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## Artemisinin: Tentative Mechanism of Action and Resistance

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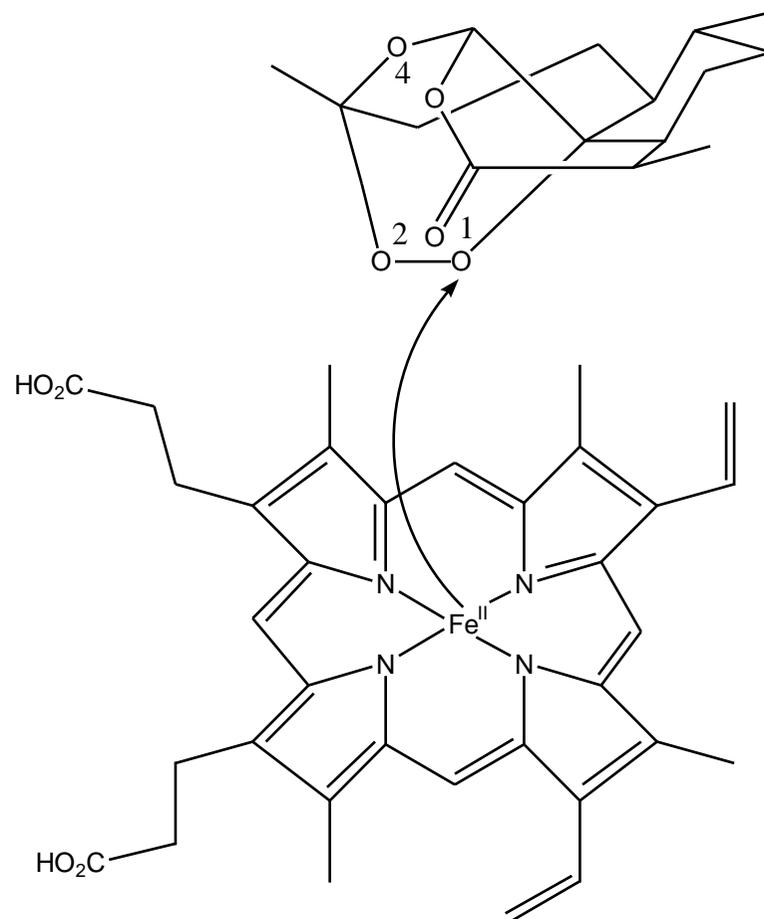
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# Artemisinin: Tentative Mechanism of Action and Resistance



**Abstract:** The sesquiterpene lactones constitute a large class of secondary plant metabolites, which carry  $\alpha$ -methylene- $\gamma$ -lactone groups as common structural element and display a number of bioactivities. Every year, 1–2 million people (mainly children) living in the tropics and subtropics die of malaria. Several malaria eradication projects were proposed (World Health Organization; Bill & Melinda Gates Foundation). Lactone artemisinin is the most effective treatment vs. malaria, the most infectious disease in the world today. Artemisinins are derived from extracts of sweet wormwood (*Artemisia annua*) and are established for the treatment of malaria, e.g., highly drug-resistant strains. They resulted in a significant advance in the treatment of malaria since the discovery and first use of quinine over 300 years ago. Their efficacy extends to phylogenetically unrelated parasitic infections, e.g., schistosomiasis. They showed potent and broad anticancer properties in cell lines and animal models. What hope does the drug offer for the future? Peroxide linkage  $-O_1-O_2-$  in the endoperoxide ring *triggers* artemisinin to *explode*. Artemisinins present structures different from quinoline. Artemisin is poorly soluble in water. When creating first generation of its derivatives in 1970s, overriding goal was to improve solubility characteristics, so that artemisinins be more easily formulated and efficiently delivered.

