another way to increase dosing on predominately trophozoite stages is to increase frequency to every 12 h, as is dosed with artemether-lumefantrine (Fig. 1*C*) (15). A recent clinical study in Palin, which compared every 12- vs. 24-h artesunate dosing, showed a small but not statistically different trend to faster clearance with every 12-h dosing of artesunate (16, 17). Unfortunately, changing the dosing does not fully reverse the delayed clearance rate seen in Cambodia. This work could also explain in the published genetic studies why single "twin" *P. falciparum* isolates have a variance in parasite clearance rate (3) from different human patients, which also could either be from timing of artemisinin dosing with predominate age in hours of the parasite biomass or from host factors, such as hemoglobin E (18).

Klonis et al. show that older laboratory isolates of malaria have almost 10-fold different response rates to drug pulses of artemisinins during the 48-h erythrocyte cycle.

Pulse-dose drug-inhibition studies at synchronized ring stages, as shown by Klonis et al. (6), are now the best way at present to segregate the phenotype of delayed parasite clearance rate, as continuous 48-h drug inhibition studies do not segregate parasites. A practical implication is that artemisinins dosed more frequently or with a longer half-life will improve time above inhibition concentration (16) as long as the toxic effects of bone marrow suppression do not appear (19). Another implication is that the mechanism of action or activation of drug may be different at different times in the erythrocyte life cycle, which would explain the time-dependent pulse drug IC_{50}. Pharmacodynamics is still everything.

1 Dondorp AM, et al. (2009) Artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* 361(5):455–467.
2 Cheeseman IH, et al. (2012) A major genome region underlying artemisinin resistance in malaria. *Science* 336(6077):79–82.
3 Anderson TJ, et al. (2010) High heritability of malaria parasite clearance rate indicates a genetic basis for artemisinin resistance in western Cambodia. *J Infect Dis* 201(9):1326–1330.
4 Takala-Harrison S, et al. (2013) Genetic loci associated with delayed clearance of *Plasmodium falciparum* following artemisinin treatment in Southeast Asia. *Proc Natl Acad Sci USA* 110(1): 240–245.
5 Witkowski B, et al. (2013) Reduced artemisinin susceptibility of *Plasmodium falciparum* ring stages in western Cambodia. *Antimicrob Agents Chemother* 57(2):914–923.
6 Klonis N, et al. (2013) Altered temporal response of malaria parasites determines differential sensitivity to artemisinin. *Proc Natl Acad Sci USA* 110:5157–5162.
7 Klonis N, et al. (2011) Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and digestion. *Proc Natl Acad Sci USA* 108(28):11405–11410.
8 ter Kuile F, White NJ, Holloway P, Pasvol G, Krishna S (1993) *Plasmodium falciparum*: In vitro studies of the pharmacodynamic properties of drugs used for the treatment of severe malaria. *Exp Parasitol* 76(1):85–95.
9 O'Neill PM, Posner GH (2004) A medicinal chemistry perspective on artemisinin and related endoperoxides. *J Med Chem* 47(12): 2945–2964.
10 Goo YK, et al. (2010) Artesunate, a potential drug for treatment of *Babesia* infection. *Parasitol Int* 59(3):481–486.
11 Jones-Brando L, D'Angelo J, Posner GH, Yolken R (2006) In vitro inhibition of *Toxoplasma gondii* by four new derivatives of artemisinin. *Antimicrob Agents Chemother* 50(12):4206–4208.
12 Rosenthal AS, et al. (2009) Malaria-infected mice are cured by a single oral dose of new dimeric trioxane sulfones which are also selectively and powerfully cytotoxic to cancer cells. *J Med Chem* 52(4):1198–1203.
13 Nagamune K, Moreno SN, Sibley LD (2007) Artemisinin-resistant mutants of *Toxoplasma gondii* have altered calcium homeostasis. *Antimicrob Agents Chemother* 51(11):3816–3823.
14 Eckstein-Ludwig U, et al. (2003) Artemisinins target the SERCA of *Plasmodium falciparum*. *Nature* 424(6951):957–961.
15 Bhullar K, et al. (2012) Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS ONE* 7(4):e34953.
16 Saralamba S, et al. (2011) Intrahost modeling of artemisinin resistance in *Plasmodium falciparum*. *Proc Natl Acad Sci USA* 108(1): 397–402.
17 Das D, et al. (2013) Effect of high-dose or split-dose artesunate on parasite clearance in artemisinin-resistant *falciparum* malaria. *Clin Infect Dis* 56(5):e48–e58.
18 Amaratunga C, et al. (2012) Artemisinin-resistant *Plasmodium falciparum* in Pursat province, western Cambodia: A parasite clearance rate study. *Lancet Infect Dis* 12(11):851–858.
19 Stepniewska K, et al. (2009) Population pharmacokinetics of artesunate and amodiaquine in African children. *Malar J* 8:200.