ARTEMISININS AND SYNTHETIC PEROXIDES AS HIGHLY EFFICIENT ANTIMALARIALS

Dejan M. Opsenica[1*], Bogdan A. Šolaja[2*]

[1]*Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia,*
[2]*Faculty of Chemistry, University of Belgrade, Studentski trg 16, P.O. Box 51, 11158 Belgrade, Serbia*

dopsen@chem.bg.ac.rs; bsolaja@chem.bg.ac.rs
*To whom correspondence should be addressed

Malaria is devastating disease and global public health problem, with nearly half world population exposed to risk. Illness is caused by five *Plasmodium* species, *P. falciparum*, *P. ovale*, *P. vivax*, *P. malaria* and *P. knowlesi*, from which *P. falciparum* is the most serious one causing cerebral malaria and is the major reason for malaria mortality. Vaccine against malaria is not expected in the near future and chemotherapy remains as most feasible alternative for treatment of the disease. The development of widespread drug-resistance to chloroquine (CQ), the most successful antimalarial drug up to date, has resulted in severe health issues for countries in malaria endemic regions. Organic peroxides, like artemisinins, 1,2,4-trioxanes, 1,2,4-trioxolanes, 1,2,4,5-tetraoxanes and their chimeras, are the best choice for malaria treatment nowadays. These therapeutics are fast acting, non-toxic, low costing and without reported data of parasite resistance. Stability of peroxide bonds enables synthetic comfort and resulting in diversity of synthesized structures. The most important classes of peroxide antimalarials with promising representatives are reviewed and possible mechanisms of action were presented in details.

Key words: antimalarials; artemisinin; peroxides; trioxanes; trioxolanes; tetraoxaneS; chimeras

АРТЕМИСИНИ И СИНТЕТИЧКИ ПЕРОКСИДИ КАКО МНОГУ ЕФИКАСНИ АНТИМАЛАРИЦИ

Маларијата е многу тежок светски проблем на јавното здравство, бидејќи околу половина од свтетската популација е изложена на ова заболување. Болеста е предизвикана од пет видови *Plasmodium*, *P. falciparum*, *P. ovale*, *P. vivax*, *P. malaria* и *P. knowlesi*, од кои најопасен е *P. falciparum* кој предизвикува церебрална маларија и е главна причина за смртноста кај маларијата. Вакцина против маларија не се очекува во блиска иднина и хемотерапијата останува единствена практична алтернатива во лекувањето и третирањето на болеста. Развивањето на сè поголемата резистентност на лекот хлорохин (CQ), досега најуспешен познат лек против маларија, доведува до сериозни здравствени проблеми во државите со области на ендемска појава на маларија. Органските пероксиди-артемисини, 1,2,4-триоксани, 1,2,4-триоксолани, 1,2,4,5-тетраоксани и нивните разновидни форми, во денешно време се најдобар избор за третирање на маларијата. Овие терапевтици имаат брзо дејство, нетоксични се, евтини и не е позната резистентност на паразитот спрема нив. Стабилноста на нивните пероксидни врски овозможува синтетизирање и добивање разновидни синтетички форми. Во прилогов подетално се дискутирани најважните класи и најпогодните претставници на пероксидни антималарици, како и можниот механизам на нивното дејство.

Клучни зборови: антималарици; артемисинин; пероксиди; триоксани; триоксолани; тетраоксани; химери

1. INTRODUCTION

Malaria is a massive global public health problem in more than 100 countries, inhabited by some 40% of the world's population, with 300–500 million clinical cases and over one million deaths each year [1]. Every 30 seconds a child somewhere dies of malaria. In any given year, nearly 10% of the global population will suffer a case of malaria, and more then half of the cases are caused by *Plasmodium falciparum* and the rest are caused by *Plasmodium vivax* [2]. While a vaccine against malaria continues to be elusive [3], chemotherapy remains the most viable alternative towards treatment of the disease. During last few years the situation has become urgent in many ways, but mainly because the malaria parasite has developed multiple drug resistance (MDR) to most used drugs, such as quinine, chloroquine (CQ), mefloquine (MFQ), primaquine, and others. The resistance is most serious with CQ, the most widely and cheapest drug used in treatment, because of the development of CQ-resistant (CQR) strains of *P. falciparum* (*P.f.*) – Indochina W2, Brazil IEC-306, FCR3, and K1. Combating malaria is even more complex because of the contemporary development of resistance by the mosquito vector to currently used insecticides [4]. For the reasons indicated above, the World Health Organization (WHO) has designated malaria as a major health problem that urgently needs the mobilisation of scientific, industrial, and political communities [5].

Artemisinin (ART, **1**), the active principle of plant *Artemisia annua* L., due to its high activity against CQR strains [6], opened new possibilities for combating this pestilence. Since the early 1980's hundreds of semisynthetic and synthetic peroxides were developed and tested for their antimalarial activity results, which have been extensively reviewed [7, 8, 9]. The therapeutic significance of this drug class lies in its noticeable lack of parasite resistance and absence of toxicity during patient treatment. The only evidence of *in vitro* resistance was found in French Guiana [10] and the Thailand-Cambodia border area, where certain ART de-

rivatives were used in uncontrolled and illegal self-medication.

This review summarizes recent achievements in the area of peroxide drug development for malaria chemotherapy.

2. THE LIFE CYCLE OF THE MALARIA PARASITE

Protozoa of the genus *Plasmodium* cause malaria. Four species of *Plasmodium* cause the disease in humans, *P. falciparum*, *P. malaria*, *P. ovale* and *P. vivax*. Of these, *P. falciparum* may cause the condition known as cerebral malaria, and is responsible for the majority of fatal outcomes.

The pathogenesis [11] and life cycle of the malaria parasite is complex [12] and consists of two stages: the sexual stage (sporogony), which takes place within the mosquito, and the asexual stage (schizogony), which takes place in the host [7c, 13]. The illness is started when the infected female mosquito of the genus *Anopheles* feeds on the blood of the uninfected vertebrate host. In less than one hour sporozoites travel to the liver, invade hepatocytes, and undergo exoerythrocytic schizogony. After some time, depending on the plasmodium species, the schizonts transforms into merozoites, which after being released into the blood stream invade erythrocytes. After significant reorganization of membrane proteins of the occupied erythrocyte [12e], merozoites undergo erythrocytic schizogony, which comprises *young rings* (12h after erythrocyte infection), *mature rings* (18h), *early trophozoites* (24h), *mature trophozoites* (30h), *early schizonts* (36h) and *mature schizonts* (42h). At the end of erythrocytic schizogony, parasites return into merozoite form, but enormously multiplied causing splattering of the host. Released merozoites invade new red blood cells and start a new erythrocytic schizogony cycle. Erythrocytic schizogony occurs every 2–3 days, depending on Plasmodium species. Each cycle is accompanied by typical malaria symptoms such as fever, chills, headache and exhaustion. After several cycles, some

of the merozoites undergo sexual development and transform into gametocytes.

Gametocytes remain in the erythrocytes and are consumed by an anopheles mosquito. In the mosquito, female and male gametocytes join and form a zygote. Within 18 to 24 hours, the zygote transforms into a slowly motile ookinete. Between 7 and 15 days, depending on the Plasmodium species, and ambient temperature, a single oocyst forms more than 10,000 sporozoites. The motile sporozoites migrate into the salivary glands and accumulate in the acinar cells. When the infected mosquito bites a susceptible vertebrate host, a new parasite cycle starts. In Table 1 the length of the *P. falciparum* life cycle is presented.

T a b l e 1

Life cycle stages of P. falciparum with time lengt

1	Ookinete formation	24 to 48 hours
2	Oocyst maturation	9 days
	Invasion of salivary glands (1+2)	10 days
3	Circulation of sporozoites in the blood stream	1 hour max.
4	Hepatic schizogony	6 days
5	Erythrocytic schizogony	48 hours
6	Gametocytogony	10 days
	Complete cycle (1 to 6)	27 days

The malaria parasite has limited capacity for *de novo* amino acid synthesis, and its survival is dependent on haemoglobin proteolysis. The parasite digests haemoglobin in the food vacuole (FV), supplying itself with the amino acids necessary for nutrition, and liberates free haem [Fe(II)PPIX], which is subsequently oxidized to haematin [Fe(III)PPIX]. Free haematin can damage cellular metabolism by inhib-

iting enzymes, by peroxidation of membranes, and by producing the reactive oxygen species (ROS) in the acidic environment of the FV [14]. In order to protect itself the parasite eliminates haematin by sequestering it as hemozoin. Hemozoin is a noncovalent aggregate of several units of haematin linked via coordinate bonds formed between the Fe(III) of one haematin and the carboxylate side chain of the adjacent one [15]. Hemozoin is insoluble, and accumulates in the lymphatic tissue, liver, bone marrow, and brain. It was found that haem [Fe(II)PPIX] cannot polymerize to hemozoin and is an effective inhibitor of Fe(III)PPIX polymerization, even better so than CQ [16].

3. ARTEMISININ AND DERIVATIVES

The active ingredient of *Artemisia annua* L. was identified as *artemisinin* **1** (ART, *qinghaosu* – *QHS*, Figure 1), a sesquiterpene lactone with the endoperoxide function [6]. The very same compound was also isolated in Belgrade by Professor Milutin Stefanović's group in the early 1970's, however, the wrong structure was proposed (Figure 1) [17]. The structure of ART was elucidated in 1979 by X-ray analysis, which was supported by total synthesis [18]. Since then, many total syntheses have been achieved [19, 20], including the newest one by Hao and co-workers [21]. Furthermore, large scale synthesis of ART was developed by continuous flow synthesis starting from artemisinic acid [22]. ART is an erythrocytic schizonticid and exhibits rapid activity against all types of human and most animal malaria. It is effective against both the CQS and CQR strains of *P. falciparum* [(IC_{50}) D6 = 9.0 nM, W2 = 6.7 nM, TM91C235 = 13.0 nM] [23], and has been successfully used for the treatment of severe cerebral malaria [7c]. ART has poor solubility in water and it is administrated as a water or oil suspension. Better results were achieved when it was administered intramuscularly (i.m.) as a suspension in oil than orally (p.o.) in water.
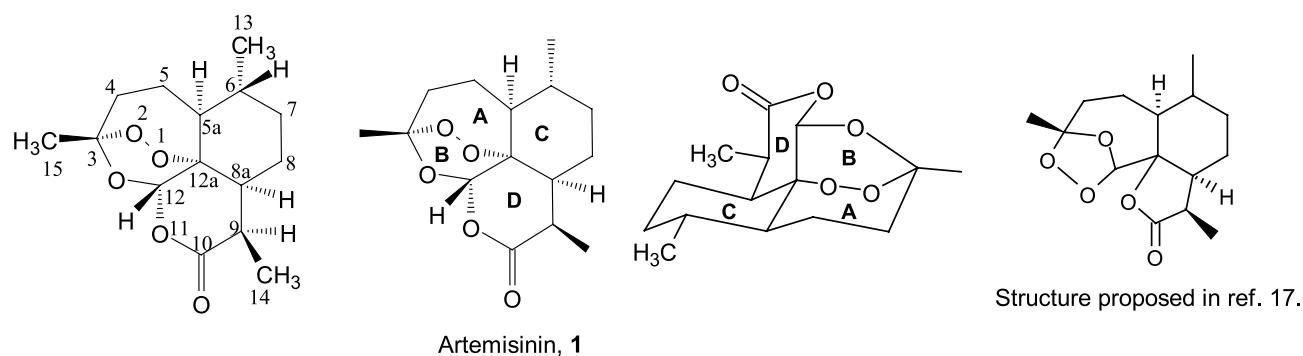
Fig 1. Structure of artemisinin

Metabolites isolated after *p.o.* administration are devoid of peroxide function and have no antimalarial activity, strongly suggesting that the peroxide function is a critical part of the pharmacophore. This was demonstrated by a complete lack of activity of derivative **2** [24] (Figure 2). The high activity of deoxo derivative **3** [25] reveals that lactone ring D is not required for activity, as was confirmed with a number of derivatives (*vide infra*). Similarly, tricyclic derivative **4** (*vide infra*), which is nearly as active as ART showed that ring A is also not required. On the other hand, the low activity of isostere **5** clearly shows the importance of the ketal moiety for good activity [26]. SAR of ARTs showed that the high complexity of the structure is not necessary, and more important, further optimization and structural simplification is possible for activity improvement. In addition, it was assumed that the good activity of ART was due, at least partially, to its amphiphilic structure, which facilitates cell membrane permeability.
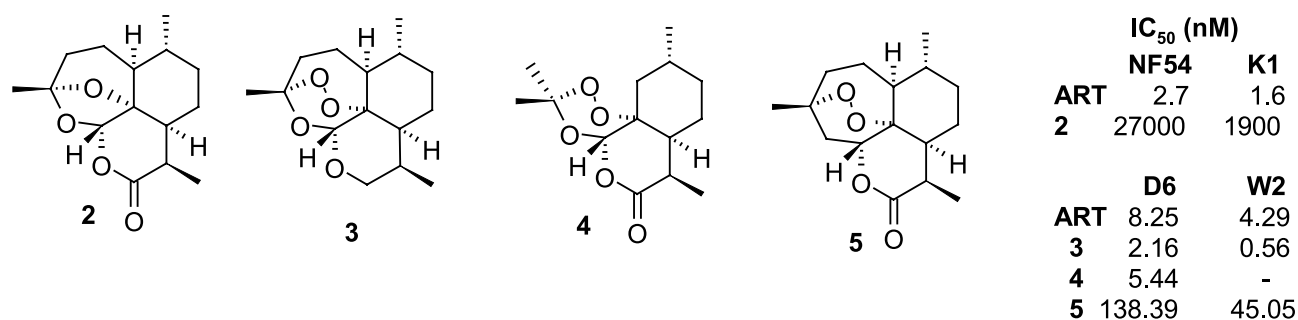