**Keywords:** Artemisinin resistance; Sesquiterpene lactone; Endoperoxide; Anticancer property; Drug development

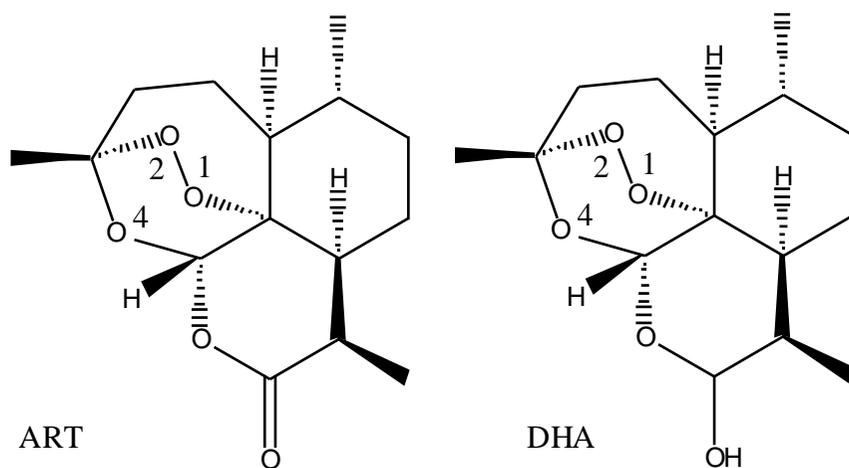


## Introduction

Malaria is caused by the bite of a female mosquito (*Anopheles*), resulting in the malaria parasite, *Plasmodium*, entering the human blood stream. Once an erythrocyte became invaded with the parasite, several rounds of asexual reproduction ensue, leading to its eventual rupture. The cyclic release of parasites from the erythrocytes causes the intermittent symptoms of fever, shivering and anaemia, which are characteristic of malaria. Of the four species of *Plasmodium* that infect humans, the most dangerous is *Plasmodium falciparum*, which accumulates in the capillaries of vital organs (e.g., brain, kidney, intestine, lungs). Cerebral malaria, *P. falciparum* accumulation in the brain, is responsible for most deaths associated with malaria. During the erythrocyte cycle, the parasite uses the host's haemoglobin (Hb) as food. Protein Hb is broken down by enzymes and the parasite assimilates the amino acids that are released. The digestive process liberates haem, Fe-containing porphyrin, which is normally buried within Hb molecule.



The free haem molecule, bearing an exposed Fe<sup>II</sup> atom at its centre, provides the unique line of attack for the drug peroxidic-sesquiterpene-lactone (STL) artemisinin (ART, *qinghaosu*, QHS). The remarkable story of the discovery of ART and establishment of its antimalarial activity by Chinese scientists represents one of the great discoveries in medicine in the latter half of the 20<sup>th</sup> century. The ART derivatives (ARTDs) present structures different from the classical quinoline.



The STLs constitute a large class of secondary plant metabolites, which carry  $\alpha$ -methylene- $\gamma$ -lactone (ML) groups as common structural element and display a number of bioactivities (e.g., cytotoxic, antineoplastic, cardiovascular, antimicrobial). What did it make STLs reach cancer clinical trials (A. Ghantous, H. Gali-Muhtasib, H., Vuorela, N. A. Saliba and N. Darwiche, What made sesquiterpene lactones reach cancer clinical trials? *Drug Discov. Today*, **15** ( 2010) 668-678)?

In an earlier publication, quantitative structure–activity relationships (QSARs) of natural STLs were reported as inhibitors of Myb-dependent gene expression (G. Castellano, L. Redondo and F. Torrens, QSAR of natural sesquiterpene lactones as inhibitors of Myb-dependent gene expression, *Int. J. Mol. Sci.*, submitted for publication).

The aim of this presentation is to initiate a debate by suggesting a number of questions (Q) that can arise when treating malaria with ARTDs and providing, when possible, answers (A) and/or hypotheses (H).



