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Heme as Trigger and Target for Trioxane-Containing Antimalarial Drugs†

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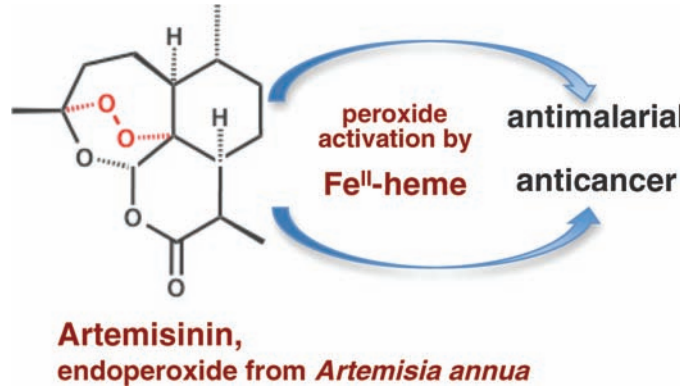

Heme is not only just the binding site responsible for oxygen transport by hemoglobin, but it is also the prosthetic group of many different heme-containing enzymes, such as cytochromes P450, peroxidases, catalase, and several proteins involved in electron transfer. Heme plays a key role in the mechanism of action of many different antimalarial drugs. In degrading the host's hemoglobin, the malaria parasite *Plasmodium* and several other heme-eating parasites are faced with this redox-active metal complex. Heme is able to induce the toxic reductive cascade of molecular oxygen, which leads to the production of destructive hydroxyl radicals. *Plasmodium* detoxifies heme by converting it into a redox-inactive iron(III) polymer called hemozoin.

Artemisinin, a natural drug containing a biologically important 1,2,4-trioxane structure, is now the first-line treatment for multidrug-resistant malaria. The peroxide moiety in artemisinin reacts in the presence of the flat, achiral iron(II)-heme; the mechanism does not reflect the classical "key and lock" paradigm for drugs. Instead, the reductive activation of the peroxide function generates a short-lived alkoxy radical, which quickly rearranges to a C-centered primary radical. This radical alkylates heme via an intramolecular process to produce covalent heme−drug adducts. The accumulation of nonpolymerizable redox-active heme derivatives, a consequence of heme alkylation, is thought to be toxic for the parasite. The alkylation of heme by artemisinin has been demonstrated in malaria-infected mice, indicating that heme is acting as the trigger and target of artemisinin. The alkylation of heme by artemisinin is not limited to this natural compound: the mechanism is invoked for a large number of antimalarial semisynthetic derivatives. Synthetic trioxanes or trioxolanes also alkylate heme, and their alkylation ability correlates well with their antimalarial efficacy.

In addition, several reports have demonstrated the cytotoxicity of artemisinin derivatives toward several tumor cell lines. Deoxy analogues were just one-fiftieth as active or less, showing the importance of the peroxide bridge. The involvement of heme in anticancer activity has thus also been proposed. The anticancer mechanism of endoperoxide-containing molecules, however, remains a challenging area, but one that offers promising rewards for research success. Although it is not a conventional biological target, heme is the master piece of the mechanism of action of peroxide-containing antimalarial drugs and could well serve as a target for future anticancer drugs.

Introduction

Among the landmarks of the vast number of publications related to heme-containing proteins, the discovery of hemoglobin as an oxygen-carrier protein by Hünefeld[1] in 1840 and its X-ray structure by Perutz et al.[2] in 1959 should be mentioned. Heme not only is the active site for oxygen-binding hemoglobin but also is the

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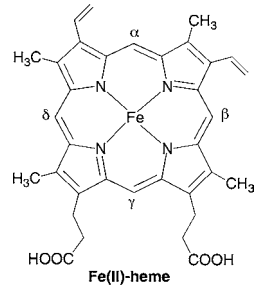

FIGURE 1. Structure of heme.

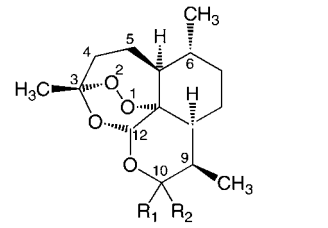

FIGURE 2. Structures of artemisinin **1**, dihydroartemisinin **2**, artemether **3**, and artesunate **4**.

prosthetic group of many different heme-containing enzymes, cytochromes P450,[3] peroxidases,[4] catalase,[5] and several heme-containing proteins involved in electron transfer.[6] All these metalloproteins or metalloenzymes have the same flat iron-tetrapyrrolic motif, namely, heme (see Figure 1 for structure), as the active site with a fast interaction with molecular oxygen when the central iron is reduced to the oxidation state +II.

Heme is not only a prosthetic group of proteins or enzymes, but it plays also an essential role in the mechanism of action of many different antimalarial drugs. *Plasmodium* is a hematophagous parasite responsible for deadly forms of malaria disease. During the infection of erythrocytes, the parasite is able to digest 60−80% of the hemoglobin to collect amino-acids for its own protein production. The released heme is efficiently polymerized by the parasite to hemozoin, the malaria pigment that is easily recognized by optical microscopy within infected erythrocytes.[7] Hemozoin is the result of the aggregation of heme dimers[8] mediated by different parasitic proteins including HRP (histidine-rich protein) and the recently characterized HDP (heme detoxification protein).[9,10] A lipidic environment has also been proposed to play a role in the nucleation of hemozoin crystals.[11] So, heme is intimately related to malaria and to many antimalarial drugs able to interact with free heme such as quinine, chloroquine, and artemisinin.

This Account is focused on heme as a trigger and target of peroxide-containing antimalarial drugs. After a rapid summary of current hypothesis on the mechanism of action of artemisinin, we will try to address the following questions: (i) How are trioxane antimalarials able to alkylate heme *in vitro*? (ii) Is heme alkylation observable *in vivo*? (iii) What are the possible correlations with heme alkylation by trioxane antimalarials and their mechanisms of action? (iv) Is heme activation of trioxane-containing drugs limited to malaria or can it be at the origin of new antitumoral drugs?

Mode of Action of Artemisinin: Main Hypotheses

Artemisinin is extracted from the leaves of *Artemisia annua* and is now widely used for the treatment of multidrug-resistant strains of *Plasmodium falciparum* in endemic zones.[12] *Artemisia* plant has been identified in traditional Chinese pharmacopoeia for the treatment of fevers since the Ming Dynasty.[13,14] The research of antimalarial drugs was prompted in China by the Vietnam war. China was supporting North Vietnam, and malaria greatly reduced the combat strength. The determination of artemisinin structure and its early clinical development was performed during the "Cultural Revolution" with tasks identified by the authorities as "Project 523". Many unknown researchers have been involved in that antimalarial program.