	IC$_{50}$ (nM)	
	NF54	**K1**
ART	2.7	1.6
2	27000	1900
	D6	**W2**
ART	8.25	4.29
3	2.16	0.56
4	5.44	-
5	138.39	45.05

Fig. 2. Structures of derivatives that illustrated SAR

3.1. *First generation of artemisinin derivatives*

The first semisynthetic derivatives of ART were simple ethers (**7**), esters (**8**), and carbonates (**9**) of dihydroartemisinin (DHA, **6**) (Scheme 1) [6, 7a]. The DHA lactol, which is easily obtained from **1**, and is the first metabolite of ART, is twice as active as ART but exhibits a relatively high degree of neurotoxicity. DHA is effective against severe cerebral malaria, but suffers from poor oral bioavailability, high recrudescence, and noteworthy is the report on its neurotoxicity [27], especially when used in higher doses in continuity [28].
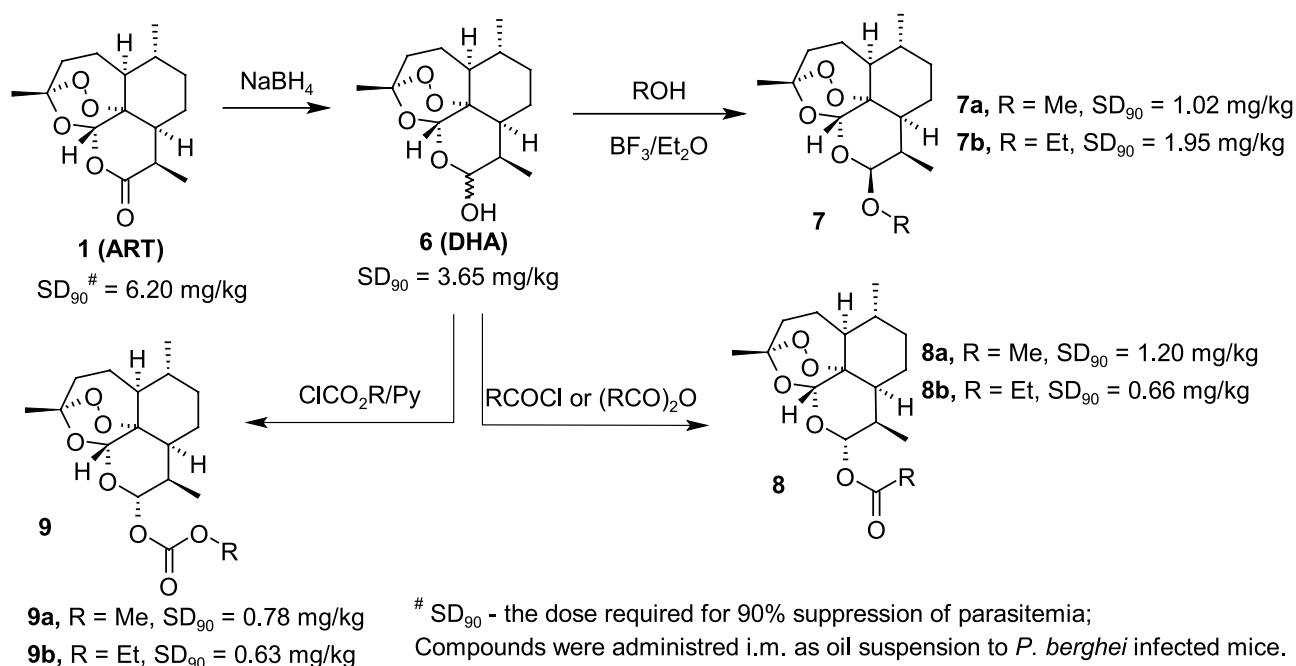
Scheme 1. Transformations of ART into first generation derivatives

Artemether **7a** and arteether **7b** were designed to increase lipid solubility, PK profile and antimalarial activity in comparison to ART and DHA. Both are fast acting blood schizonticides and are especially active against CQR strains. Administration in high doses can produce neurotoxic effects, probably due to its metabolitic conversion to DHA. Later, it was shown that neurotoxicity occurs when the compounds are administered in at least five times higher doses than is recommended. Today, artemether **7a** is the most widely used derivative and is applied as oil solution for i.m. injection (Artenam®, Artemos®), or recently in combination with lumefantrine (Coartem®).

Although carbonates **9** exhibited higher *in vivo* activity then ART, DHA and **7**, there is no reported clinical application, probably because of their low stability under physiological conditions, due to their fast hydrolysis to DHA.

For the treatment of severe forms of malaria, water-soluble derivatives of ART (Figure 3) are indispensable, such as sodium-artesunate **8c** and artelinic acid derivatives **10** [29]. The derivatives can be administered intravenously

(i.v.), which enables faster and more efficacious antimalarial effects compared to less polar derivatives that are administered i.m as an oil suspension. Artesunate **8c** rapidly diminishes parasitemia and is very efficacious in the restoration to consciousness of comatose cerebral malaria patients [30]. Its shortcoming is high recrudescence of the disease and is normally used in ACT[1] with MFQ [7e] and amodiaquine (Arsucam®). Na-artesunate is used as freshly prepared solution in dextrose or saline because of rapid hydrolysis to DHA.
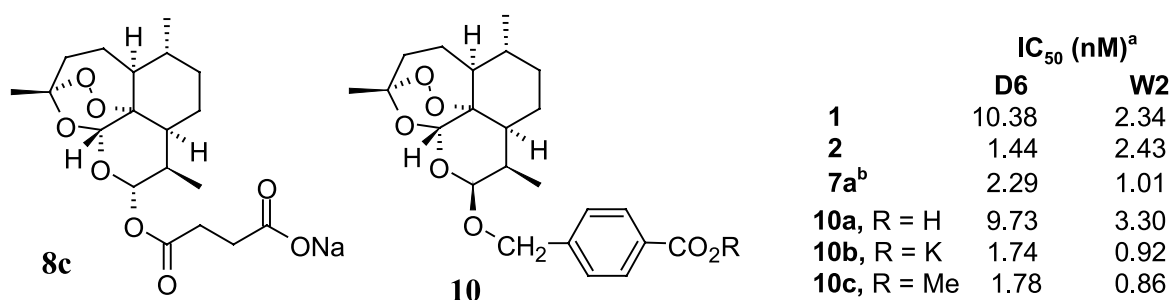
Artelinic acids **10a** possesses C(10) β-ether linkages and thus are hydrolytically more stable than artesunate **8c**. The acid **10a** expresses *in vitro* activity comparable to ART and **8c** against the D6 and W2[2] strains of *P. falciparum,* but shows superior *in vivo* activity against *P. berghei* [29]. Moreover, **10a** has a longer plasma-life [29], higher plasma concentration, higher binding

---

[1] ACT – **a**rtemisinin **c**ombination **t**herapy

[2] D6 (*Sierra Leone strain*) is resistant to mefloquine and susceptible to chloroquine, pyrimethamine and sulfadoxine; W2 (*Indochina strain*) is resistant to chloroquine, quinine, pyrimethamine and sulfadoxine, and susceptible to mefloquine.

capacities, and lower toxicity in comparison to other first generation semisynthetic ART derivatives [7c]. Although methyl ester **10c** exhibits higher *in vitro* activity, it is less suitable because of its low solubility in water. Artelinic acid **10a** was relatively well tolerated in rats; for example, 50 mg kg^{-1} intramuscular doses of **10a** given for 7 consecutive days caused only mild anorectic toxicity. In one study [31], single intravenous

dose LD$_{50}$ values for **10a** and artesunate **8c** were 120 and 350 mg kg^{-1}, respectively. During neurotoxicity tests 36 mg kg^{-1} intramuscular doses of **10a** were administered for 7 consecutive days to rats, and no behavioral abnormalities or brain damage were detected [32]. However, recently [33], neuronal injury became evident when 160 mg kg^{-1} of **10a** was administered orally to rats for 9 consecutive days.

	IC$_{50}$ (nM)^a	
	D6	**W2**
1	10.38	2.34
2	1.44	2.43
7a^b	2.29	1.01
10a, R = H	9.73	3.30
10b, R = K	1.74	0.92
10c, R = Me	1.78	0.86

^{a} Data taken from reference 20; ^{b} Data taken from ref. 22.

Fig. 3. Structures and antimalarial activities of derivatives **8a** and **10**

Recently, a new group of ether derivatives **11** and **12** (Figure 4) were synthesized to examine the role of side chain elongation on antimalarial activity [34]. It was shown that elongation of the side chain increased activity, but not at the level of parent arteether **7b** (Table 2). The most active derivatives were **11e**

and **11f**, which exhibited 100% suppression under 6 mg kg^{-1} × 4 day doses. Derivative **11f** was more active, with 100% suppression and 5/6 cured mice under 6 mg kg^{-1} × 4 day doses. Interestingly, corresponding acids **12** were significantly less active, similarly to artesunic acid **8d** being less active in comparison to **7b**.
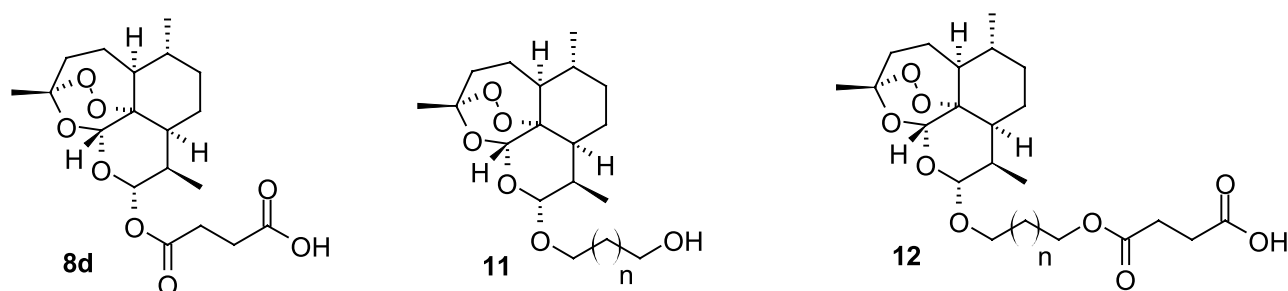

**Fig. 4.** Structures of derivatives **8d**, **11** and **12**

T a b l e   2

Antimalarial activity of selected compounds 11 *against multidrug resistant strain P. yoelii nigeriensis*

Compound	n	mg kg^{-1} × 4 day	% suppression of parasitemia on day 4	Cured/Treated
11a	0	48	100	12/12
		24	100	11/12
		12	100	9/11
		6	100	3/6
11b	1	48	100	5/5
		24	100	5/5
		12	100	9/11
		6	100	6/12
		3	98.3	0/6
11c	2	48	100	5/5
		24	100	5/5
		12	100	17/17
		6	100	11/12
		3	99.5	0/6
11d	3	48	100	5/5
		24	100	5/5
		12	100	5/5
		6	100	1/5
11e	4	48	100	6/6
		24	100	6/6
		12	100	6/6
		6	100	9/12
		3	100	0/6
11f	6	48	100	6/6
		24	100	6/6
		12	100	6/6
		6	100	5/6
7b		6	100	6/6
		3	100	0/6
8d		48	100	6/6
		24	100	0/5

First generation derivatives suffer from short plasma life and CNS toxicity caused by rapid metabolism into DHA [35]. Ethers **7** were metabolized by cytochrome P-450 forming C(α)-hydroxyl derivative **13**, which was further transformed into DHA (Scheme 2). Esters **8** simply hydrolyze to DHA. In addition, all of these derivatives possess acetal or hemiacetal groups in the D ring, and therefore are readily hydrolyzed under acidic conditions after *p.o.* administration. The longer plasma half-life of artelinic acid **10a,** most likely, is caused by steric hindrance and poorer accessibility to P-450. Consequently, second generation semisynthetic derivatives of ART were designed to overcome these disadvantages.
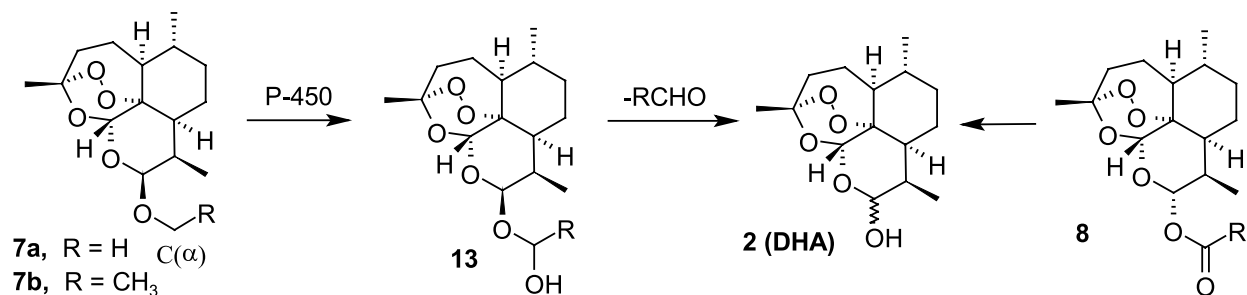
Scheme 2. Metabolic transformations of derivatives **7** and **8**

3.2. *Second generation of artemisinin derivatives*

Suppression of metabolic transformations was attempted in several ways. One was modification of artelinic acid **10a** by introducing substituents on the α-carbon (Figure 5) [35]. The compounds were more active against W2 than against the D6 clone, and it was found that electronic effects, lipophilicity, and steric factors have considerable influence on antimalarial activity. Configuration at the C(α) also contributed to activity, i.e. (*S*)-isomers were more

active than (*R*)-isomers. The most active compound **14** was 10, 20 and 40 times more active then **7b**, ART and **10a**, respectively.