# Results and discussion

## *The Discovery of Artemisinin*

Against a scene of rising chloroquine resistance and on-going wars in Cambodia and Vietnam, Chinese Government Project May 23 (523), 1967, began an examination of plants in traditional Chinese medicine (TCM) with aim of finding a new antimalarial (Anonymous, Antimalaria studies on Qinghaosu, *Chin. Med. J.*, **92** (1979) 811-816; Y. Y. Tu, M. Y. Ni, Y. R. Zhong, L. N. Li, S. L. Cui, M. Q. Zhang, X. Z. Wang and X. T. Liang, [Studies on the constituents of *Artemisia annua* L. (authors' translation)] [Article in Chinese], *Acta Pharm. Sinica*, **16** (1981) 366-370; Y.-Y. Tu, M.-Y. Ni, Y.-R. Zhong, L.-N. Li, S.-L. Cui, M.-Q. Zhang, X.-Z. Wang, Z. Ji and X.-T. Liang, Studies on the constituents of *Artemisia annua* Part II, *Planta Med.*, **44** (1982) 143-145). *Artemisia annua* (sweet/annual wormwood) was one of several hundred species listed in TCM *Pharmacopoeia* to be considered. *Artemisia annua* is cool (yin) and can be used to treat internal heat (yang). First report of *A. annua* appeared (TCM materia medica *Ben Cao Gang Mu*, 1596) treating hot and cold because of intermittent fever illness. One bunch of leaves, collected in spring or summer, was taken with two sheng (0.4L) of water and pounded with a pestle and mortar to express the juice. This procedure was intended to improve recovery of essential oils (EOs) from leaves, in which active principal ART is concentrated. However, when making hot water extracts of *A. annua* according to ancient texts, no activity was observed vs. mice infected with malarial *P. berghei*. Cold ethereal extracts of *A. annua* showed activity; ART was isolated and characterized (1973).



Zhang proposed Qs, Hs and As on Project 523 (J. Zhang (Ed.), *A Detailed Chronological Record of Project 523 and the Discovery and Development of Qinghaosu (Artemisinin)*, Strategic Book, Houston, TX, 2013).

Q1. How could the Chinese have achieved such high-quality results?

H1. High temperature in preparing *A. annua* destroyed plant active ingredient.

H2. (Li, 1974). Mixing crushed ART tablets with water, the drug is administered to comatose cerebral malaria patients *via* a feeding tube *via* nose.

H3. (Li, 1974). Parasites are destroyed faster than *via* intravenous quinine.

H4. Preliminary clinical trial H: ART is a fast-acting antimalarial drug.

H5. Untrue H: An N-hetero-ring is necessary for an effective antimalarial.

Q2. Who did it discover what first?

Q3. Did the extracted crystals contain active antimalarial substance?

Q4. Who did it discover what and when?

Q5. Was ART treatment effective?

H6. (Project 523, 1975). ARTDs development and new compounds in ART combination therapy (ACT) for other diseases.

Q6. What is the action of the peroxide group in ART?

Q7. If peroxide was effective vs. malaria, would other peroxides be efficient?



H7. Creating effective ARTDs, it is necessary to keep ART peroxide/nucleus.

H8. (Project 523, 1977). Increasing the solubility of the water-soluble ARTDs.

Q8. How would one know ARTD-dihydroartemisinin (DHA) chemical structure?

Q9. *Who does it know which glass is sterile?*

Q10. Why did not World Health Organization (WHO) support more effective and clinically tested artemether rather than less efficient arteether?

A10. Unfounded rumours: artemether metabolized releasing methanol, increasing toxicity.

Q11. How could decision to drop artemether and develop arteether happen?

Q12. Why could Chinese discover/develop ART/ARTDs and could not they produce a final world-class-standards drug from their manufactures?

A12. China's isolation, not lack of ability or intelligence, was at fault.

Q13. How to develop collaboration to create a pathway for Chinese drugs?

Q14. Are accurate/reliable methods for measuring blood levels of ACT drugs?

Q15. Is the therapeutic efficacy of ACT credible?

Q16. Did ARTDs affect development of babies born to treated pregnant?

A16. ARTDs were appropriate in treating uncomplicated *falciparum* severe malaria in women in pregnancy 2<sup>nd</sup>/3<sup>rd</sup> trimester?



H9. Untrue H (Project 523, 1978): ART had no effect on gametocytes in *Anopheles* mosquitoes.

H10. An alternative method exists for controlling malaria spread eliminating gametocytes infectivity.

Q17. Why did Li team decide to devote their research to new ACTs?

H11. (Li, 1991). He indicated immediate use of artesunate in all epidemic and endemic areas of Vietnam.