Artemisinin is one of the few examples of natural sesquiterpene containing a 1,2,4-trioxane as a pharmacophore. The removal of one O-atom of the peroxide bridge is associated with complete loss of the antimalarial activity of the drug (see Figure 2 for structure of artemisinin, dihydroartemisinin, artemether, and artesunate).[15−17] After more than two decades of intensive studies, the mechanism of action of artemisinin is still controversial. Three main hypotheses have been considered: (i) generation of reactive oxygen species (ROS) inducing reduction of the level of antioxidants such as glutathione[18,19] or specific mitochondrial activation,[15,20] (ii) inhibition of PfATP6, a Pf-sarco/endoplasmic reticulum calcium ATPase homologous to the mammalian sarco/endoplasmic reticulum ATPase (SERCA1a),[21] and (iii) alkylation of heme by a C-centered radical resulting from the reductive activation of the endoperoxide function.[22−26]

In the present Account, the role of ROS directly generated by the peroxide will not be extensively described. However, it should be mentioned that free heme itself (released from hemoglobin), if not quickly polymerized to hemozoin, is a cat-

alytic source of ROS. Fe(II)-heme is indeed readily oxidized by dioxygen to generate Fe(III)-heme and superoxide anion $O_2^{\bullet -}$, which dismutates to produce hydrogen peroxide and dioxygen. The produced Fe(III)-heme is easily reduced to Fe(II)-heme by all biological reductants (glutathione, NADPH, ascorbic acid, etc.), thus generating reduced oxygen species in a catalytic fashion. In addition, hydroxyl radicals can be produced by the reductive activation of hydrogen peroxide by Fe(II)-heme.[27] Such a catalytic cycle is dependent on the regeneration of glutathione by the glutathione reductase, explaining the antimalarial activity of glutathione reductase inhibitors.[28,29] A dramatic production of ROS may alter membrane potential and electron transport chain of parasite mitochondria.[20]

As an alternative pathway, Krishna and colleagues[21] reported that artemisinin inhibits PfATP6, a calcium-dependent ATPase overexpressed in *Xenopus* oocytes, whereas deoxyartemisinin had no effect on PfATP6 activity. An antagonistic interaction was also reported between artemisinin and thapsigargin, a specific SERCA1a inhibitor. However, the inhibitory constant of OZ277, an antimalarial synthetic trioxolane as active as artemisinin, on PfATP6 is very weak (7700 nM) compared to that of artemisinin (79 nM), creating doubt on the validity of calcium carrier protein as a general target for endoperoxidic antimalarials.[30,31] Additional studies suggested that mutations on PfATP6 were able to modulate the affinity of artemisinin for the protein.[32,33] However, contradiction was brought out by a genetic analysis indicating the absence of mutation in *atp6* genes of artemisinin-resistant *P. falciparum* and *P. chabaudi*.[34,35]

To determine whether the binding affinity of artemisinin and synthetic peroxides to PfATP6 can be correlated to their *in vitro* antiplasmodial activity, we performed a docking study of a series of structurally different antimalarial drugs, into the thapsigargin binding cleft of a PfATP6 model. This study included peroxide- and quinoline-based antimalarials. The predicted binding affinity for PfATP6 of these drugs correlates neither with their antimalarial activity nor with the reported inhibition of the protein overexpressed in *Xenopus laevis* oocytes reported by Krishna et al. In addition, the binding energy could not be correlated with the stereochemistry of the drugs, but is probably mainly due to hydrophobic properties rather than stereospecific interactions with a chiral protein pocket.[36]

It should be noted that the PfATP6 inhibition by artemisinin in *Xenopus* oocytes was reported to be iron-dependent, although the role of iron was not investigated.[21] Results of docking study concern the affinity of drugs with PfATP6, but they do not take into account the possible iron(II) induced reaction of peroxide-containing drugs with PfATP6. When discussing the role of iron in Ca^{2+}-pump inhibition, Karlish and co-workers carried out the Fe^{2+}-catalyzed oxidative cleavage of a mammalian SERCA by the Fenton reaction.[37,38] Further docking study should include a possible iron site in the protein structure. A recently reported method of purification of ATPases will also facilitate further direct studies of the action of peroxide-containing antimalarial drugs with isolated PfATP6.[39]

How Are Trioxane Antimalarials Able to Alkylate Heme *in Vitro*?

The flat nonchiral heme is a target out of the "key and lock" paradigm for drugs. However, it is present at a 20 mM concentration in erythrocytes and contains a redox-active metal center.

Alkylating species generated by ferrous iron-mediated homolytic cleavage of the endoperoxide function of artemisinin, in particular the alkyl radical centered at C4 of artemisinin (or related 1,2,4-trioxanes), were early proposed to be important, and the reactivity with iron salts was extensively studied.[40] However, at pH 7 under aerobic conditions, "free" iron ions should be $[Fe(OH)_3]_n$, which is highly insoluble ($K_s = 10^{-39}$), and the concentration of "free" iron in living cells is then close to zero. Then reaction of artemisinin with heme is more relevant than reaction with "free" iron salts.

Heme as Trigger and Target for Trioxane-Containing Antimalarial Drugs. After preliminary experiments with synthetic metalloporphyrins,[24] we have reported that iron(III)-heme [iron(III)-protoporphyrin(IX)] incubated with artemisinin, in the presence of a reducing agent able to generate iron(II)-heme, was readily converted in high yield to heme−artemisinin covalent adducts (**5** and **6**, Figure 3). These adducts result from alkylation of the four *meso* positions of the macrocyclic ligand by the C4-alkyl radical derived from artemisinin.[25] The reductive cleavage of artemisinin is initiated by an electron transfer from the low-valent iron(II)-heme to the antibonding σ^* LUMO orbital of the peroxide bond. This reductive activation generates a short-lived alkoxy radical which quickly rearranges, via β-fragmentation, to a C4-centered primary radical thermodynamically facilitated by concomitant formation of an ester functionality. Intramolecular addition of this akylating species occurs without regioselectivity on the four *meso* carbons of the protoporphyrin ligand. After demetalation of the heme moiety, complete NMR characterization of these adducts was obtained.[41]

The alkylating ability of artemisinin is not limited to this natural compound. A number of active semisynthetic deriva-