Replacing the *O*-alkyl group with an *O*-phenyl group should prevent the oxidative dealkylation described in Scheme 2 (Figure 5) [36]. Derivative **15** was *in vitro* as active as artemether, but had outstanding *in vivo* antimalarial activity that was higher than clinically used sodium artesunate. Moreover, introducing a CF₃-group metabolically stabilized the compound compared to its parent compound (i.e., H instead of CF₃).
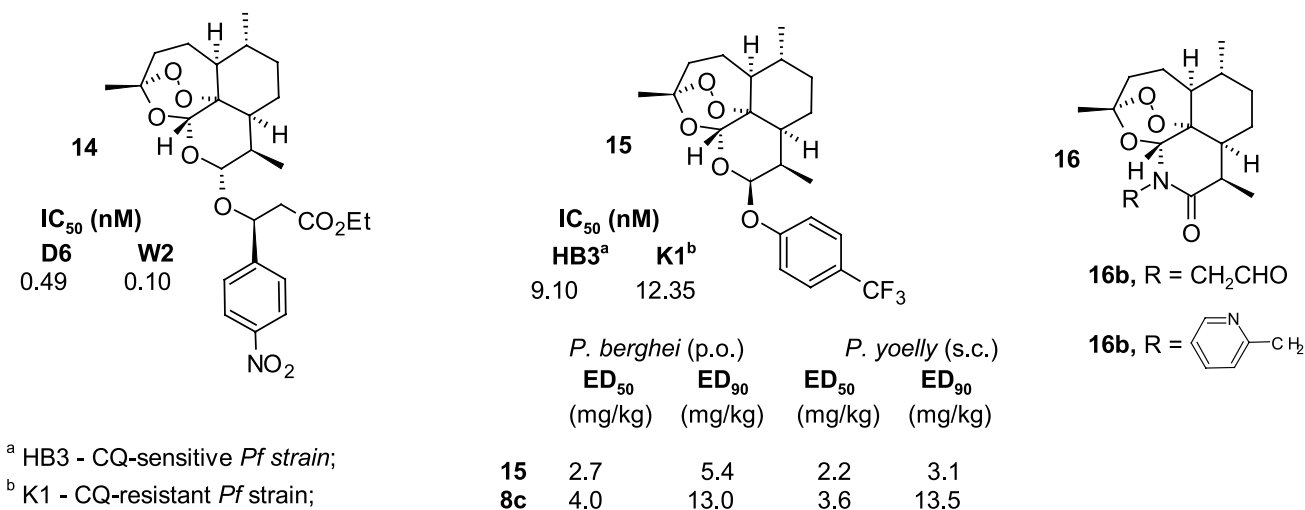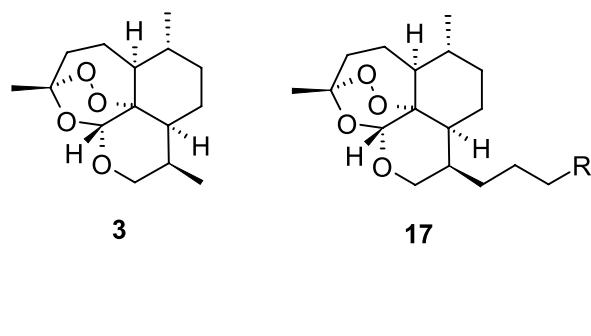

	P. berghei (p.o.)		*P. yoelly* (s.c.)	
	ED$_{50}$ (mg/kg)	ED$_{90}$ (mg/kg)	ED$_{50}$ (mg/kg)	ED$_{90}$ (mg/kg)
15	2.7	5.4	2.2	3.1
8c	4.0	13.0	3.6	13.5

[a] HB3 - CQ-sensitive *Pf strain*;
[b] K1 - CQ-resistant *Pf* strain;

ED$_{50}$ = effective dose - dose that decreases parasitemia by 50%

Fig. 5. Structures and antimalarial activities of derivatives **14–16**

Introducing a lactam ring instead of lactone or acetal moieties also stabilized compounds under physiological conditions. Many 11-azaartemisinins **16** (Figure 5) were described [37] and they exhibited significantly higher antimalarial activity.

Moreover, the possibility of changing the substituents on the nitrogen enables the fine-tuning of the activity. New compounds **16a** and **16b** were 26 and 22 times more active then ART, respectively [37b].

The superior activity of deoxoartemisinin **3** [21] encouraged the synthesis of a series of C(9)-substituted derivatives **17** (Figure 6) [38, 39]. Derivatives **17a-h** were 21–33 times more active then ART against the W2 clone and 50–70 times more active against the D6 clone, clearly demon-strating that the removal of the lactone carbonyl provides excellent potency enhancement [38]. Those and some new derivatives [39] were tested *in vivo* both s.c. and *p.o.* The activity of derivative **17a** was superior to that of ART, curing all mice at 8 mg kg^{-1}/day s.c. dose.

17a, R = CH$_3$
17b, R = C$_6$H$_5$
17c, R = *p*-ClC$_6$H$_4$
17d, R = *p*-FC$_6$H$_4$
17e, R = *p*-MeOC$_6$H$_4$
17f, R = 3,4-Cl$_2$C$_6$H$_3$
17g, R = 3,4-CF$_3$C$_6$H$_3$
17h, R = 3,4-F$_2$C$_6$H$_3$

	IC$_{50}$ (nM)	
	D6	W2
ART	8.25	4.29
3	2.16	0.56
17b	2.42	3.62
17c	4.67	6.39
17g	1.53	13.98
17h	2.01	15.91

Fig. 6. Structures and antimalarial activities of derivatives 3 and 17

A series of C(10) carbon heterocyclic sub-stituted derivatives (Figure 7) of deoxyartemisi-nin were synthesized using a short and efficient synthetic procedure [40]. The compounds were tested *in vitro* against CQS NF54 *P.f.* strain and demonstrated activity similar to that of ART. Derivative **18a** was the most active in the series with an IC$_{50}$ = 1.4 nM. The compounds **19a-d** were also more stable at physiological condi-tions then ART, and at the same time, retained very good to excellent activity [41]. Some of these derivatives are significantly more active (IC$_{50}$ = 1.3–3.2 nM) than ART (IC$_{50}$ = 9.9 nM) against the CQS NF54 *P.f.* strain.

18a, R$_1$ = H
18b, R$_1$ = CH$_3$
18c

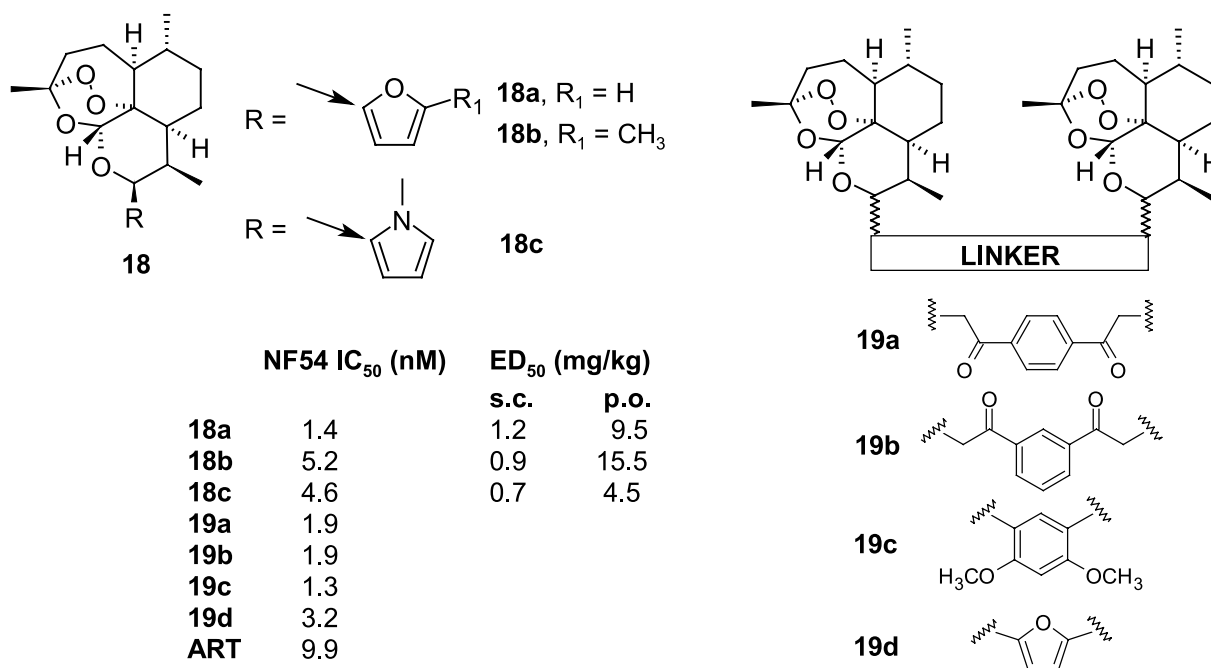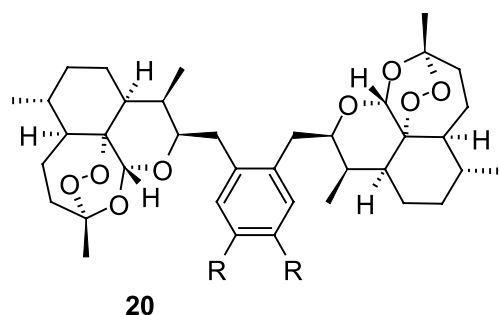|	NF54 IC$_{50}$ (nM)	ED$_{50}$ (mg/kg)	
		s.c.	p.o.
18a	1.4	1.2	9.5
18b	5.2	0.9	15.5
18c	4.6	0.7	4.5
19a	1.9		
19b	1.9		
19c	1.3		
19d	3.2		
ART	9.9		

Fig. 7. Structures and antimalarial activities of derivatives **18** and **19**

Hydrolytically stable C(10)-nonacetal artemisinin dimers **20** (Figure 8), which possess phthalate derivatives as linkers, showed higher *in vitro* antimalarial activities against the CQS NF54 *P. f.* strain than ART, with **20a** and **20b** being the most active [42]. These two dimers were 3 times and 37 times more efficacious then artesunate **8c** when administered s.c., and **20b** was 1.5 times more efficacious then **8c** (*p.o.*).

	NF54	ED$_{50}$ (mg/kg)	
	IC$_{50}$ (nM)	s.c.	p.o.
20a R = CO$_2$Me	1.6	0.71	
20b R = CH$_2$OH	0.77	0.06	2.6
ART	6.6		
8c		2.2	4.0

Fig. 8. Structures and antimalarial activities of dimers **20**

A new generation of ART dimers with excellent *in vivo* activity in *P. berghei* infected mice was developed [43, 44]. Eleven new derivatives **21–27** (Figure 9) showed curative activity at 3 × 30 mg kg^{-1} oral doses. At this dose the average mouse survival period was ≥3 times longer in comparison to artesunate **8c** (>30 days for derivatives **21–27** *vs*. 7 days for **8c**) [44].
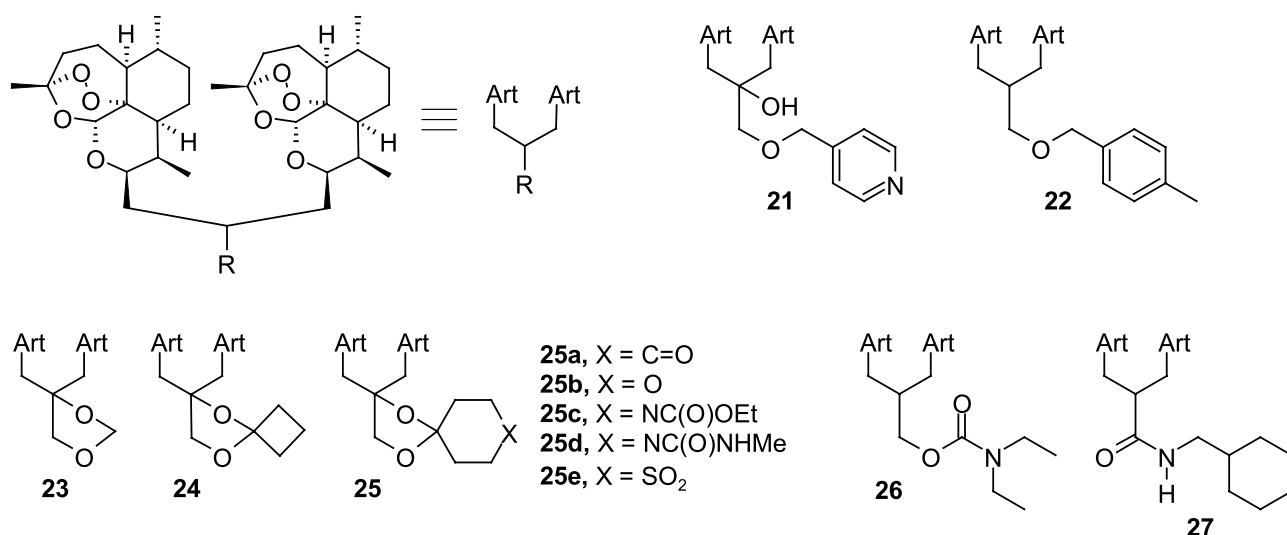

Fig. 9. Structures of derivatives **21–27**

4. ARTEMISONE (ARTEMIFONE®)

With the aim to prevent metabolic transformation of artemisins to DHA and with the drive to improve PK characteristics, several 10-(alkylamino)artemisinins **28** were designed (Figure 10) [45, 46]. All of the tested compounds showed excellent *in vivo* activities against *P. berghei*, with **28c** as the most ac-

tive derivative being almost 25 times (s.c.) and 7 times (*p.o.*) more active than artesunate **8c**. Unfortunately, **28c** suffers from serious neurotoxicity even at low doses, thus pointing out once again that more lipophilic compounds are more toxic [46]. However, derivative **28e** (artemisone, Artemifone®) showed no toxicity [47] and had tractable physicochemical properties.
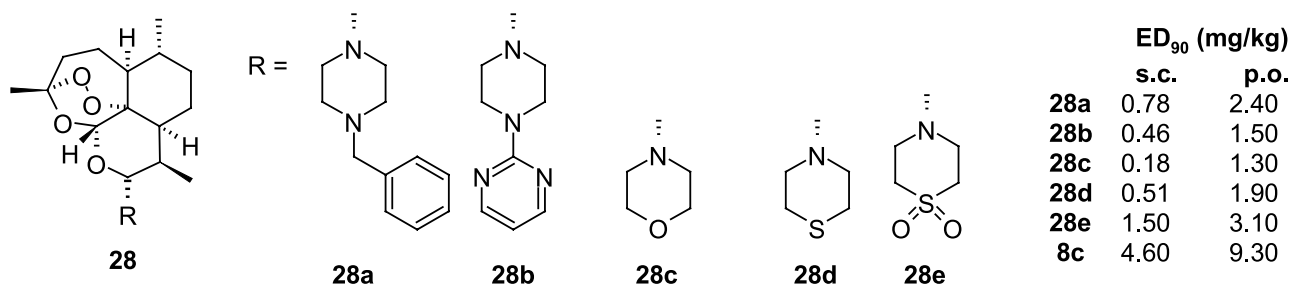