H12. (Li). Large amounts of antimalarial drugs during Indochina War (1960/70s) rendered Vietnam most severe multi-drug-resistant *P.f.* malaria endemic area.

H13. Artequick ACT is an alternative to bring under control malaria epidemics.

Q18. How, during *Cultural Revolution* with repressed science and limited technical resources, could China made such important scientific progress?

Q19. Key Q: How to solve the drug resistance problem?

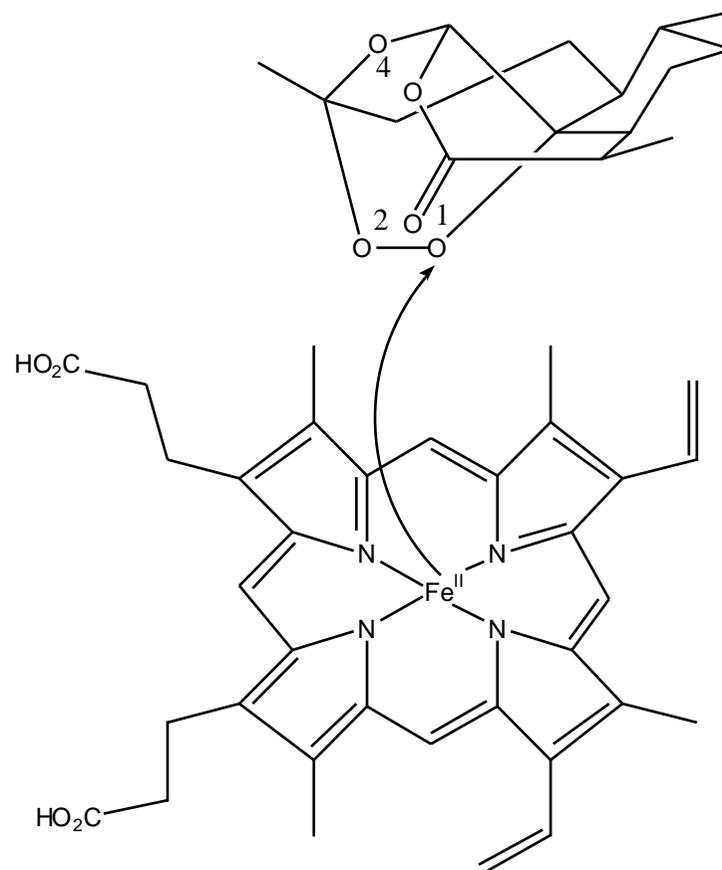
H14. To solve the problem, it is needed to find new drugs with new structures.

H15. Project 523 units have the ability to accomplish assignment as in a major military campaign.



## *Tentative Mechanism of Action of Artemisinin*

Unusual structural 1,2,4-trioxane ring, is the basis for its unique antimalarial action. The pharmacophoric peroxide  $-O_1-O_2-$  linkage in the endoperoxide ring *triggers* ART to *explode* (but only in the vicinity of the *Plasmodium* parasite). The endoperoxide bond is cleaved when it comes into contact with  $Fe^{II}$ , releasing reactive radicals, which ultimately destroy the parasite.



Substantial quantities of reactive Fe<sup>II</sup> accumulate inside an infected erythrocyte as a result of the liberation of the haem group, which is a by-product of the digestion of Hb. However, the effective *delivery* of ART *bomb* to the infected erythrocytes, where it can be detonated, suffers from one fundamental problem, which the Chinese scientists encountered when attempting to prepare their herbal teas from *A. annua*: ART is poorly soluble in water (and oil). When creating the first generation of ARTD drugs in 1970s, the overriding goal was to improve its solubility characteristics, so that ARTDs be more easily formulated and efficiently delivered.