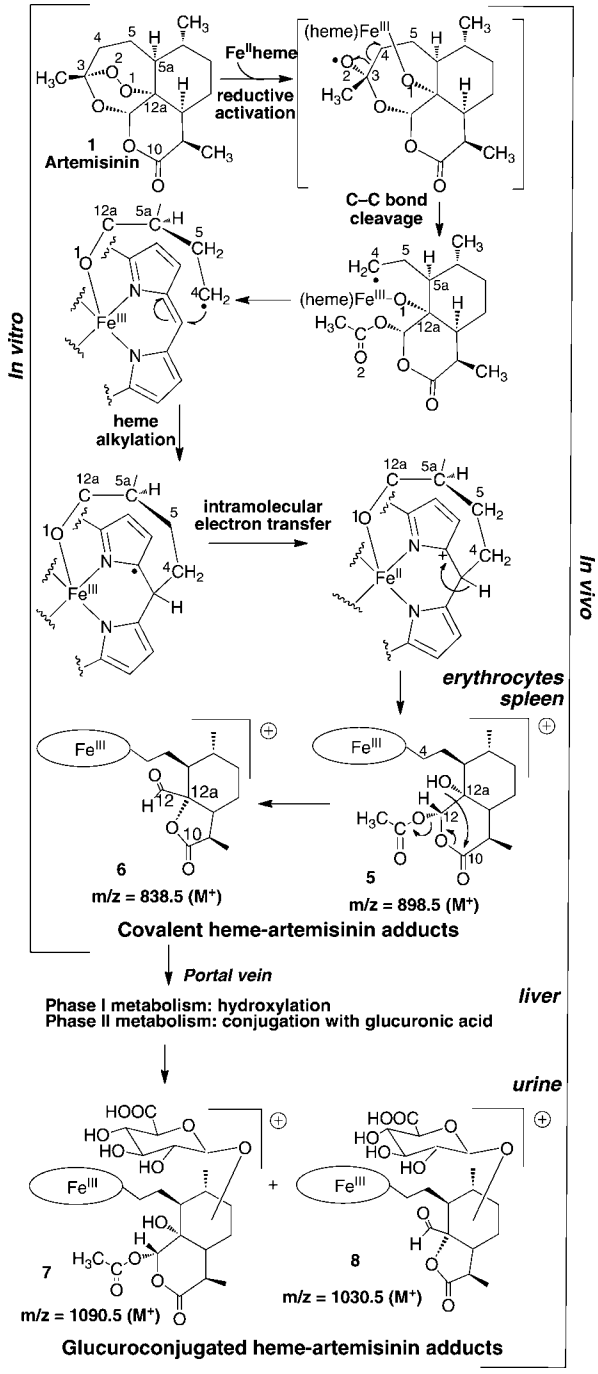

FIGURE 3. Alkylation of heme by artemisinin *in vitro* and *in vivo*.

tives modified at position C10 (either with α or β configuration) were found to react in the same way.[42−45] Synthetic trioxanes or trioxolanes were also able to alkylate heme or a synthetic heme model, and their alkylation ability correlates well with their antimalarial efficacy.[46−49] The ovals stand for the tetrapyrrolic ring of heme.

Is Heme Alkylation Observable in Vivo?

Is the formation of heme−artemisinin adducts a laboratory curiosity or is it biologically relevant and related to the mechanism of action of the drug *in vivo*? To address this question, we orally or intraperitoneally treated *Plasmodium vinckei* infected mice with artemisinin at pharmacological doses. The covalent adducts heme−artemisinin **5** and **6** (Figure 3) were identified in the spleen of all treated mice, and the hydroxylated and glucuroconjugated derivatives of heme−artemisinin adducts **7** and **8** were identified in the urine. These adducts were not detected after treatment of healthy mice.[50]

The alkylation of heme occurred also in infected mice treated with the trioxaquine DU1301, an efficient 1,2,4-trioxane based antimalarial drug which was designed to have alkylating properties.[51] The presence of these heme−drug adducts should therefore be considered as the signature of the strong alkylating capacity of the trioxane-containing drugs triggered by the presence of the parasite in mice. This result confirms that artemisinin and trioxaquines share similar heme-alkylating properties and supports the importance of this ability for their antimalarial activity. In addition, artemisinin and the citrate salt of DU1301 are both active on the same stages of synchronized *P. falciparum* parasites (ring, trophozoite, and gametocyte),[52] suggesting that their common alkylating reactivity might a key factor of their antimalarial activity. The role of heme alkylation in the mechanism of action of artemisinins is still a matter of debate.[53]

Possible Correlations between Heme Alkylation and the Mechanism of Action of Trioxane Antimalarial

In vitro, heme polymerizes to β-hematin, a synthetic analogue of hemozoin. Heme−artemisinin adducts treated *in vitro* under β-hematin synthesis conditions were unable to polymerize. The bulky artemisinin residue linked at the *meso*-positions of the porphyrin ring probably prevented the coordination of the propionate side chain on the iron atom, an initial step required for the polymerization process.[54] Monomeric heme−artemisinin adducts, that retain the redox activity of any heme derivative in a glutathion rich medium, may therefore activate molecular oxygen, leading to the production of toxic reduced oxygen species (Figure 4, route 1). Unlike artemisinin itself, heme−artemisinin adducts were able to completely inhibit the polymerization of heme *in vitro*, suggesting that free heme may also accumulate in parasites treated by artemisinin, thus increasing oxidative stress.[54] It was also suggested that heme−artemisinin adducts were able to inhibit the formation of β-hematin in the presence of histidine-rich protein.[55] Trioxaquines have been designed to combine the quinoline nucleus of chloroquine and the 1,2,4-trioxane of artemisinin.[56] Beside their alkylating ability toward heme, tri-

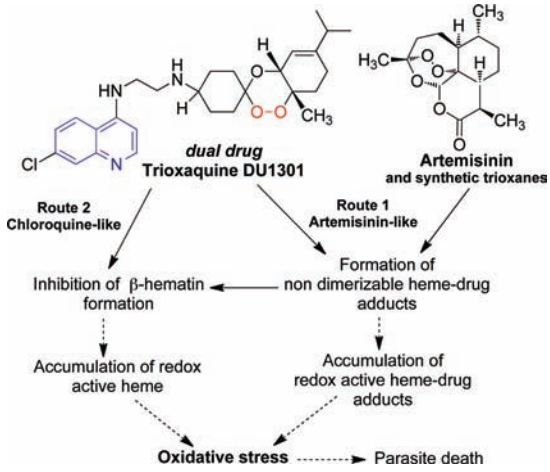

FIGURE 4. Possible relation between heme alkylation by endoperoxide drugs and antimalarial mechanism of action.