		ED$_{90}$ (mg/kg)	
		s.c.	p.o.
28a		0.78	2.40
28b		0.46	1.50
28c		0.18	1.30
28d		0.51	1.90
28e		1.50	3.10
8c		4.60	9.30

Fig. 10. Structures and antimalarial activities of derivatives **28**

A detailed antimalarial efficacy and drug-drug interaction study of artemisone **28e** was performed [48]. It was shown that the *in vitro* antimalarial activities of artemisone against 12 different *P. falciparum* strains were comparable (exhibiting a mean IC$_{50}$ value of 0.83 nM), independent of their drug-susceptibility profile to other antimalarial classes [46, 48]. During examination of the *in vitro* drug–drug interaction against drug sensitive 3D7 and multidrug resistant K1 strains, it was noticed that artemisone shows slight antagonistic effects with CQ, amodiaquine, tafenoquine, atovaquone or pyrimethamine, and slight synergism with MFQ. *In vivo* screening using the 4-day Peters test against drug-susceptible (NY), primaquine-resistant (P) and sulfadoxine/pyrimethamine-resistant (KFY) lines of *P. berghei*, CQR (NS) and artemisinin-resistant lines of *P. yoelii* NS and drug-susceptible *P. chabaudi* (AS) showed artemisone has superior ED$_{50}$ and ED$_{90}$ activity in comparison with artesunate **8c**. Artemisone exhibited 7 times greater activity (lower dosage) than artesunate (artemisone ED$_{90}$ = 12.13 mg kg^{-1} *vs*. artesunate ED$_{90}$ = 87.50 mg kg^{-1})

against the *P. yoelii* artemisinin-resistant line. The above results appear quite important in light of the recent isolation of artemether-resistant *P. falciparum* strains from humans [10] and emerging evidence for resistance *in vivo*, suggesting more potent derivatives may be efficacious where first generation compounds fail.

During *in vivo* drug–drug interaction examinations against the drug-susceptible *P. berghei* NY and the MFQ-resistant *P. berghei* N1100 lines, artemisone showed synergism with MFQ against both parasite lines. In combination with CQ, no interaction against drug-susceptible *P. berghei* NY parasites was detected, however, a synergistic effect against the CQR line, *P. yoelii* NS, was observed.

No DHA had been produced after 30 min when isotopically labelled artemisone **28e*** (Scheme 3) was incubated with human liver microsomes, but only dehydrogenated **31** and mono-hydroxylated metabolites **29**, **30** and **32**, **33**, with *syn*-hydroxy and peroxide groups (Scheme 3) [46], clearly distinguishing artemisone and the compounds from the first generation artemisinins. Isolated artemisone metabo-

lites were tested against the *P. falciparum* K1 strain and were also found to be potent antimalarials, with **30** and **31** being the most active (**30**: IC_{50} = 5.51 nM and **31**: IC_{50} = 4.26 nM, artemisone: IC_{50} = 1.99 nM) [46, 49].

29: R¹ = OH, R² = H
30: R¹ = H, R² = OH

31: R¹ = R² = H
32: R¹ = OH, R² = H
33: R¹ = H, R² = OH
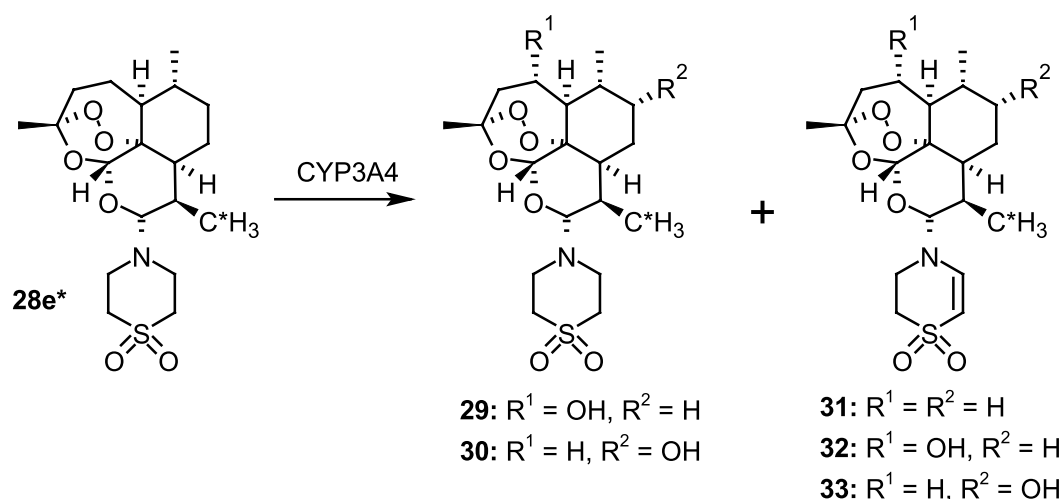
Scheme 3. Metabolic transformation of **28*** and structures of obtained metabolites

In preclinical studies, artemisone showed enhanced efficacy and improved PK in comparison to artesunate and did not demonstrate neurotoxicity *in vitro* and *in vivo* [46, 49], which is a characteristic of the artemisinin derivatives that currently are used in clinical treatment [50]. Artemisinin was well tolerated during administration of artemisone as a single dose (10 mg to 80 mg) or multiple doses (40 mg or 80 mg given once daily for 3 days). It appears that artemisone is devoid of time dependent PK (unlike artemisinin and artemether), with comparable C_{max}, AUC, and $t_{1/2}$ values after the first and third doses following the 3-day regime. Although not being as active *in vitro* as the parent drug, the relatively high concentrations of metabolites obtained after artemisone administration probably add to the overall parasiticidal effect of artemisone [49]. *In vivo* testing on non-immune *Aotus* monkeys infected with *P. falciparum* showed that a single dose of artemisone (10 mg kg⁻¹) in combination with single doses of MFQ (12.5 mg kg⁻¹ or 5 mg kg⁻¹) cleared parasitemia by day 1, with complete cure for all four monkeys tested [51]. With a single dose of

mefloquine (2.5 mg kg⁻¹) parasitemia was cleared by day 1, but without cure. For 3 days of treatment with a combination of artemisone (10 mg kg⁻¹day⁻¹) and amodiaquine (20 mg kg⁻¹day⁻¹), all three monkeys tested were cured in contrast to those that were administered the individual drugs for 3 days. From this study, it is clear that various total dosages of artemisone (20 to 90 mg kg⁻¹) alone, administered over 1 to 3 days, were unable to cure non-immune *Aotus* monkeys infected with *P. falciparum*. However, cure can be achieved when artemisone is combined with a single, sub-curative dose of MFQ, or with a 3-day treatment course of amodiaquine (or clindamycin).

5. 1,2,4-TRIOXANES

A very important finding was the high antimalarial activity of 1,2,4-trioxanes **34–39** [52–55] (Figure 11), which are structurally much simpler than natural or semisynthetic ARTs. Observed antimalarial activity data significantly contribute to understanding the

minimum structure requirements for exhibiting good anti-plasmodial activity. Small stereo or structural differences have significant contribution to the activity, e.g., epimers **34** showed different *in vitro* activity [52], or in the case of derivatives **35** where methyl substituents are replaced with spirocyclopentyl groups [52]. Contrary to observed stereoselectivity, the enantiomers of **36a** and **36c** or **36b** and **36d** showed very close activities, suggesting that *cis*-fusion significantly contributes to the activity [53]. Introducing fluorine atoms greatly enhanced activity. As measured in the 4-day Peters suppressive test, derivative **36b** (**Fenozane B07**) showed an ED_{50} of 2.5 mg kg^{-1}day^{-1}, an order of magnitude more effective than **36a** with an ED_{50} of 25 mg kg^{-1}day^{-1}. Other derivatives of this group, like ethers, alcohols or acids are far less active when compared with Fenozane. Probably, epimers **37** are the most interesting examples where the change of the configuration at one stereocentre dramatically changed the activity [54]. A similar structure-activity relationship, SAR, could be developed for **38** and **39** [55].
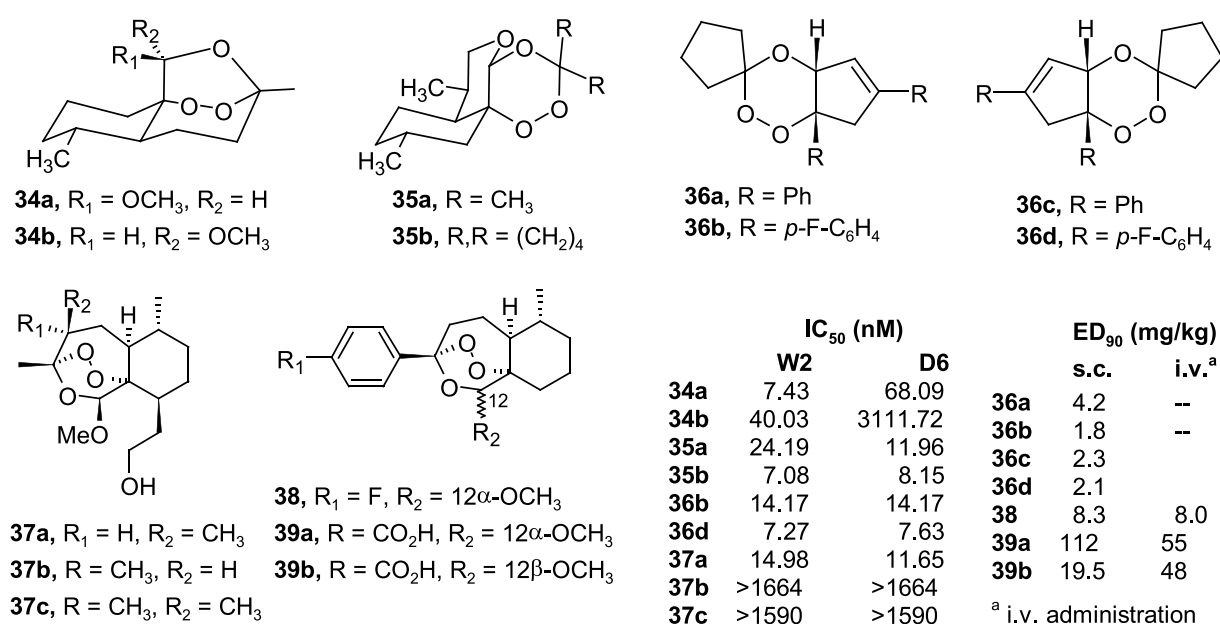

34a, R$_1$ = OCH$_3$, R$_2$ = H
34b, R$_1$ = H, R$_2$ = OCH$_3$

35a, R = CH$_3$
35b, R,R = (CH$_2$)$_4$

36a, R = Ph
36b, R = *p*-F-C$_6$H$_4$

36c, R = Ph
36d, R = *p*-F-C$_6$H$_4$

37a, R$_1$ = H, R$_2$ = CH$_3$
37b, R = CH$_3$, R$_2$ = H
37c, R = CH$_3$, R$_2$ = CH$_3$

38, R$_1$ = F, R$_2$ = 12α-OCH$_3$
39a, R = CO$_2$H, R$_2$ = 12α-OCH$_3$
39b, R = CO$_2$H, R$_2$ = 12β-OCH$_3$

	IC$_{50}$ (nM)			ED$_{90}$ (mg/kg)	
	W2	D6		s.c.	i.v.[a]
34a	7.43	68.09	36a	4.2	--
34b	40.03	3111.72	36b	1.8	--
35a	24.19	11.96	36c	2.3	
35b	7.08	8.15	36d	2.1	
36b	14.17	14.17	38	8.3	8.0
36d	7.27	7.63	39a	112	55
37a	14.98	11.65	39b	19.5	48
37b	>1664	>1664			
37c	>1590	>1590	[a] i.v. administration		

Fig. 11. Structures and antimalarial activities of derivatives **34–39**

Trans-fused derivatives **40–43** (Figure 12) also exhibited high activities [56]. They caused 96–100 % suppression of parasitemia on day 4 after 96 mg kg^{-1}day^{-1} *p.o.* dose, with spirocycloheptane **42** as the most active. Although trioxanes **40–43** are somewhat less effective than *β*–arteether under the same test conditions (100 % of suppression at 48 mg kg^{-1}day^{-1}), the obtained results suggest that *trans*-fusion may also provide good antimalarial activity, and that some other structural aspects additionally should be taken into consideration. Accordingly, *cis*-fused trioxane **44** (Figure 12) showed IC$_{50}$ and IC$_{90}$ values of 893 nM and 1845 nM, respectively, against a W2 *P. falciparum* clone, while under the same screening conditions, *cis*-fused trioxane **36a** also showed 15 nM and 36 nM efficacies, respectively [57].