### *Artemisinin (Qinghaosu): An Antimalarial Drug from China*

The herb *A. annua* was used for many centuries in TCM as a treatment for fever and malaria (D. L. Klayman, *Qinghaosu* (artemisinin): An antimalarial drug from China, *Science*, **228** (1985) 1049-1055). In 1971, Chinese chemists isolated, from the leafy portions of the plant, the substance responsible for its reputed medicinal action. The compound, called ART, is an STL that bears a peroxide group and, unlike most other antimalarials, lacks an N-containing heterocyclic ring. The ART was used successfully in several thousand malaria patients in China, e.g., with both chloroquine-sensitive and -resistant strains of *P. falciparum*. The ARTDs (e.g., DHA, artemether, water-soluble Na artesunate) appear to be more potent than ART. Na artesunate acts rapidly in restoring to consciousness comatose patients with cerebral malaria. The ART and ARTDs offer promise as a totally new class of antimalarials. Klayman proposed the following H.

H1. (Qinghaosu Antimalaria Coordination Research Group, 1979). The ART presents no practical effect on early or persistent exoerythrocytic tissue stage.



## *Artemisinin and a New Generation of Antimalarial Drugs: Resistance*

The following Qs were raised on ART-resistant malaria, challenges and public health.

Q1. Why ARTD compounds with their elaborate functionality are so effective?

Q2. How are parasites becoming more tolerant to ARTDs?

Brown proposed Qs and Hs on ART new generation of antimalarial drugs (G.

Brown, Artemisinin and a new generation of antimalarial drugs, *Educ. Chem.*, (7) (2006) 1-7).

Q3. What hope does ART drug offer for the future?

H1. The pestle-and-mortar procedure betters EOs recovery from leaves.

Q4. Could the 1,2,4-trioxane ring embedded in the structure of ART be stable?

Q5. Could ART ever really be useful as a drug?

Q6. How long can the situation of no documented clinical case of resistance to ARTDs last?

H2. (Jambou, 2005). Resistance is arising in areas with uncontrolled use of ARTDs.



Krishna reviewed and proposed Hs and Qs on ARTDs and growing importance in medicine (S. Krishna, L. Bustamante, R. K. Haynes and H. M. Staines, Artemisinins: Their growing importance in medicine, *Trends Pharmacol. Sci.*, **29** (2008) 520-527; S. Krishna, S. Ganapathi, I. C. Ster, M. E. M. Saeed, M. Cowan, C. Finlayson, H. Kovacsevics, H. Jansen, P. G. Kremsner, T. Efferth and D. Kumar, A randomised, double blind, placebo-controlled pilot study of oral artesunate therapy for colorectal cancer, *EBioMedicine*, **2** (2015) 82-90).

H3. Safe and cheap ARTDs drug class saving lives at risk from malaria are important in oncology.

H4. ARTDs rival acetylsalicylic acid (ASA) in their anti-disease properties.

Q7. Why have ARTDs potential to rival ASA in their anti-disease properties?

Q8. What are the mechanisms of antimalarial action of ARTDs?

Q9. (Haynes, 2007). What is Fe<sup>II</sup>-species role in ARTDs antimalarial actions?

Q10. Is there a single target for ARTDs in *Plasmodium* or multiple targets?

Q11. (Krishna, 2006). How do ARTDs work in light of emerging evidence of *in vitro* resistance?

Q12. How might structurally related drugs, e.g., synthetic trioxolanes, work?

Q13. What is the basis for resistance development by parasites to this class of antimalarial?

H5. (Peters, Richards, 1984). Murine malaras are models for understanding resistance mechanisms to different antimalarial classes.



They proposed Hs on action mechanisms and molecular targets.

H6. Haem association in insoluble haemozoin (Hz) is aided by histidine-rich protein II.

H7. ARTDs act on parasite Hb-digestion processes in *food vacuole*.

H8. SERCA H:  $\text{Ca}^{2+}$  pump localized to endoplasmic reticulum PfATP6, *P.f.* SERCA.

H9. ART disrupts mitochondrial membrane potential when grown in nonfermentable conditions.

H10. ARTDs target translationally controlled tumour protein (TCTP).

H11. The shared-target H for mechanisms of action.

They proposed additional Qs and Hs on ARTDs and growing medical importance.

Q14. Should ARTDs remain relegated to *in vitro* properties vs. cancers?

H12. ARTDs do not remain relegated to such large category of compounds.

Q15. How are the dosing regimes?

Q16. How is the safety of long-term use?

Q17. How might interactions with therapies be related to tumours treatment?