oxaquines DU1301 and PA1103 prevent β-hematin formation like chloroquine, thus being able to interfere with the heme detoxification process (Figure 4, route 2).[49,54] Artemisinin treatment of cultured parasites caused no mesurable change in hemozoin content.[57] However, the concentration of the heme pool that accumulates within the food vacuole during hemoglobin proteolysis may be as high as 400 mM, and free heme can damage cells at micromolar concentration. Consequently, a very small portion that escapes the aggregation process (for example, 1 molecule over 10^4) should be sufficient to kill the parasite without having a detectable effect on hemozoin content.[58]

Role of the Cytosolic Labile Iron Pool in the Mechanism of Action of Artemisinin. The cytosolic labile iron pool (LIP, also referred to as the chelatable iron pool), as possibly the first compartment of iron release from ferritin, has an impact on the iron regulatory protein (IRP) as well as iron homeostasis. The putative role of non-heme iron species from the LIP for antimalarial activity of artemisinin was based on *in vitro* antagonist activities of artemisinin and the bacterial siderophore deferoxamine (DFO).[59] The antagonist effect was also reported for chloroquine and DFO,[60] despite the fact that the mechanism of antimalarial action of chloroquine is different from that of artemisinin derivatives.[61] In addition, other series of siderophores such as catecholates had no effect on the activities of chloroquine, quinine, and artemether.[62] In addition, Pradines et al. underline that antagonism is usually assessed with construction of isobolograms which should apply to combinations of different agents "if they had similar modes of action and similar dose–response curves."[62] This is not the case for DFO, chloroquine, and artemisinin derivatives.

Artemisinin derivatives with an iron chelator covalently linked at C10 have been synthesized. However, the presence of iron chelator in vicinity of artemisinin did not potentiate the antimalarial activity of such conjugated drugs, but only decreased their stability.[63] DFO decreases the ferritin level in lysosomes and disturbs the H-/L-ferritin subunit ratio in human leukemia K562 cells, and this effect is partially reversed by chloroquine which is a lysosomotropic compound.[64] It was recently reported that DFO affects ferritin degradation by a route that is different from the iron exporter ferroportin expression or the more membrane-permeable oral iron chelators (deferriprone and desferasirox); and chloroquine, by affecting lysosomal pH, prevents ferritin loss in DFO-treated cells.[65] These data suggest that the interaction between DFO and any other treatment cannot be limited to iron deprivation and is still misunderstood.

Alkylable Target(s) Other than Heme. When incubated in *Plasmodium* cultures, radiolabeled artemisinin alkylates heme and also some unidentified but specific parasitic proteins.[66] The artemisinin-induced inhibition of a parasite homologue of the antiapoptotic protein TCTP[67] was clearly heme-dependent.[68] Alkylation by artemisinin may also be involved in the specific inhibition of malarial histidine-rich protein[9] or cysteine protease.[69] The reported iron-dependent inhibition of PfATP6 may also be due to protein alkylation.

In vitro, the C4 centered radical of artemisinin can react with the thiol function of cysteine or glutathione to alkylate the peptide through a thioether linkage,[70] indicating that a covalent heme–protein coupling may occur by this way. However, no artemisinin–protein adduct has been characterized up to now, and *in vitro* these reactions are much less efficient than heme alkylation.[71]

Artemisinin Derivatives as New Antitumoral Drugs: Is Heme Also Involved?

After early Chinese articles,[72] the cytotoxicity of artemisinin derivatives toward several tumor cell lines was reported.[73,74] Iron metabolism is likely to play a central role in the anticancer activity of artemisinin. Since cancer cells have higher iron influx via the transferrin receptor mechanism, cancer cells should be more susceptible to the cytotoxic effect of artemisinin, under conditions of high iron availability. Preloading tumor cells with iron or inclusion of holotransferrin enhances the activity of artemisinin derivatives toward human breast cancer cells.[75] In order to deliver both artemisinin and iron "as one package" to cancer cells, artemisinin-tagged transferrins, bearing ~16 artemisinins attached to the *N*-glycoside chains of transferrin, were tested on prostate carcinoma DU145 cells.[76] However, the cytotoxicity per artemisinin unit did not increase by conjugation to transferrin. In contrast to the case

of artemisinin, the cytotoxicity of dihydroartemisinin is independent of the level of transferrin receptor expression on DU145 cells. Thus, endosomal iron in the endocytosis of holotransferrin is unlikely to participate in the activation of dihydroartemisinin in the cell.[76]

More interesting than monomers, C10-artemisinin dimers or trimers were found active at submicromolar concentration (30−300 nM) in a panel of tumor cell lines.[77−81] Artemisinin dimers were >1000 times more active than artemisinin, and treatment was associated with an indiscriminate generation of oxidative stress as a consequence of heme-mediated endoperoxide cleavage, activation of caspases, and consequent apoptosis.[82] Deoxy analogues were at least 50 times less active, thus emphasizing the importance of the peroxide bridge. The involvement of heme in anticancer activity was supported by (i) a decline in expression for the ferritin transporter, (ii) upregulation of heme oxygenase HMOX1 involved in the catabolism of heme to bilirubin, and (iii) down regulation of genes involved in heme biosynthesis.

TCTP (also named fortilin) is an antiapoptotic protein overexpressed in many human cancers, which is considered as a putative target of artemisinin derivatives.[83] The reversible binding of dihydroartemisinin to TCTP ($K_d = 38\ \mu M$) results in the shortening of the half-life of TCTP, via ubiquitination and subsequent proteasome-mediated degradation. However, the heme-mediated alkylation of TCTP by dihydroartemisinin, resulting in an irreversible linkage between both entities, should be considered too.

Other hypotheses on the mechanism of action of artemisinin derivatives against cancer include (i) inhibition of angiogenesis,[84] (ii) inhibition of topoisomerase IIa,[85] (iii) inhibition of the hyperactive Wnt/β-catenin pathway of human colorectal carcinoma,[86] (iv) down regulation of estrogen receptor ERα in MCF7 human breast cancer,[87] or (v) transcriptional down regulation of cyclin-dependent kinase-4 (CDK4) expression in prostate cancer.[88]

Conclusion

The small flat heme is not only the prosthetic group of a considerable number of metalloproteins, but is also the master piece of the mechanism of action of peroxide-containing antimalarial drugs and probably also a target for future anticancer drugs. So, heme will be continue to be a unique iron complex in biology and pharmacology.