For trioxanes **45–51** (Figure 12), structures were further simplified and still retained high activity. Different starting compounds, such as geranyl acetate [58] (trioxanes **45**), cyclopropyl or phenyl allylic alcohols [59]
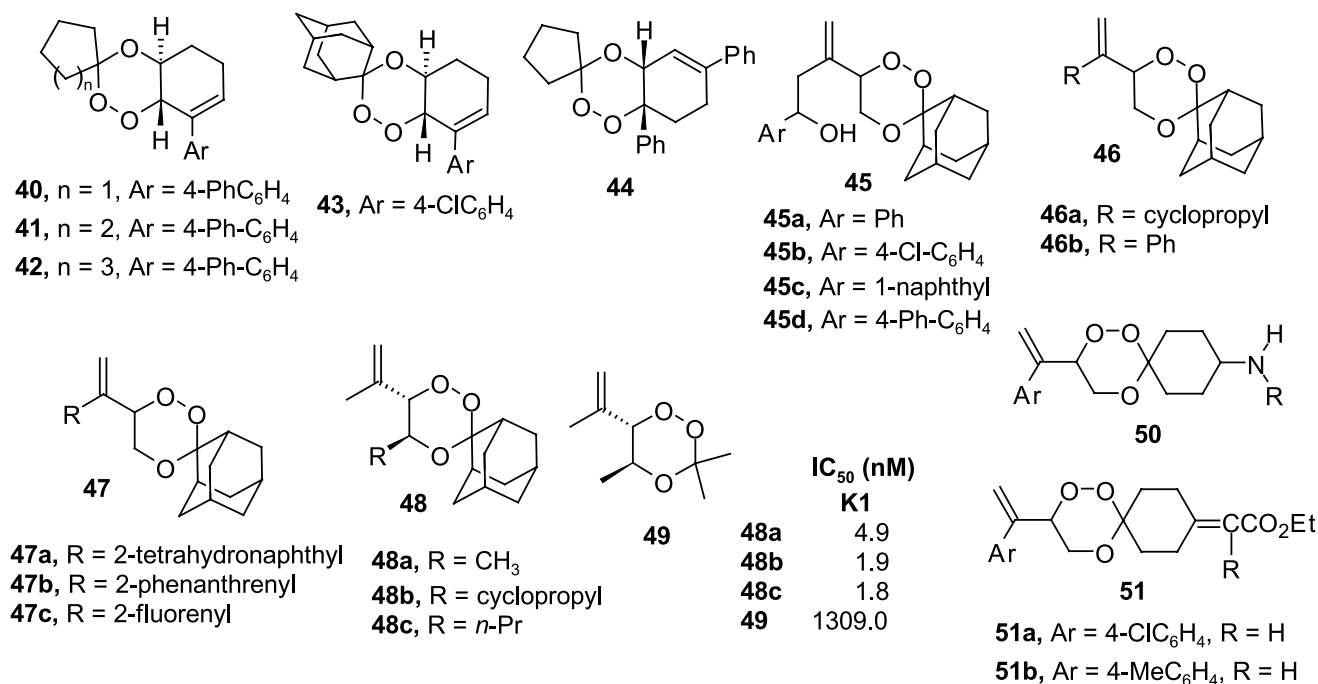
40, n = 1, Ar = 4-PhC$_6$H$_4$
41, n = 2, Ar = 4-Ph-C$_6$H$_4$
42, n = 3, Ar = 4-Ph-C$_6$H$_4$

43, Ar = 4-ClC$_6$H$_4$

44

45
45a, Ar = Ph
45b, Ar = 4-Cl-C$_6$H$_4$
45c, Ar = 1-naphthyl
45d, Ar = 4-Ph-C$_6$H$_4$

46
46a, R = cyclopropyl
46b, R = Ph

47
47a, R = 2-tetrahydronaphthyl
47b, R = 2-phenanthrenyl
47c, R = 2-fluorenyl

48
48a, R = CH$_3$
48b, R = cyclopropyl
48c, R = *n*-Pr

49

50

51
51a, Ar = 4-ClC$_6$H$_4$, R = H
51b, Ar = 4-MeC$_6$H$_4$, R = H

	IC$_{50}$ (nM) K1
48a	4.9
48b	1.9
48c	1.8
49	1309.0

Fig. 12. Structures and antimalarial activities of derivatives **40–51**

(trioxanes **46**), aryl substituted allyl alcohols (trioxanes **47**) [60] or methyl substituted allyl alcohols (trioxanes **48** and **49**) [61] were used for obtaining *β*-hydroxyhydroperoxides. These *β*-hydroxyhydroperoxides were later coupled to different carbonyl compounds, and very active trioxanes with diversified structures were obtained [62, 63]. 2-Adamantyl derivatives **45–48** were the most active against multidrug resistant *P. yoelli* in mice and exhibited 100% suppression of parasitemia on day 4 at 96 and 48 mg kg^{-1} × 4 days (Peters test) doses *p.o.* The most active 2-fluorenyl derivate **47c** showed 100% suppression of parasitemia on day 4, even at 24 mg kg^{-1} × 4 days, which appeared to be a half effective dose of arteether [60]. Intramuscular injection decreases the activities of these derivatives and thus confirmed, once again, that hydrophobic compounds show better bioavailability by oral route. Derivatives **48** and **49** with C(5)-alkyl substituted 1,2,4-trioxane rings were tested against the CQR K1 *P.f.* strain and showed activities which strongly depend on C(3)-substituents. While spiroadamantane derivates **48** were as

active as ART, gem-dimethyl derivative **49** was 270 times less active [61]. Replacing spiroadamantyl with a spirocyclohexyl group bearing an ionizable arylamino moiety, as in **50** [62] or other polar groups as in **51** [63], also resulted in loss of the activity. Although some derivatives such as **51a** and **51b** [63] exhibit 100 % suppression of parasitemia on day 4 at 96, 48, and 24 mg kg^{-1} × 4 day doses *p.o.*, in general, these compounds are less potent than adamantyl derivatives. These results convincingly introduce the adamantyl-spiro-1,2,4-trioxane motif as significant contributor to good antimalarial activity.

The antimalarial activities of a series of steroids possessing the 1,2,4-trioxane moiety (**52–54** (Figure 13), respectively) were also tested [64]. Only pregnane-based trioxanes **53a-f** exhibited good activity. They showed 100% suppression of parasitemia on day 4 and 40–100 % protection at 96 mg kg^{-1} × 4 day doses, with **53b** being the most efficacious. Cholestane- and tigonenin-based derivatives were much less successful with 15–73% suppression.
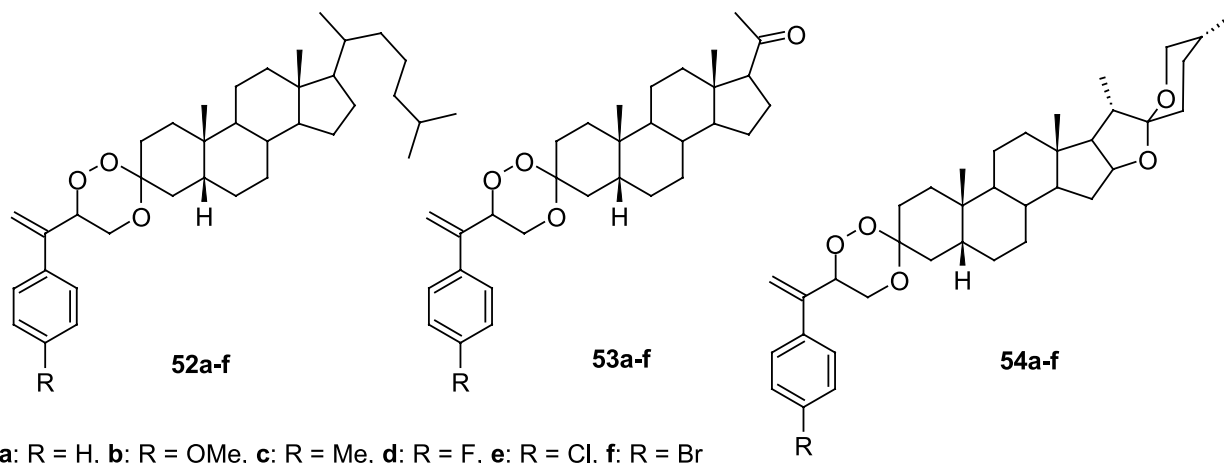
a: R = H, b: R = OMe, c: R = Me, d: R = F, e: R = Cl, f: R = Br

Fig. 13. Structures of steroid based 1,2,4-trioxanes **52–54**
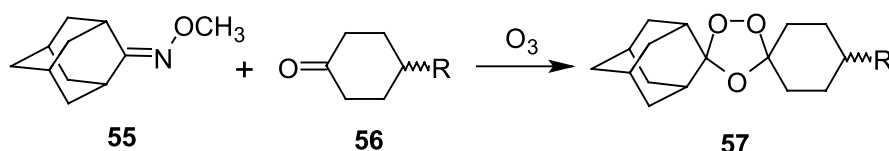
6. 1,2,4-TRIOXOLANES (ARTEROLAN)

1,2,4-Trioxolanes, ozonides, a very well known class of organic compounds, appear as intermediates during scission of olefins into carbonyls during ozonolysis. It was an unexpected and surprising discovery [65] that ozonides are relatively stable and that many express excellent activity against malaria parasites, like the structurally similar 1,2,4-trioxanes.

6.1. *First generation 1,2,4-trioxolanes (**OZ209, OZ277 and OZ339**)*

1,2,4-trioxolane **57** was obtained by Griesbaum co-ozonolysis of suitable methyl oximes **55** and ketones **56** (Scheme 4) [66, 67]. Synthesis of a vast array (Figures 14 and 15) of differently substituted derivatives is en-

abled with the applied method and the stability of the 1,2,4-trioxolane moiety to reduction and alkylation conditions [68].

Usually, 1,2,4-trioxolane antimalarials are prepared in 1 to 4 steps, depending on the modifications required [66, 67, 69, 70, 71] affording the final products in yields up to 75 %. The vast majority of these antimalarials are achiral, which greatly facilitates production in the developmental step. The advantage of ozonide (OZ) antimalarials is the use of the adamantane moiety that has lipophilic functionality, allowing the opposite part of the molecule to be fine-tuned using a number of polar functional groups, preferably basic in nature [65, 66, 68, 70, 71]. Many of the so obtained OZ compounds are active in all stages of development of the malaria parasite, and are more active than artemether and artesunate, both *in vivo* and *in vitro*.

Scheme 4. Griesbaum co-ozonolysis

When compared with other OZ compounds, trioxolanes **OZ209** (**59** as mesylate, Figure 14) and **OZ277** (**60** as tosylate, **Arterolan**, Figure 14) showed superior PK results, such as prolonged half-life and enhanced bioavailability after a single oral dose. Compound **OZ209** had somewhat better antimalarial results and a lower recrudescence level. However, **OZ277** was chosen as the development candidate, primarily because of its improved toxicological profile and reduced concentrations in brain tissue after oral dosing [65]. For example, 2 hours after dosing, both **OZ209** and **OZ277** were distributed throughout the liver, kidney, lung and heart, while after 18 hours, **OZ277** was detected only in the lungs and in several-fold lower concentrations than **OZ209**.

Unlike **OZ209**, which was quantified in brain tissue after both 2 h and 18 h, **OZ277** could not be quantified in this organ. In view of potential neurotoxicity issues, these findings were taken as a considerable advantage of **OZ277** over **OZ209**. Trioxolane **OZ277** ap-

peared quite stable to metabolic transformation ($t_{1/2} = 1.4$ h, *p.o.* in healthy rats) [65]. The other derivatives **58**, **61–67** (Figure 14) afforded further insight into SAR in the context of the physico-chemical, biopharmaceutical, and toxicological profiles of trioxolanes [70].

Recently [72], the same authors revealed data for a series of OZ compounds with weak base functional groups, which were responsible for high antimalarial efficacy in *P. berghei*-infected mice. Their antimalarial efficacy and ADME profiles are equal or superior to **OZ277**. One of the most promising is **OZ339** (Table 3, as the tosylate salt). Despite the obvious difference in *in vitro* activity, both ozonides eradicate parasitemia below the detectable level one day after administration (99.9 %, 1×10 mg kg^{-1}, and 3×3 mg kg^{-1}). The drug candidate **OZ277** is a powerful fast-acting antimalarial with a 67 % cure record at a 3×10 mg kg^{-1} dosage (mice) [65]. However, at a 3×3 mg kg^{-1} dosage, the

	IC$_{50}$ (nM)	
	K1	NF54
58	105.46	139.58
59	1.33	1.43
60	2.55	2.32

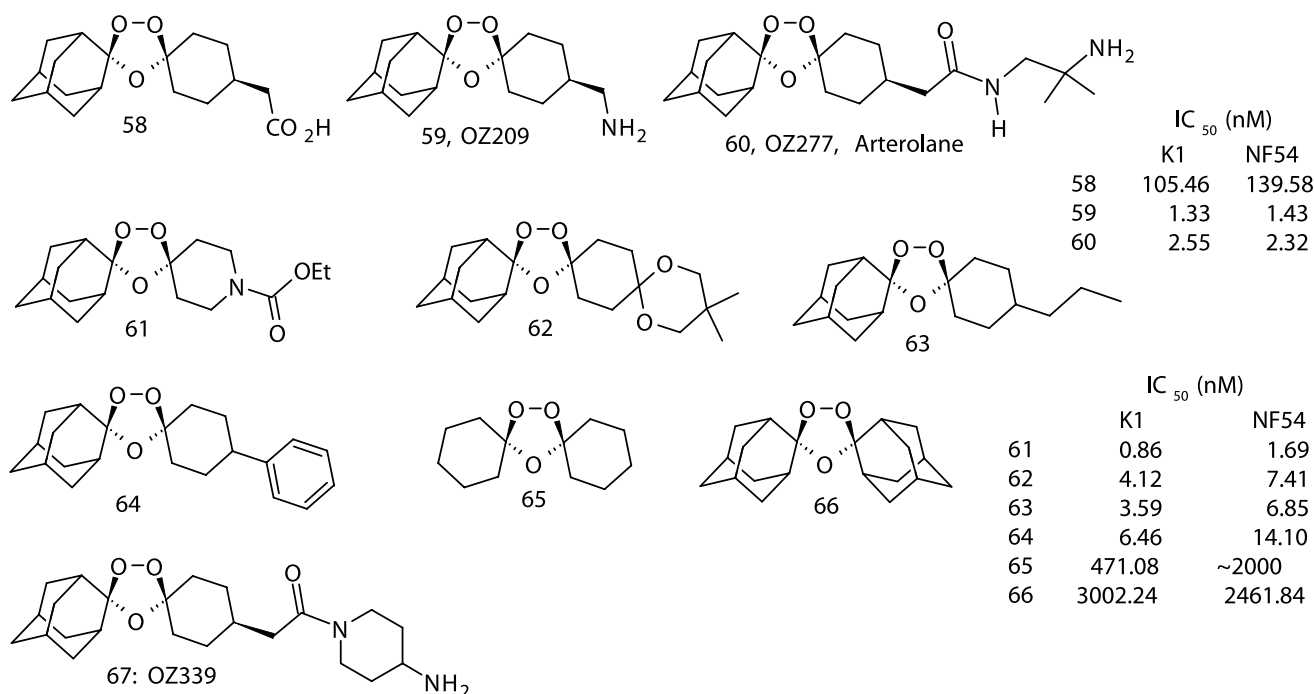|	IC$_{50}$ (nM)	
	K1	NF54
61	0.86	1.69
62	4.12	7.41
63	3.59	6.85
64	6.46	14.10
65	471.08	~2000
66	3002.24	2461.84

Fig. 14. Structures of trioxolanes 58–67.

same compound cured no mice, while trioxolane **OZ339** cured 3/5 mice with an excellent survival time of 27 days (**OZ277** had 2.4 times lower survival time). These good PK characteristics were underlined by the favourable *p.o.* bioavailability data for **OZ339**, 78% *vs* 26% for **OZ277**. In all experiments, artesunate showed inferior activity. Inhibition assays revealed that **OZ339**, like **OZ277**, did not inhibit CYP3A4, CYP2C9, and CYP2D6 at concentrations up to 50 μM. Finally, preliminary toxicological experiments indicated that **OZ339** was minimally toxic (liver) and, similarly to **OZ277**, demonstrated no detectable signs of neurotoxicity.