H13. Interactions with existing therapies are related to tumours treatment.



## *Art-induced Dormancy in P. falciparum, Duration, Recovery and Failure*

Cheng proposed Hs and Qs on ART-induced dormancy in *P. falciparum* (F. Teuscher, M. L. Gatton, N. Chen, J. Peters, D. E. Kyle and Q. Cheng, Artemisinin-induced dormancy in *Plasmodium falciparum*: Duration, recovery rates, and implications in treatment failure, *J. Infect. Dis.*, **202** (2010) 1362-1368).

H1. Temporary arrest of growth of ring-stage parasites (*dormancy*) after exposure to ARTs explains recrudescence.

H2. ART-induced arrest of growth occurs, being a key factor in *P. falciparum* malaria treatment failure.

H3. The failures are not because of the development of parasite resistance.

Q1. (Giao, 2001). Is there a place for ART monotherapy for treatment of uncomplicated *falciparum* malaria?

H4. (White, 1997; Giao, 2001). Recrudescence is because short ART half-life results in plasma drug concentrations not remaining above minimum inhibitory concentration (MIC).

Q2. How may parasite dormancy effect ART treatment failure?

Q3. How do long parasites remain dormant after ART exposure?

Q4. What proportion of dormant parasites does recover?

Q5. How is the dynamics of the recovery?

Q6. Are companion drugs in ACT formulations effective vs. dormant parasites?



- H5. Dormancy facilitates the development of resistance to ART drugs.
- H6. Understanding of dormancy helps to reveal ART-resistance mechanisms.
- H7. Some W2 parasites recover from dormancy after day 20.
- H8. Dormancy theory (DT, Kyle, Webster, 1996): Dormancy is specific to ARTDs, given that it could not be induced by other antimalarials, e.g., quinine.
- Q7. How long do dormant parasites survive and recover?
- H9. Dormant-parasites recovery was continuing at end of experimental period.
- H10. Repeated-treatment continuation over a longer period results in a reduction of recovery rate.
- H11. (White, 1999). Antimalarials should no longer be used alone to protect from resistance emergence.
- H12. Delay and decay in recovery were caused by mefloquine or DHA killing parasites that continued growing after first treatment and dormant parasites recovering early after treatment.
- H13. (Lewis, 2007). Dormancy facilitates ART-resistance development, giving that microbes persister cells be associated with resistant mutants emergence.
- H14. Dormancy is a mechanism used by *P.f.* to survive ART treatment.
- H15. Dormancy-resistance development link requires urgent attention.



Nosten proposed Hs/Qs on *waking the sleeping beauty* and ART-induced dormancy (F. Nosten, Waking the sleeping beauty, *J. Infect. Dis.*, **202** (2010) 1300-1301).

H16. DT: Some parasites exposed at ring *in vitro* become metabolically inactive resuming growth after drug removal.

H17. Soon dormant-parasites recovery explains some recurrences in patients during weeks after treatments.

H18. Parasites proportion recovering from dormancy stage is dose dependent.

Q8. Is dormancy observed in nature?

Q9. How may it contribute to malaria recurrences in ARTs-treated patients?

Q10. Have different ARTDs or other peroxides different effects on dormancy?

Q11. Have different partner drugs dissimilar effects on the proportion of parasites recovering from dormant stages?

Q12. Can this observation be related to resistance to ARTs?

H19. With a correct prescription, ARTDs were universally effective vs. *P.f.*

Q13. Could *P.f.* West Cambodia phenotype be explained by DT?

H20. Decline in parasitemia levels after treatment in Cambodia does not indicate recovery of a transiently dormant-parasites minority.



Q14. Are both observations (dormancy phenomenon, slow-clearance phenotype) totally unrelated?

Q15. Is observation that parasites must be exposed to DHA *at ring stage* random or are some parasites genetically primed to become dormant?

Q16. Are parasites that recover from dormancy more *tolerant* to DHA?

Q17. Have they adapted?

Q18. Could these effects on the ring stage explain the delayed clearance?