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BIOGRAPHICAL INFORMATION

Bernard Meunier was born in 1947 and received degrees from the Universities of Montpellier (R. J. P. Corriu, 1971) and Paris-Orsay (H. Felkin, 1977). After a postdoc at Oxford University (1977−1978), he joined the "Laboratoire de Chimie de Coordination du CNRS" in Toulouse in 1979. He has been Director of Research at the CNRS, Associate Professor at the Ecole Polytechnique (1993−2006), and President of the CNRS (2004−2006). He is currently CEO of Palumed, a small company founded in 2000. Author of 360 publications and 32 patents, he has been a member of the French Academy of Sciences since 1999 and of the Polish Academy of Sciences since 2005.

Anne Robert was born in 1959 and received degrees from the Chemical Engineering School of Toulouse and the University of Toulouse. She joined the research group of Bernard Meunier at the Laboratoire de Chimie de Coordination in 1985. She is currently Director of Research of the CNRS. Her major research interests are the chemical models of heme enzymes and the mechanism of action of peroxide-based antimalarial drugs.

FOOTNOTES

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†This manuscript is dedicated to the known and unknown Chinese chemists of "Project 523" who identified artemisinin in the 1970s.

REFERENCES

1 Hünefeld, F. L. *Die Chemiscus in der tierischen organisation*; Brockhaus: Leipzig, 1840.
2 Perutz, M. F.; Rossmann, M. G.; Cullis, A. F.; Muirhead, H.; Will, G.; North, A. C. T. Structure of haemoglobin: a three-dimensional Fourier synthesis at 5.5 Å resolution obtained by X-Ray analysis. *Nature* **1960**, *185*, 416–422.
3 Meunier, B.; de Visser, S. P.; Shaik, S. Oxidation reactions catalyzed by cytochrome P450 enzymes. *Chem. Rev.* **2004**, *104*, 3947–3980.
4 Dawson, J. H. Probing structure-function relations in heme-containing oxygenases and peroxidases. *Science* **1988**, *240*, 433–439.
5 Fita, I.; Rossmann, M. G. The active center of catalase. *J. Mol. Biol.* **1985**, *185*, 21–37.
6 Edwards, S. L.; Kraut, J. Crystal structure of nitric oxide inhibited cytochrome *c* peroxidase. *Biochemistry* **1988**, *27*, 8074–8081.
7 Francis, S. E.; Sullivan, D. J.; Goldberg, D. E. Hemoglobin metabolism in the malaria parasite, *Plasmodium falciparum. Annu. Rev. Microbiol.* **1997**, *51*, 97–123.
8 Pagola, S.; Stephens, P. W.; Bohle, D. S.; Kosar, A. D.; Madsen, S. K. The structure of malaria pigment beta-hematin. *Nature* **2000**, *404*, 307–310.
9 Sullivan, D. J.; Gluzman, I. Y.; Goldberg, D. E. *Plasmodium* hemozoin formation mediated by histidine-rich proteins. *Science* **1996**, *271*, 219–222.
10 Jani, D.; Nagarkatti, R.; Beatty, W.; Angel, R.; Slebodnick, C.; Andersen, J.; Kumar, S.; Rathore, D. HDP - a novel heme detoxification protein from the malaria parasite. *PLoS Pathog.* **2008**, *4* (e1000053), 1–15.
11 Egan, T. G. Recent advances in understanding the mechanism of hemozoin (malaria pigment) formation. *J. Inorg. Biochem.* **2008**, *102*, 1288–1299.
12 Klayman, D. L. Qinghaosu (artemisinin): an antimalarial drug from China. *Science* **1985**, *228*, 1049–1055.

13 Li, Y.; Wu, Y. L. An over four millennium story behind qinghaosu (artemisinin) - a fantastic traditional Chinese herb. *Curr. Med. Chem.* **2003**, *10*, 2197–2230.

14 Cui, L.; Su, X.-Z. Discovery, mechanisms of action and combination therapy of artemisinin. *Expert Rev. Anti-Infect. Ther* **2009**, *7*, 999–1013.

15 Meshnick, S. R.; Taylor, T. E.; Kamchonwongpaisan, S. Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. *Microbiol. Rev.* **1997**, *60*, 301–315.

16 Cumming, J. N.; Ploypradith, P.; Posner, G. H. Antimalarial activity of artemisinin (qinghaosu) and related trioxanes: mechanism(s) of action. *Adv. Pharmacol.* **1997**, *37*, 253–297.

17 Jefford, C. W. Peroxidic antimalarials. *Adv. Drug Res.* **1997**, *29*, 271–325.

18 Krungkrai, S. R. Y.; Yuthavong, Y. The antimalarial action on *Plasmodium falciparum* of qinghaosu and artesunate in combination with agents which modulates oxidant stress. *Trans. R. Soc. Trop. Med. Hyg.* **1987**, *81*, 710–714.

19 Olliaro, P. L.; Haynes, R. K.; Meunier, B.; Yuthawong, Y. Possible modes of action of artemisinin-type compounds. *Trends Parasitol.* **2001**, *17*, 122–126.

20 Wang, J.; Huang, L.; Li, J.; Fan, Q.; Long, Y.; Li, Y.; Zhou, B. Artemisinin directly targets malarial mitochondria through its specific mitochondrial activation. *PLoS One* **2010**, *5*, e9582.

21 Eckstein-Ludwig, U.; Webb, R. J.; van Goethem, D. A.; East, J. M.; Lee, A. G.; Kimura, M.; O'Neill, P. M.; Bray, P. G.; Ward, S. A.; Krishna, S. Artemisinins target the SERCA of *Plasmodium falciparum*. *Nature* **2003**, *424*, 957–961.

22 Hong, Y. L.; Yang, Y. Z.; Meshnick, S. R. The interaction of artemisinin with malarial hemozoin. *Mol. Biochem. Parasitol.* **1994**, *63*, 121–128.

23 Jefford, C. W.; Vicente, M. G. H.; Jacquier, Y.; Favarger, Y.; Mareda, J.; Millasson-Schmidt, P.; Brunner, G.; Burger, U. The deoxygenation and isomerization of artemisinin and artemether and their relevance to antimalarial action. *Helv. Chim. Acta* **1996**, *79*, 1475–1487.

24 Robert, A.; Meunier, B. Characterization of the first covalent adduct between artemisinin and a heme model. *J. Am. Chem. Soc.* **1997**, *119*, 5968–5969.

25 Robert, A.; Cazelles, J.; Meunier, B. Characterization of the alkylation product of heme by the antimalarial drug artemisinin. *Angew. Chem., Int. Ed.* **2001**, *40*, 1954–1957.

26 Laurent, S. A.-L.; Robert, A.; Meunier, B. C10-modified artemisinin derivatives: efficient heme-alkylating agents. *Angew. Chem., Int. Ed.* **2005**, *44*, 2060–2063.