T a b l e 3

Comparative data for fast-acting antimalarials: **60 (OZ277), 67 (OZ339)** *and sodium-artesunate* **8c**

Comp.	IC$_{50}$ (ng/ml)		Activity (%) *p.o.*		*In vivo* activity (3×3 mg kg^{-1}, *p.o.*)[e]			
	K1	NF54	(1×10 mg kg^{-1})[c]	ER[d]	Activity (%)	Survival (days)	Cure[f] (%)	$t_{1/2}$ (*i.v.*, min)
67 (OZ339)[a,b]	0.35	0.39	>99.9	0.45	>99.9	27.0	60	83
60 (OZ277)[a,b]	1.0	0.91	99.9	0.32	>99.9	11.4	0	76
8c (AS)[b]	1.3	1.6	67	0.43	70	9.2	0	40[g]

[a] Tosylate salt.

[b] Taken from ref. 70.

[c] Groups of five *P. berghei*-infected NMRI mice were treated orally on day +1 (1×10 mg kg^{-1}). Activity measured on day +2.

[d] Predicted hepatic extraction ratios (ER) using human microsomes.

[e] Groups of five *P. berghei*-infected NMRI mice were treated orally on days +1, +2, and + 3 (3×3 mg kg^{-1}). Activity measured on day +4.

[f] Percent of mice alive on day 30 with no evidence of blood parasite.

[g] Taken from ref. 66. N.D. not dosed.

As mentioned above, the tolerance of the 1,2,4-trioxolane moiety to diverse reaction conditions [67] enabled the synthesis of a significant number of derivatives. Many of them showed very good antimalarial activity, like derivatives **68–72** (Figure 15) [73], piperidine derivatives **73–75** [74] and derivatives which contain aliphatic and aromatic amino functional groups or azole heterocycles as substituents (**76–82**) (Figure 15) [71]. Lack of activity of 83 indicates the essential contribution of the spiro-adamantane system to the antimalarial properties of this class of compounds.

Many of the examined derivatives exhibited excellent *in vitro* results, but failed during *in vivo* tests, toxicity trials or metabolic stability and bioavailability tests. More lipophilic trioxolanes tend to have better oral activities and are metabolically less stable than their polar counterparts. Such behaviour is consistent with results obtained for other classes of synthetic peroxides. Trioxolanes with a wide range of neutral and basic groups had good antimalarial profiles unlike derivatives with acidic groups. Based on the collected extensive screening results the authors concluded that the *in vitro* activities of 1,2,4-trioxolanes are not (always) a reliable predictor of *in vivo* potency [73]. Rather, their experiments in *P. berghei*-infected mice confirmed that *in vivo*
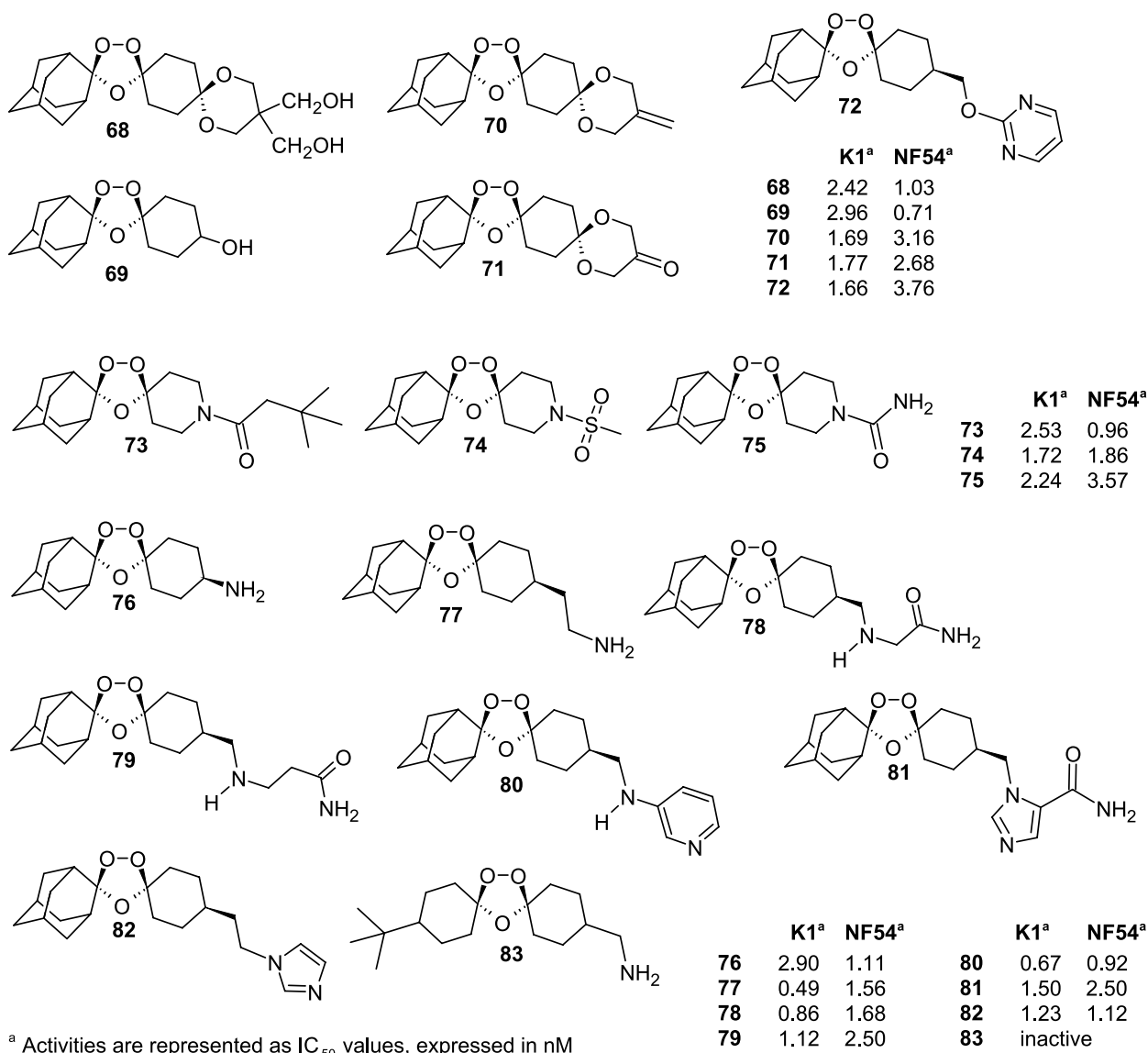
	K1[a]	NF54[a]
68	2.42	1.03
69	2.96	0.71
70	1.69	3.16
71	1.77	2.68
72	1.66	3.76

	K1[a]	NF54[a]
73	2.53	0.96
74	1.72	1.86
75	2.24	3.57

	K1[a]	NF54[a]		K1[a]	NF54[a]
76	2.90	1.11	80	0.67	0.92
77	0.49	1.56	81	1.50	2.50
78	0.86	1.68	82	1.23	1.12
79	1.12	2.50	83	inactive	

[a] Activities are represented as IC$_{50}$ values, expressed in nM

Fig. 15. Structures and antimalarial activities of derivatives **68–83**

results were essential for compound differentiation and selection for further metabolic and pharmacokinetic profiling [72].

Following the recommendation of WHO for R&D of artemisinin-based combination therapies (ACT), **OZ277** entered Phase III clinical trials in combination with piperaquine (arterolane maleate + piperaquine phosphate) [72]. In 2011, Ranbaxy received permission from the Indian Drug Regulator, Drug Controller General of India (DCGI), to manufacture and market this drug in India as Synriam®,

for the treatment of uncomplicated malaria in adults [75].

6.2. Second generation of 1,2,4-trioxolane: **OZ439**

Although entire anti-malarial profiles show superior properties of **OZ277** and **OZ339** in comparison to DHA, the search for an ozonide with significantly increased half-life continued.

As a result, very recently the screening results of second generation of ozonide antimalarials were revealed [76]. Of the several very active derivatives of undisclosed structure, it appears that the most promising antimalarial candidate is **OZ439** [76] (Table 4). The initial results indicate that this compound provides a single-dose oral cure in a murine malaria model at 20 mg kg^{-1}, a situation not known for any of peroxide antimalarials, except for artelinic acid at more than 7 times higher dose. The second-generation ozonide **OZ439** completed Phase I studies and is currently undergoing Phase II clinical trials. The prolonged blood stability, significantly high bioavailability (76 % for **OZ439** *vs* 13% for **OZ277**) and improved PK characteristics (Table 4) led to positioning of **OZ439** as current major OZ drug candidate – postinfection cure (3×5 mg kg^{-1}day^{-1} and 20 mg kg^{-1} single dose), and exclusive prophylactic characteristics (Table 4). The absence of metabolic products significantly contributes overall to **OZ439** relative to other peroxide antimalarial drugs [76].
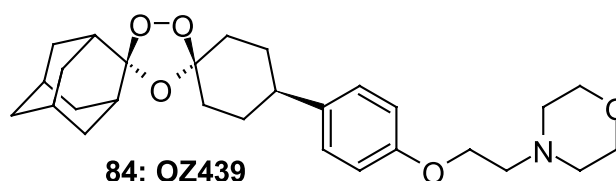
84: OZ439

T a b l e 4

Comparative data for first and second generation of ozonide antimalarials:
60 (OZ277) vs. 84 (OZ439)

Compound	IC$_{50}$ (ng/ml)		Postinfection (%) *p.o.*[c]		*In vivo* prophylactic activity *p.o.* (1×30 mg kg^{-1})[e]			
	K1	NF54	1×30 mg kg^{-1}	Cure[d]	Activity (%)	Survival (days)	Cure[d] (%)	$t_{1/2}$ (*p.o.*, min)
60 (OZ277)[a,b]	0.71	0.63	99.9	0	0	7	0	55
84 (OZ439)[b]	1.6	1.9	>99.9	100	99.8	>30	100	1380
8c (AS)[b]	1.2	1.5	92	0	21	7	0	40[f]

[a] Tosylate salt.

[b] Taken from ref. 76.

[c] Groups of *P. berghei*-infected ANKA mice (*n* =5) were treated orally on day +1 (1×30 mg kg^{-1}). Activity measured on day +3.

[d] Percent of mice alive on day 30 with no evidence of blood parasite.
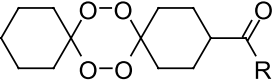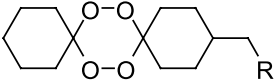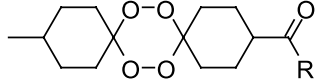
[e] Groups of *P. berghei*-infected ANKA mice (*n* =5) were treated orally on day $^{-1}$ (1×30 mg kg^{-1}). Activity measured on day +3.

[f] Intravenous, taken from ref. 66.

7. 1,2,4,5-TETRAOXANES

3,6-Substituted derivatives of 1,2,4,5-tetraoxacyclohexane (1,2,4,5-tetraoxane) have been known for many years and were used for different purposes [77]. They are readily formed by acid-catalyzed [78] peroxyacetalization of carbonyl compounds with hydrogen peroxide or *bis*-trimethylsilylperoxide [79–81] or other procedures [82]. The discovering that inexpensive tetraoxane 3,6-dicyclohexylidenes exhibit pronounced antimalarial activity opened new possibilities in combating this disease. A real breakthrough was achieved with steroidal tetraoxanes – with these derivatives the high potency of tetraoxanes for treatment of *P. falciparum* CQR strains was shown [80, 98] For that reason, many efforts have been made in finding better procedures for synthesizing and designing new derivatives with improved activities [83]. Many of them have been reviewed and their antimalarial activities analyzed [7f, 7g, 84].

85: R = NHCH$_2$CH$_2$N(CH$_3$)$_2$

92: R = OCH$_3$
93: R = NH$_2$
94: R = NHCH$_2$CH$_2$N(CH$_3$)$_2$

86: R = OH
87: R = N$_3$
88: R = NH$_2$
89: R = NHC$_6$H$_{11}$
90: R = NHPr^i
91: R = NHCH$_2$Ph

	IC$_{50}$ (nM)		
	D6	W2	TM91C235
85	11.24	9.72	7.24
86	15.18	6.54	12.62
87	9.87	6.07	11.29
88	12.84	7.79	19.75
89	11.18	6.17	10.78
90	9.40	7.54	9.27
91	13.82	8.19	13.89
92[a]	12.90	6.10	16.0
93[a]	13.60	8.40	18.80
94[a]	14.90	6.10	22.60

[a] Compounds were tested as mixture of diastereoisomers

Fig. 16. Structures and antimalarial activities of derivatives **85–94**.