### *Art Discovery from the Chinese Herbal Garden and Differential Sensitivity*

Miller and Su proposed Qs and A on ART discovery from the Chinese herbal garden (L. H. Miller and X. Su, Artemisinin: Discovery from the Chinese herbal garden, *Cell*, **146** (2011) 855-858).

Q1. Without a publication record, who should be credited with ART discovery?

A1. Major credit must go to Tu (Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences).

Q2. Important unanswerable Q. How effective ART or ARTDs will be in future?

Q3. What drug to adopt next if resistance to ART becomes a problem?

Q4. However, who is going to develop these new drugs?

Q5. How effective is any treatment or control measure in reducing mortality?



Tilley proposed Hs, paradox (Pa) and Qs on ART activity vs. *P.f.* (N. Klonis, M. P. Crespo-Ortiz, I. Bottova, N. Abu-Bakar, S. Kenny, P. J. Rosenthal and L. Tilley, Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and digestion, *Proc. Natl. Acad. Sci. U.S.A.*, **108** (2011) 11405-11410).

H1. (O'Neill, Posner, 2004). Hb parasite uptake and hydrolysis are needed for ART activity.

H2. (Haynes, 2010). Activation of ARTs is metal independent.

H3. (Eckstein-Ludwig, 2003; Krishna, 2010). ART inhibits Ca<sup>2+</sup> ATPase (PfATP6).

H4. Hb digestion plays a critical role in action mechanism of ART drug class.

Pa1. One effect of ART is to inhibit uptake of Hb, the source of ART activator.

H5. An Hb degradation product is needed for the antimalarial activity of ART.

H6. (Becker, 2004). Detoxification is barely sufficient to prevent damage.

H7. If parasites evolve mechanisms to delay Hb uptake or hydrolysis, they circumvent ART action.

H8. Effect is clinically relevant because of short ART-antimalarials half-lives.

H9. ART-mediated Hb-uptake inhibition drives some parasites to quiescence.

H10. Slowed growth is a general stress response.

H11. Dormant state allows parasite to tolerate afforded short drug exposure.



- H12. Growth stalling at a stage before Hb uptake is initiated or short-term inhibition of Hb uptake and degradation is sufficient to permit parasite survival.
- H13. (Noedl, 2008; Dondorp, 2009; Witkowski, 2010). Delayed Hb uptake explains postponed parasites clinical clearance by ARTs in Southeast Asia.
- H14. Inhibitors of Hb hydrolysis are not suitable in combination with ARTs.
- H15. Unique chemotherapy modes facilitating Hb uptake, hydrolysis or intracellular oxidative stress enhance ARTs and circumvent evolving tolerance.



They proposed Hs and Q on altered temporal response and sensitivity (N. Klonis, S. C. Xie, J. M. McCaw, M. P. Crespo-Ortiz, S. G. Zaloumis, J. A. Simpson and L. Tilley, Altered temporal response of malaria parasites determines differential sensitivity to artemisinin, *Proc. Natl. Acad. Sci. U.S.A.*, **110** (2013) 5157-5162).

H16. The parasite experiences a saturable effective drug dose.

H17. Young ring-stage parasites exhibiting no Hb uptake show low sensitivity.

H18. Youngest rings exhibited ARTs hypersensitivity due to stress from synchronization protocol rather than an intrinsic sensitivity difference.

H19. Standard 3d drug assays do not always reveal important differences in parasite-strains sensitivity to short-lived ARTs.

H20. Long exposure times conceal what results clinically relevant differences in drug sensitivities.

H21. Population average sensitivity is not enough to class a strain as resistant.

H22. Parameter  $t_{50}^e$ , a parasite-population average property, is not a predictor of the fraction of parasite population surviving drug.

H23. Parameter  $t_{50}^e$  is only poorly correlated with delayed parasite clearance.

H24. A tightly synchronized infection of mid-ring parasites exists at beginning.

H25. The parasites remain tightly synchronized during treatment.

H26. Infection D10-strain treatment reduces parasite load to 0.004% after 48h.



H27. Assays *via* short pulses *in vitro*, combined with simple analytical model, provide a correlate with parasite clearance times in the field.