27 Becker, K.; Tilley, L.; Vennerstrom, J. L.; Roberts, D.; Rogerson, S.; Ginsburg, H. Oxidative stress in malaria parasite-infected erythrocytes: host-parasite interactions. *Int. J. Parasitol.* **2004**, *34*, 163–189.

28 Zhang, Y. A.; Hempelmann, E.; Schirmer, R. H. Glutathione reductase inhibitors as potential antimalarial drugs. *Biochem. Pharmacol.* **1988**, *37*, 855–860.

29 Friebolin, W.; Jannack, B.; Wenzel, N.; Furrer, J.; Oeser, T.; Sanchez, C. P.; Lanzer, M.; Yardley, V.; Becker, K.; Davioud-Charvet, E. Antimalarial dual drugs based on potent inhibitors of glutathione reductase from *Plasmodium falciparum*. *J. Med. Chem.* **2008**, *51*, 1260–1277.

30 Uhlemann, A. C.; Wittlin, S.; Matile, H.; Bustamante, L. Y.; Krishna, S. Mechanism of antimalarial action of the synthetic trioxolane RBX11160 (OZ277). *Antimicrob. Agents Chemother.* **2007**, *51*, 667–672.

31 Crespo, M. d. P.; Avery, T. D.; Hanssen, E.; Fox, E.; Robinson, T. V.; Valente, P.; Taylor, D. K.; Tilley, L. Artemisinin and a series of novel endoperoxide antimalarials exert early effects on digestive vacuole morphology. *Antimicrob. Agents Chemother.* **2008**, *52*, 98–109.

32 Uhlemann, A. C.; Cameron, A. U.; Eckstein-Ludwig, U.; Fishbarg, J.; Iserovich, P.; Zuniga, F. A.; East, M.; Lee, A.; Brady, L.; Haynes, R. K. A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. *Nat. Struct. Mol. Biol.* **2005**, *12*, 628–629.

33 Jambou, R.; Legrand, E.; Niang, M.; Khim, N.; Lim, P.; Volney, B.; Ekala, M. T.; Bouchier, C.; Esterre, P.; Fandeur, T.; Mercereau-Pujalon, O. Resistance of *Plasmodium falciparum* field isolates to in-vitro artemether and point mutations of the SERCA-type PfATPase6. *Lancet* **2005**, *366*, 1960–1963.

34 Afonso, A.; Hunt, P.; Cheesman, S.; Alves, A. C.; Cunha, C. V.; Do Rosario, V.; Cravo, P. Malaria parasites can develop stable resistance to artemisinin but lack mutations in candidate genes *atp6* (encoding the sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase) *tctp*, *mdr1*, *and cg10*. *Antimicrob. Agents Chemother.* **2006**, *50*, 480–489.

35 Dondorp, A. M.; Nosten, F.; Yi, P.; Das, D.; Phyo, A. P.; Tarning, J.; Lwin, K. M.; Ariey, F.; Hanpithakpong, W.; Lee, S. J.; Ringwald, P.; Silamut, K.; Imwong, M.; Chotivanich, K.; Lim, P.; Herdman, T.; An, S. S.; Yeung, S.; Singhasivanon, P.; Day, N. P.; Lindegardh, N.; Socheat, D.; White, N. J. Artemisinin resistance in *Plasmodium falciparum* malaria. *N. Engl. J. Med* **2009**, *361*, 455–469.

36 Bousejra-El Garah, F.; Stigliani, J.-L.; Coslédan, F.; Meunier, B.; Robert, A. Docking studies of structurally diverses antimalarial drugs targeting PfATP6: no correlation between in silico binding affinity and *in vitro* antimalarial activity. *ChemMedChem* **2009**, *4*, 1469–1479.

37 Shin, J. M.; Goldshleger, R.; Munson, K. B.; Sachs, G.; Karlish, S. J. Selective Fe^{2+}-catalyzed oxidative cleavage of gastric H^+, K^+-ATPase. Implication for the energy transduction mechanism of P-type cation pumps. *J. Biol. Chem.* **2001**, *276*, 48440–48450.

38 Montigny, C.; Jaxel, C.; Shainskaya, A.; Vinh, J.; Labas, V.; Møller, J. V.; Karlish, S. J. D.; Le Maire, M. Fe^{2+}-catalyzed oxidative cleavage of Ca^{2+}-ATPase reveal novel features in its pumping mechanism. *J. Biol. Chem.* **2004**, *279*, 43971–43981.

39 Cardi, D.; Montigny, C.; Arnou, B.; Jidenko, M.; Marchal, E.; Le Maire, M.; Jaxel, C. Heterologous expression and affinity purification of eukaryotic membrane proteins in view of functional and structural studies: the example of the sarcoplasmic reticulum Ca(2+)-ATPase. *Methods Mol. Biol.* **2010**, *601*, 247–267.

40 Posner, G. H.; Oh, C. H.; Wang, D.; Gerena, L.; Milhous, W. K.; Meshnick, S. R.; Asawamahasakda, W. Mechanism-based design, synthesis, and *in vitro* antimalarial testing of new 4-methylated trioxanes structurally related to artemisinin: the importance of a carbon-centered radical for antimalarial activity. *J. Med. Chem.* **1994**, *37*, 1256–1258.

41 Robert, A.; Coppel, Y.; Meunier, B. NMR characterization of covalent adducts obtained by alkylation of heme with the antimalarial drug artemisinin. *Inorg. Chim. Acta* **2002**, *339*, 488–496.

42 Robert, A.; Meunier, B. Alkylating properties of antimalaric artemisinin derivatives and synthetic trioxanes when activated by a reduced heme model. *Chem.—Eur. J.* **1998**, *4*, 1287–1296.

43 Rodriguez, M.; Delpon-Bonnet, D.; Bégué, J.-P.; Robert, A.; Meunier, B. Alkylation of manganese(II) tetraphenylporphyrin by antimalarial fluorinated artemisinin derivatives. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1059–1062.

44 Laurent, S. A.-L.; Robert, A.; Meunier, B. C10-modified artemisinin derivatives: efficient heme-alkylating agents. *Angew. Chem., Int. Ed.* **2005**, *44*, 2060–2063.

45 Bousejra-El Garah, F.; Meunier, B.; Robert, A. Artemisone: an efficient heme alkylating agent. *Eur. J. Inorg. Chem.* **2008**, 2133–2135.

46 Laurent, S. A.-L.; Loup, C.; Mourgues, S.; Robert, A.; Meunier, B. Heme alkylation by artesunic acid and trioxaquine DU1301, two antimalarial trioxanes. *ChemBioChem* **2005**, *6*, 653–658.