After the first report where they were described [85], mixed tetraoxanes[3] drew significant attention. Since then, some new procedures for their synthesis [86, 87], or the synthesis of gem-dihydroperoxides as key precursors [88] were developed with the aim to improve the yields of tetraoxane compounds.

A new group of mixed dicyclohexylidene tetraoxanes, bearing polar, neutral, or basic groups, demonstrated high activities against both CQS and CQR *P.f.* strains (Figure 16) [89]. The goal was to obtain the simplest amphiphilic structures of the kind and to minimize the influence of steric effects on antimalarial activity. Besides that, stability under basic, acidic, oxidative, reductive and reductive amination conditions revealed significant stability of the tetraoxane moiety that enabled the synthesis of a variety of derivatives [89c]. Interestingly, the most active compounds within the group of dicyclohexylidene tetraoxanes **85–94** have very close activities disregarding the presence of neutral (**87**, **92**), polar protic (**86**) or basic ionizable groups (**85**, **88–91**, **94**). Such conduct impedes profound SAR analysis. In the present set of derivatives, amines **88** and **89** were the most active *in vivo*. They both cured 5/5 mice at 300 mg×kg^{-1}day^{-1} doses s.c. in a modified

Thompson test. With 150 mg×kg^{-1}day^{-1} doses s.c. **88** retains the same efficiency, but **89** was less active with 4/5 cured mice.

A structurally similar group was synthesized [90] using the procedure previously applied to this class of compounds (Figure 17) [85, 89a]. The compounds were screened against CQS *P.f.* strain 3D7 and the most active derivatives **95–101** have the activities within the same range as ones described above. The least active derivatives **100** and **101** confirmed the superiority of the 3,6-dispiro-1,2,4,5-tetraoxane structural motif. Adamantyl derivatives **98** and **99** [90] showed 100 % inhibition by *p.o.* administration at 30 mg kg^{-1} doses, and derivative **99** had superior ED values against *P. berghei* (ANKA) as compared to artemether: ED$_{50}$ = 3.18 mg kg^{-1} and ED$_{90}$ = 3.88 mg kg^{-1} for **99**; ED$_{50}$ = 5.88 mg kg^{-1} and ED$_{90}$ = 10.57 mg kg^{-1} for artemether.

The ability of adamantyl substituent to stabilize the structure and to improve the antimalarial activity was additionally exemplified with a new series of amphiphilic adamantyl-based mixed tetraoxanes (Figure 18) [91]. The derivatives that contain polar sulphonamide exhibit activities in 3–30 nM range, with **102–105** as the most active compounds against 3D7 *P. f.* strain. In addition, derivative **102** was tested versus seven additional strains of *P. falciparum* and exhibited activity in the 1.9 – 3.8 nM range.

---

[3] Term "mixed tetraoxanes" describes 1,2,4,5-tetraoxacyclohexanes possesing nonidentical substituents at positions 3 and 6.

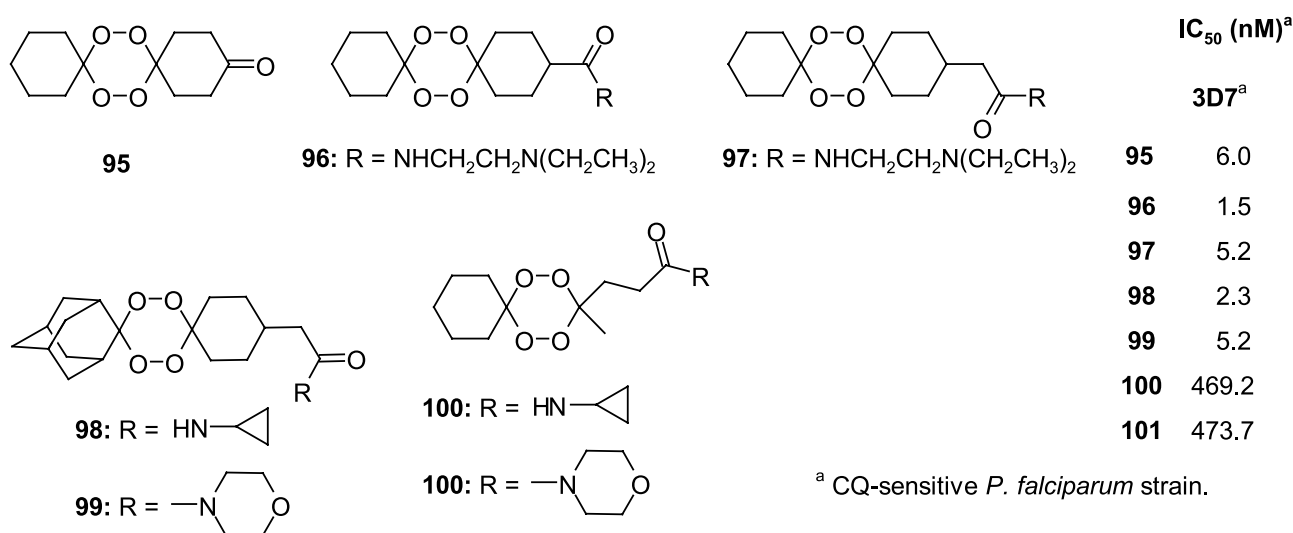|	IC$_{50}$ (nM)^a
	3D7^a
95	6.0
96	1.5
97	5.2
98	2.3
99	5.2
100	469.2
101	473.7

^{a} CQ-sensitive *P. falciparum* strain.

Fig. 17. Structures and antimalarial activities of derivatives **95–101**

Compounds **102** and **104** were noticeably active *in vivo* (*p.o.*, *P. berghei* ANKA): ED$_{50}$ = 6.61 mg kg^{-1} for **102** and ED$_{50}$ = 7.93 mg kg^{-1} for **104**, with ED$_{50}$ = 8.42 mg kg^{-1} for ART for comparison).

Further structure optimization brings up derivative **106 (RKA182)** as a lead compound, with selected formulation as its ditosylate salt [92]. Synthesis of **106** were developed on high scale employing Re$_2$O$_7$ as a mild Lewis acid catalyst during peroxyacetalysation with hydrogen peroxide [93]. The compound exhibits superior *in vitro* activity, which is additionally confirmed by *in vivo* and ADME results. It shows 100%

suppression activity after 30 mg kg^{-1} dose on day 4 in Peters test, and *in vivo* activity with an ED$_{50}$/ED$_{90}$ of 1.33/4.18 mg kg^{-1} (*P. berghei* ANKA), which is superior to artemether and artesunate, and comparable to artemisone. In an assessment of efficacy in *P. berghei*-infected mice, the administration of 3 consecutive 10 mg kg^{-1} oral doses of **106** and artesunate results in the survival of 22 mice in comparison to 9 for artesunate. Even though no specific metabolites of **106** were identified, Phase I metabolic pathways for the compound class were identified as adamantane/cyclohexane hydroxylation, *N*-oxide formation and *N*-dealkylation. With the
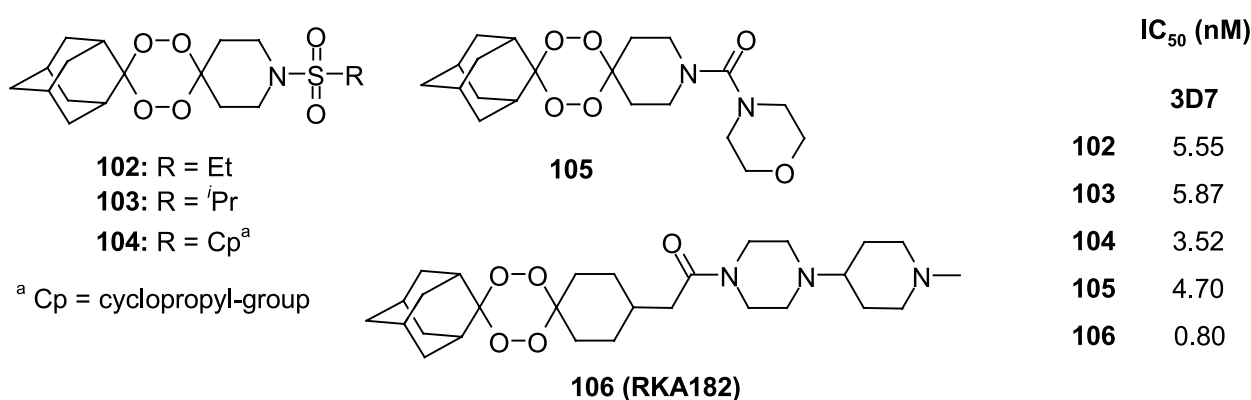

	IC$_{50}$ (nM)
	3D7
102	5.55
103	5.87
104	3.52
105	4.70
106	0.80

Fig. 18. Structures and antimalarial activities of derivatives **102–106**

exception of weakly inhibiting CYP2C19 (IC_{50} at 59 mM), **106** did not inhibit any of the major human CYP450 isoforms at concentrations up to 100 mM. In PK experiments in rats conducted over 6 h, **106** (1 mg kg^{-1} intravenous and 10 mg kg^{-1} oral doses) had an oral C_{max} of 90 ng/ml, intravenous and oral half-lives of 0.80 and 2.4 h, CL of 136 ml/min/kg, Vd of 9.5 l/kg, and an oral bioavailability of 40%. In toxicity experiments, the maximum tolerated dose of 14 in rats was 400 mg kg^{-1}.

Some other types of tetraoxanes were less successful. Such examples are symmetrically substituted 3,3,6,6-tetraalkyl-1,2,4,5-tetraoxanes derived from acyclic ketones [94] and symmetric and nonsymmetric 1,2,4,5-tetraoxane derivatives of substituted benzaldehydes (Figure 19) [95]. Compounds were tested as mixtures of corresponding isomers and in general exhibited rather poor antimalarial activity against both CQS and CQR *P. falciparum* strains. Within the series of acyclic derivatives, compounds **107–109** were the most active, but still they were 1.6 – 2.2 times less active than corresponding tetraoxanes **110** derived from cyclohexane, or 40–50 times less active than artemether [94]. Within the benzaldehyde series compound **111** was the most active, while the other members were significantly less active with IC_{50}'s in the range 1.4–17 μM [95], which is far less then corresponding 1,2,4,5-tetraoxanes or 1,2,4-trioxanes considered as ac-

tive. Nonetheless, these results offer valuable basic information for the correlation between the structure and antimalarial activity of the simple peroxides.

1,2,4,5,7,8-Hexaoxonanes are customary side products of the synthesis of 1,2,4,5-tetraoxacyclohexanes (Figure 20). Their activities against K1 and NF54 *P. f.* strains showed that hexaoxonanes **112–118** are convincingly less active than the corresponding tetraoxanes, with 3-4 orders of magnitude lower IC_{50}'s [96]. Since peroxide bonds are essential for antimalarial activity, such poor efficiency was attributed to steric hindrance. Analogous behaviour was noticed with asymmetric 1,2,4,5,7,8-hexaoxonane **118** as well [89c].

The first described steroidal 1,2,4,5-tetraoxane antimalarials **120** and **12**1 were derivative from 5α-cholestane-3-one (Figure 21) [81]. Although tetraoxane **107** was less active in comparison to ART (IC_{50} (D6) = 155 nM), the result of this pioneering research clearly showed that even complex molecules such as steroids could be good carriers of the tetraoxane pharmacophore. Replacing cholestane with derivatives of cholic acid significantly improved antimalarial activity (Figure 21) [80, 97]. Compounds obtained as a series of diastereomers were named as *cis*-C(2)C(2a) and *trans*-C(2)C(2a) and they showed moderate to high activity against both CQS and CQR strains. The most active were primary amide **122** (IC_{50}(W2) = 18.79 nM) and
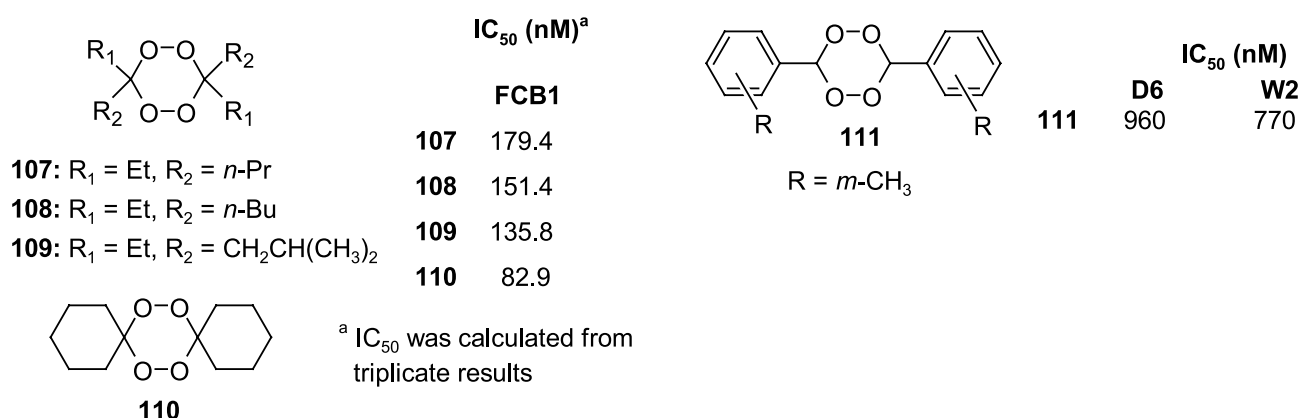