H28. (Ter Kuile, 1993; Skinner, 1996). Hypersensitive rings (hRings) presence in a culture with ring-stage parasites is responsible for variability in reports of drug sensitivity of ring-stage parasites.

H29. Clinically relevant differences in strains responses are not picked up in standard assays.

H30. It is not sufficient to measure one parameter, e.g., 50% lethal dose  $LD_{50}(t^e)$ , representing a parasite-population average property.

Q6. (Teuscher, 2010; Witkowski, 2010; Tucker, 2012). Are dormant or tolerant-parasites subpopulations involved in ARTs resistance?

H31. (Mok, 2011). *P. falciparum* resistance is associated with an altered transcription temporal pattern.

H32. (Cheeseman, 2012). Resistance in *P.f.* is linked to a region of chromosome 13.



## *A Molecular Mechanism of Art Resistance in P. Falciparum Malaria*

Haldar proposed Hs and Qs on endoplasmic reticulum (ER) phosphatidylinositol 3-phosphate (PI3P) lipid binding targeting malaria proteins to host cell (S. Bhattacharjee, R. V. Strahelin, K. D. Speicher, D. W. Speicher and K. Haldar, Endoplasmic reticulum PI(3)P lipid binding targets malaria proteins to the host cell, *Cell*, **148** (2012) 201-212).

H1. (Boddey, 2010; Russo, 2010). Cleavage is due to a resident ER protease plasmepsin V.

H2. (Boddey, 2010; Russo, 2010). Plasmepsin-V cleavage is host-targeting (HT) mechanism.

H3. Lipid binding and protein export are linked.

H4. The HT signals of multiple, important malarial effector proteins bind PI3P.

H5. PI3P binding is a generalized property of a wide range of malaria secretome effectors exported to erythrocyte.

H6. The PI3P is present in highly localized secretory regions within parasite.

H7. PI3P-enriched regions are likely to be present early in secretory pathway, in ER, but not concentrated in Golgi or periphery.

Q1. Is HT signal on endogenous ER of a PI3P-associated effector protein?

H8. Analysis of most N-terminal peptide indicated cleavage at AAAA site for wild type and mutant.



- H9. The PI3P binding is the mechanism of export to the host cell.
- H10. Green fluorescent protein (GFP) is recognized by signal peptidase and released from membrane.
- H11. Lipid binding plays a major role in targeting malaria parasite proteins to host erythrocyte.
- H12. Nanomolar affinity displayed by HT signal of virulence determinants containing R/KxLxE motif indicates a new PI3P-binding mode.
- H13. The HT-signal lipid interactions occur early in ER.
- H14. At steady state, a detectable amount of endogenous precursor carrying HT signal was not processed by plamepsin V.
- H15. Export mechanism is an efficient, early sorting event in ER, dependent on high-affinity binding to PI3P in ER lumen.
- H16. Association by PI3P facilitates protease cleavage by plamepsin V.
- H17. Cleavage *per se* does not provide specificity for HT rather it releases protein from ER membrane.
- H18. HT-independent export of parasite protein reporters to host can occur.
- H19. Non HT-dependent export to the erythrocyte is based on charge.
- Q2. How does the erythrocyte recognize putative protein cargo?



H20. PI3P and plamepsin V recycle back to ER once HT signal sorting and cleavage are completed.

Q3. Is phosphatidylinositol 3-kinase (PI3K) recruited in secretory pathway?

H21. (Tawk, 2010). Blood cell infection increases PI3P levels.

Q4. What is the overall ratio of PI3P in ER to total cellular PI3P?

H22. The PI3P also functions in *P. infestans* ER.

H23. PI3P binding in ER is a generalized mechanism for pathogenic secretion in eukaryotic pathogens.

H24. Aspects of such mechanism are targeted to disrupt pathogen–host interactions underlying disease.



## Conclusions

Despite much research, ART remains the only known natural product to contain a 1,2,4-trioxane ring and *A. annua* continues to be the only known natural source. Phytochemical investigation of the species revealed an abnormally wide range of other endoperoxides and hydroperoxides, many of which were not tested for their antimalarial activity. Novel DHA-coumarin hybrids were designed, synthesized and their cytotoxicities were assayed *via* click chemistry.



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