47 Cazelles, J.; Robert, A.; Meunier, B. Alkylating capacity and reaction products of antimalarial trioxanes after activation by a heme model. *J. Org. Chem.* **2002**, *67*, 609–619.

48 Creek, D. J.; Charman, W. N.; Chiu, F. C. K.; Prankerd, R. J.; Dong, Y.; Vennerstrom, J. L.; Charman, S. A. Relatioship between antimalarial activity and heme alkylation for spiro- and dispiro-1,2,4-trioxolane antimalarials. *Antimicrob. Agents Chemother.* **2008**, *52*, 1291–1296.

49 Coslédan, F.; Fraisse, L.; Pellet, A.; Guillou, F.; Mordmüller, B.; Kremsner, P. G.; Moreno, A.; Mazier, D.; Maffrand, J.-P.; Meunier, B. Selection of a trioxaquine as an antimalarial drug candidate. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 17579–17584.

50 Robert, A.; Benoit-Vical, F.; Claparols, C.; Meunier, B. The antimalarial drug artemisinin alkylates heme in infected mice. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 13676–13680.

51 Bousejra-El Garah, F.; Claparols, C.; Benoit-Vical, F.; Meunier, B.; Robert, A. The Antimalarial trioxaquine DU1301 alkylates heme in malaria-infected mice. *Antimicrob. Agents Chemother.* **2008**, *52*, 2966–2969.

52 Benoit-Vical, F.; Lelièvre, J.; Berry, A.; Deymier, C.; Dechy-Cabaret, O.; Cazelles, J.; Loup, C.; Robert, A.; Magnaval, J.-F.; Meunier, B. Trioxaquines are new antimalarial agents active on all erythrocytic forms, including gametocytes. *Antimicrob. Agents Chemother.* **2007**, *51*, 1463–1472.

53 Coghi, P.; Basilico, N.; Taramelli, D.; Chan, W.-C.; Haynes, R. K.; Monti, D. Interation of artemisinins with oxyhemoglobin Hb-Fe^{II}, Hb-Fe^{II}, carboxyHb-Fe^{II}, heme-Fe^{II}, and carboxyheme-Fe^{II}: significance for mode of action and implications for therapy of cerebral malaria. *ChemMedChem* **2009**, *4*, 2045–2053.

54 Loup, C.; Lelièvre, J.; Benoit-Vical, F.; Meunier, B. Trioxaquines and heme-artemisinin adducts inhibit the *in vitro* formation of hemozoin better than chloroquine. *Antimicrob. Agents Chemother.* **2007**, *51*, 3768–3770.

55 Kannan, R.; Sahal, D.; Chauhan, V. S. Heme-artemisinin adducts are crucial mediators of the ability of artemisinin to inhibit heme polymerization. *Chem. Biol.* **2002**, *9*, 321–332.

56 Dechy-Cabaret, O.; Benoit-Vical, F.; Robert, A.; Meunier, B. Preparation and antimalarial activities of trioxaquines, new modular molecules with a trioxane skeleton linked to a 4-aminoquinoline. *ChemBioChem* **2000**, 281–283.

57 Asawamahasakda, W.; Ittarat, I.; Chang, C.-C.; McElroy, P.; Meshnick, S. R. Effects of antimalarials and protease inhibitors on plasmodial hemozoin production. *Mol. Biochem. Parasitol.* **1994**, *67*, 183–191.

58 Robert, A.; Benoit-Vical, F.; Meunier, B. The key role of heme to trigger the antimalarial activity of trioxanes. *Coord. Chem. Rev.* **2005**, *249*, 1927–1936.

59 Stocks, P. A.; Bray, P. G.; Barton, V. E.; Al-Helal, M.; Jones, M.; Araujo, N. C.; Gibbons, P.; Ward, S. A.; Hughes, R. H.; Biagini, G. A.; Davies, J.; Amewu, R.; Mercer, A. E.; Ellis, G.; O'Neill, P. M. Evidence for a common non-heme chelatable-iron-dependent activation mechanism for semisynthetic and synthetic endoperoxide antimalarial drugs. *Angew. Chem., Int. Ed.* **2007**, *46*, 6278–6283.

60 Vippagunta, S. R.; Dorn, A.; Bubendorf, A.; Ridley, R. G.; Vennerstrom, J. L. Deferoxamine: stimulation of hematin polymerization and antagonism of its inhibition by chloroquine. *Biochem. Pharmacol.* **1999**, *58*, 871–824.

61 Sullivan, D. J., Jr.; Matile, H.; Ridley, R. G.; Goldberg, D. E. A common mechanism for blockade of heme polymerization by antimalarial quinolines. *J. Biol. Chem.* **1998**, *273*, 31103–31107.

62 Pradines, B.; Ramiandrasoa, F.; Basco, L. K.; Bricard, L.; Kunesch, G.; Le Bras, J. *In vitro* activities of novel catecholate siderophores against *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* **1996**, *40*, 2094–2098.

63 Kamchonwongpaisan, S.; Paitayatat, S.; Thebtaranonth, Y.; Wilairar, P.; Yuthavong, Y. Mechanism-based development of new antimalarials: synthesis of derivatives of artemisinin attached to iron chelators. *J. Med. Chem.* **1995**, *38*, 2311–2316.

64 Konijn, A. M.; Glickstein, H.; Vaisman, B.; Meyron-Holtz, E.; Slotki, I. N.; Cabantchik, Z. I. The cellular labile iron pool and intracellular ferritin in K562 cells. *Blood* **1999**, *94*, 2128–2134.

65 De Domenico, I.; McVey Ward, D.; Kaplan, J. Specific iron chelators determine the route of ferritin degradation. *Blood* **2009**, *114*, 4546–4551.

66 Asawamahasakda, W.; Ittarat, I.; Pu, Y. M.; Ziffer, H.; Meshnick, S. R. Reaction of antimalarial endoperoxides with specific parasite proteins. *Antimicrob. Agents Chemother.* **1994**, *38*, 1854–1858.

67 Li, F.; Zhang, D.; Fujise, K. Characterization of fortilin, a novel antiapoptotic protein. *J. Biol. Chem.* **2001**, *276*, 47542–47549.

68 Bhisutthibhan, J.; Pan, X. Q.; Hossler, P. A.; Walker, D. J.; Yowell, C. A.; Carlton, J.; Dame, J. B.; Meshnick, S. R. The translationally controlled tumor protein homolog and its reaction with the antimalarial drug artemisinin. *J. Biol. Chem.* **1998**, *273*, 16192–16198.

69 Sijwali, P. S.; Rosenthal, P. J. Gene disrupting confirms a critical role for the cysteine protease falcipain-2 in hemoglobin hydrolysis by *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4384–4389.

70 Wang, D. Y.; Wu, Y. L. A possible antimalarial action mode of qinghaosu (artemisinin) series compounds. Alkylation of reduced glutathione by *C*-centered primary radicals produced from antimalarial compound qinghaosu and 12-(2,4-dimethoxyphenyl)-12-deoxoqinghaosu. *Chem. Commun.* **2000**, 2193–2194.

71 Bousejra-El Garah, F.; Pitié, M.; Vendier, L.; Meunier, B.; Robert, A. Alkylating ability of artemisinin after Cu(I)-induced activation. *J. Biol. Inorg. Chem.* **2009**, *14*, 601–610.

72 Sun, W. C.; Han, J. X.; Yang, W. Y.; Deng, D. A.; Yue, X. F. Antitumor activities of 4 derivatives of artemisinic acid and artemisinin-B *in vitro*. *Acta Pharmacol. Sin.* **1992**, *13*, 541–543 (article in Chinese, English abstract available).

73 Disbrow, G. L.; Baege, A. C.; Kierpiec, K. A.; Yuan, H.; Centeno, J. A.; Thibodeaux, C. A.; Hartmann, D.; Schlegel, R. Dihydroartemisinin is cytotoxic to papillomavirus-expressing epithelial cells *in vitro* and *in vivo*. *Cancer Res.* **2005**, *65*, 10854–10861.

74 Hou, J.; Wang, D.; Zhang, R.; Wang, H. Experimental therapy of hepatoma with artemisinin and its derivatives: *in vitro* and *in vivo* activity, chemosensitization, and mechanism of action. *Clin. Cancer Res.* **2008**, *14*, 5519–5530.

75 Singh, N. P.; Lai, H. Selective toxicity of dihydroartemisinin and holotransferrin toward human breast cancer cells. *Life Sci.* **2001**, *70*, 49–56.

76 Nakase, I.; Gallis, B.; Takatani-Nakase, T.; Oh, S.; Lacoste, E.; Singh, N. P.; Goodlett, D. R.; Tanaka, S.; Futaki, S.; Lai, H.; Sasaki, T. Transferrin receptor-dependent cytotoxicity of artemisinin-transferrin conjugates on prostate cancer cells and induction of apoptosis. *Cancer Lett.* **2009**, *274*, 290–298.

77 Posner, G. H.; Ploypradith, P.; Parker, M. H.; O'Dowd, H.; Woo, S.-H.; Northrop, J.; Krasavin, M.; Dolan, P.; Kensler, T. W.; Xie, S.; Shapiro, T. A. Antimalarial, antiproliferative, and antitumor activities of artemisinin-derived, chemically robust, trioxane dimers. *J. Med. Chem.* **1999**, *42*, 4275–4280.

78 Rosenthal, A. S.; Chen, X.; Liu, J. O.; West, D. C.; Hergenrother, P. J.; Shapiro, T. A.; Posner, G. H. 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.* **2009**, *52*, 1198–1203.

79 Jung, M.; Lee, S.; Ham, J.; Lee, K.; Kim, H.; Kim, S. K. Antitumor activity of novel deoxyartemisinin monomers, dimers, and trimers. *J. Med. Chem.* **2003**, *46*, 987–994.

80 Chadwick, J.; Mercer, A. E.; Park, B. K.; Cosstick, R.; O'Neill, P. M. Synthesis and biological evaluation of extraordinarily potent C-10 carba artemisinin dimers against *P. falciparum* malaria parasites and HL-60 cancer cells. *Bioorg. Med. Chem.* **2009**, *17*, 1325–1338.

81 Alagbala, A. A.; McRiner, A. J.; Borstnik, K.; Labonte, T.; Chang, W.; d'Angelo, J. G.; Posner, G. H.; Foster, B. A. Biological mechanism of action of novel C-10 non-acetal trioxane dimers in prostate cancer cell lines. *J. Med. Chem.* **2006**, *49*, 7836–7842.

82 Stockwin, L. H.; Bingnan, H.; Yu, S. X.; Hollingshead, M. G.; ElSohly, M. A.; Gul, W.; Slade, D.; Galal, A. M.; Newton, D. L. Artemisinin dimer anticancer activity correlates with heme-catalyzed reactive oxygen species generation and endoplasmic reticulum stress induction. *Int. J. Cancer* **2009**, *125*, 1266–1275.

83 Fujita, T.; Felix, K.; Pinkaew, D.; Hutadilok-Towatana, N.; Liu, Z.; Fujise, K. Human fortilin is a molecular target of dihydroartemisinin. *FEBS Lett.* **2008**, *582*, 1055–1060.

84 Dell'Eva, R.; Pfeffer, U.; Vené, R.; Anfosso, L.; Forlani, A.; Albini, A.; Efferth, T. Inhibition of angiogenesis *in vivo* and growth of Kaposi's sarcoma xenograft tumors by the antimalarial artesunate. *Biochem. Pharmacol.* **2004**, *68*, 2359–2366.

85 Youns, M.; Efferth, T.; Reichling, J.; Fellenberg, K.; Bauer, A.; Hoheisel, J. D. Gene expression profiling identifies novel key players involved in the cytotoxic effect of artesunate on pancreatic cancer cells. *Biochem. Pharmacol.* **2009**, *78*, 273–283.

86 Li, L.-N.; Zhang, H.-D.; Yuan, S.-J.; Tian, Z.-Y.; Wang, L.; Sun, Z.-X. Artesunate attenuates the growth of human colorectal carcinoma and inhibits hyperactive Wnt/β-catenin pathway. *Int. J. Cancer* **2007**, *121*, 1360–1365.

87 Sundar, S. N.; Marconett, C. N.; Doan, V. B.; Willoughby, J. A.; Firestone, G. L. Artemisinin selectively decreases functional levels of estrogen receptor-alpha and ablates estrogen-induced proliferation in human breast cancer cells. *Carcinogenesis* **2008**, *29*, 2252–2258.

88 Willoughby, J. A.; Sundar, S. N.; Cheung, M.; Tin, A. S.; Modiano, J.; Firestone, G. L. Artemisinin blocks prostate cancer growth and cell cycle progression by disrupting Sp1 interactions with the cyclin-dependent kinase-4 (CDK4) promoter and inhibiting CDK4 gene expression. *J. Biol. Chem.* **2009**, *284*, 2203–2213.