	IC$_{50}$ (nM)^a
	FCB1
107	179.4
108	151.4
109	135.8
110	82.9

107: R_1 = Et, R_2 = *n*-Pr
108: R_1 = Et, R_2 = *n*-Bu
109: R_1 = Et, R_2 = CH$_2$CH(CH$_3$)$_2$

110

^{a} IC$_{50}$ was calculated from triplicate results

		IC$_{50}$ (nM)	
		D6	**W2**
	111	960	770

R = *m*-CH$_3$

111

Fig. 19. Structures and antimalarial activities of derivatives **107–111**

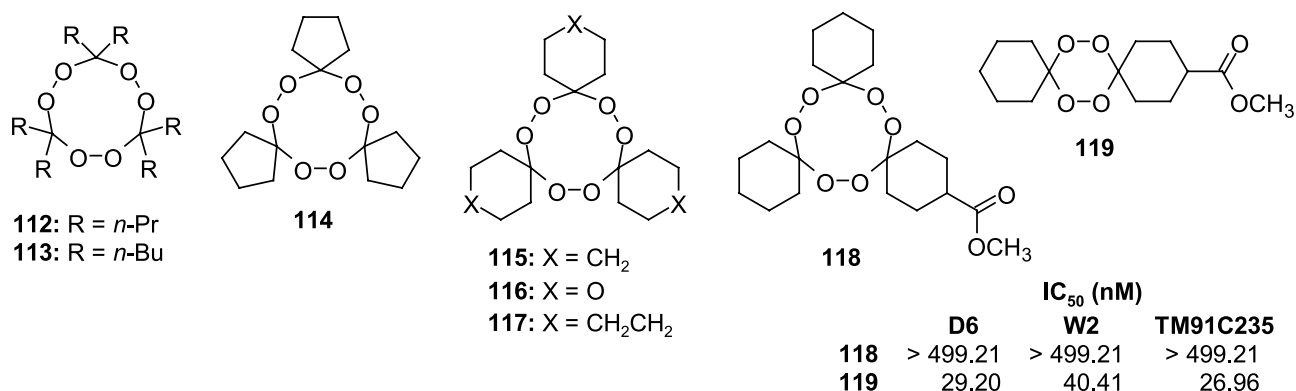|	IC$_{50}$ (nM)		
	D6	W2	TM91C235
118	> 499.21	> 499.21	> 499.21
119	29.20	40.41	26.96

Fig. 20. Structures and antimalarial activities of derivatives **112–119**
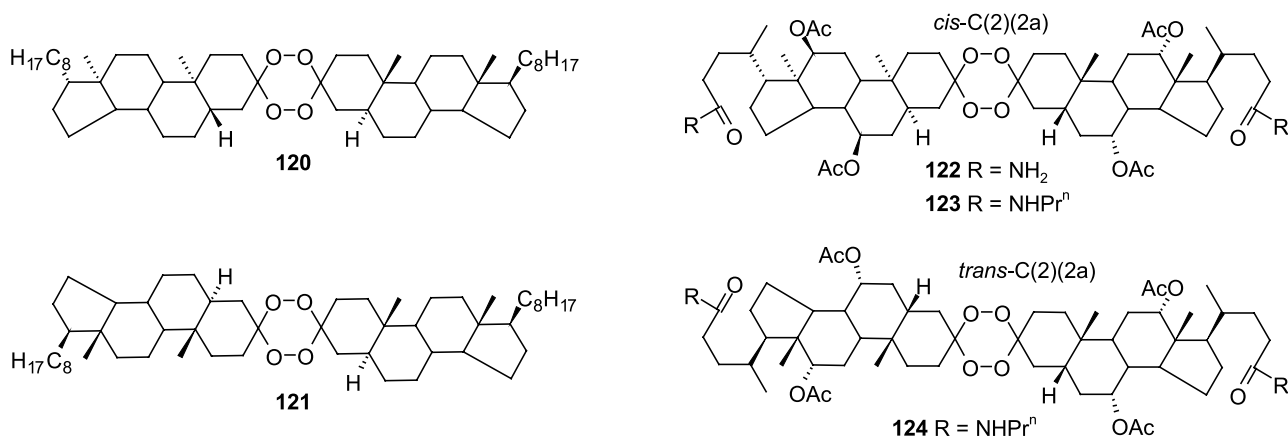

Fig. 21. Structures of derivatives **120–124**

N-propyl amides **123** and **124** (IC$_{50}$(D6) = 9.29 nM and 20.08 nM, respectively).

Replacing one steroid with a simpler alkylidene moiety enabled the synthesis of the second generation of steroidal tetraoxanes based on cholic acid derivatives. Starting from 4-alkyl [98, 99], 4-aryl [100], 4-carboxy substituted cyclohexanones [101] and the acetone [102] afforded the synthesis of a number of various derivatives (Figure 22). The stability of the tetraoxane moiety under a range of reaction conditions enabled the synthesis of diverse derivatives [89c]. Some of the shown mixed steroidal tetraoxanes exhibited impressive *in vitro* and *in vivo* antimalarial activity. Contribution of cholic acid moiety as a carrier is clearly emphasized by the pronounced

activity of derivative **132**, since *bis*-isopropylidene tetraoxane was completely inactive [103].

When administered to mice (*p.o.*, *P. berghei* KBG 173 strain) tetraoxane **126** in the Thompson test cured 4/5 mice at 200 mg kg^{-1}day^{-1} doses, while being moderately active when applied at 50 mg kg^{-1}day^{-1} (cured 2/5) [98]. The administration of tetraoxane **131** (*p.o.*) cured 2/5 mice at 320 mg kg^{-1}day^{-1} doses, however, the same compound was more efficient at 160 mg kg^{-1}day^{-1} s.c. doses, with 5/5 cured mice [102]. All cured mice had negative blood smears within all days tested (6–31). Tetraoxane **134** cured 5/5 mice at 320 mg kg^{-1}day^{-1}, and using lower doses (80 mg kg^{-1}day^{-1}) demonstrated moderate activity with 3/5 cured mice [99]. It should be

emphasized that all these compounds showed no toxic effect against tested animals at any applied concentrations. In addition, they have low activity against the Vero cell line, showing a cytotoxicity/antimalarial potency ratio 1/(1400–9500) [98]. Furthermore, the *n*-propyl amide **127**, revealed no healthy erythrocyte (RBC) membrane lysis [104], suggesting that the antimalarial activity of these compounds is the consequence of an interaction specific to infected RBCs, and is not the result of uncontrolled RBC membrane lysis.

A series of tetraoxanes based on deoxycholic derivatives were prepared with the aim

to compare them to cholic acid-derived tetraoxanes (Figure 22) [99]. In general, these compounds follow the same trends as the cholic acid derivatives: higher activity of 4″-methylcyclohexyl derivatives than nonsubstituted cyclohexyl ones and higher activity of 4″R- over 4″S-epimers. However, tetraoxanes with deoxycholic acid-derived carriers are less active then corresponding cholic acid derivatives suggesting that the C(7) acetyloxy group appreciably contributes to their antimalarial activity. Within this group of tetraoxanes, the derivatives **135** and **136** were the most active exhibiting activities comparable to ART
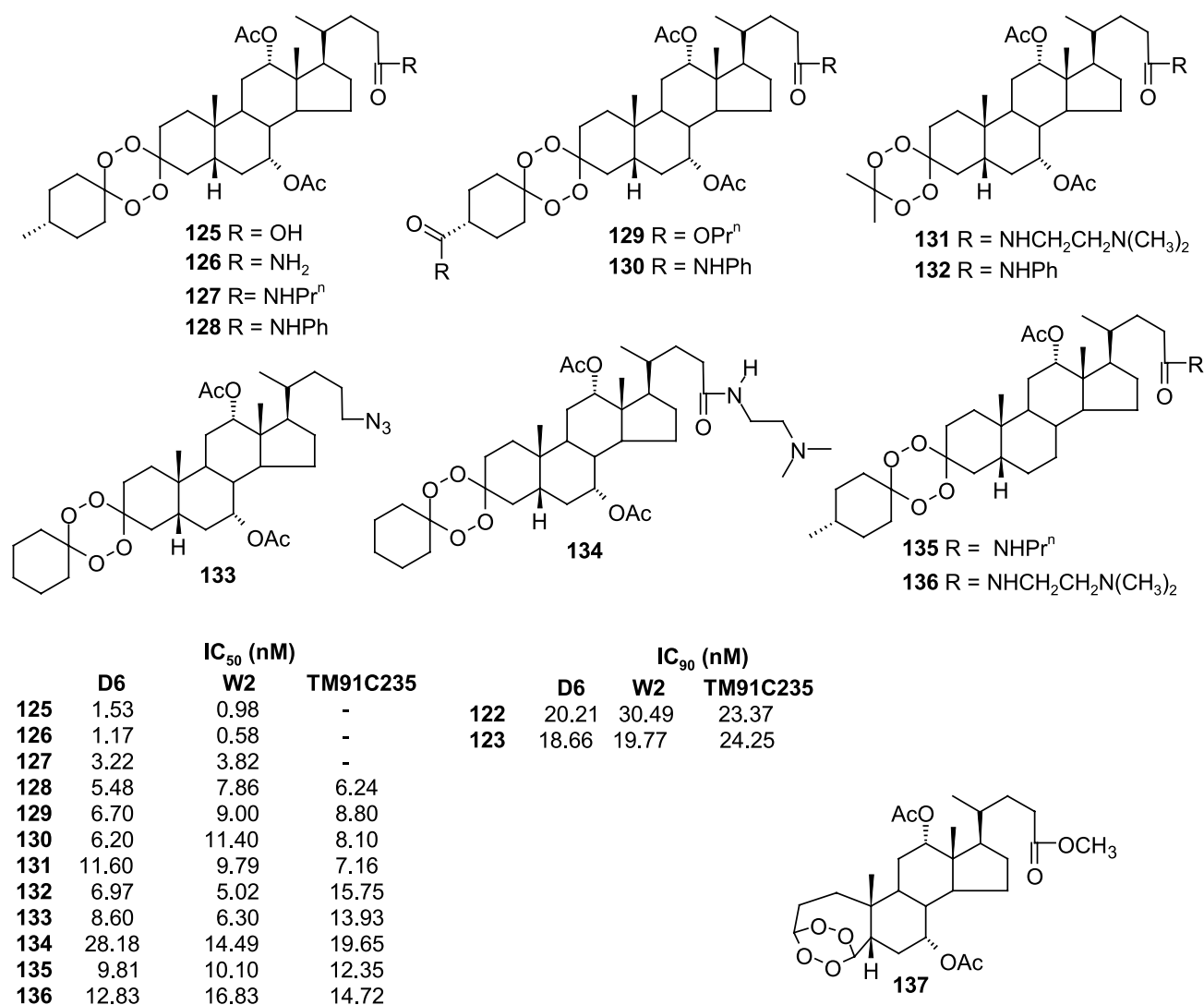

125 R = OH
126 R = NH$_2$
127 R = NHPr^n
128 R = NHPh

129 R = OPr^n
130 R = NHPh

131 R = NHCH$_2$CH$_2$N(CH$_3$)$_2$
132 R = NHPh

133

134

135 R = NHPr^n
136 R = NHCH$_2$CH$_2$N(CH$_3$)$_2$

| | IC$_{50}$ (nM) | | | | | IC$_{90}$ (nM) | | |
	D6	W2	TM91C235			D6	W2	TM91C235
125	1.53	0.98	-		**122**	20.21	30.49	23.37
126	1.17	0.58	-		**123**	18.66	19.77	24.25
127	3.22	3.82	-					
128	5.48	7.86	6.24					
129	6.70	9.00	8.80					
130	6.20	11.40	8.10					
131	11.60	9.79	7.16					
132	6.97	5.02	15.75					
133	8.60	6.30	13.93					
134	28.18	14.49	19.65					
135	9.81	10.10	12.35					
136	12.83	16.83	14.72					

137

Fig. 22. Structures and antimalarial activities of derivatives **125–137**

and mefloquine. *In vivo*, compound **136** cured 3/5 mice at 160 mg kg⁻¹day⁻¹ doses (*p.o.*, *P. berghei* KBG 173 strain), however, at lower doses of 40 mg kg⁻¹day⁻¹, the activity sharply declined with no cured mice.

The only intramolecular steroidal 1,2,4,5-tetraoxane **137** (Figure 22) tested up to now exhibited moderate *in vitro* antimalarial activity against *P. falciparum* strains (IC_{50}(D6) = 0.63 µM; IC_{50}(W2) = 0.52 µM) [105]. Recently, Terent'ev et al. published the synthesis of diverse intramolecular tetraoxanes from *β*-diketones and hydrogen-peroxide in the presence of strong mineral acids [106].

8. CHIMERIC PEROXIDE – QUINOLINE COMPOUNDS

A new concept for treating malaria was introduced with chimeric peroxides, the compounds that possess covalently bonded two well-known pharmacophores – the quinoline functionality and peroxide moiety. The compounds were designed with the aim to overcome resistance of the parasite to CQ-based drugs and to take advantage of the PK properties of trioxanes, ozonides and tetraoxanes. The initial set of derivatives was more active against CQR strains, with compound 138 as the most active against laboratory strains [107] and human isolates (Figure 23) [108].

D6 (nM)		W2 (nM)	
IC_{50}	IC_{90}	IC_{50}	IC_{90}
4.52	17.27	2.13	6.11

n = 1, 2 or 3
Ar = phenyl or 4-biphenyl

n = 1, 2 or 3

	IC_{50} (nM)		
	FcB1[a]	FcM29[a]	Nigerian[a]
138	9.0	18.0	2.0
138[b]	27.0	27.0	22.0
140[b]	15.0	6.0	17.0
141[b]	112.0	108.0	176.0
142	24.0	10.0	-
143[b]	365.0	188.0	536.0
CQ[c]	116.0	155.0	19.0

[b] Tested as dicitrate salt; [c] Tested as diphosphate salt.

IC_{50} (nM)		% Activity (10 mg/kg)	
K1	NF54	p.o.	s.c.
11.98	10.74	75	99.95

[a] **FcB1 -** Colombia CQR strain *P.f.*; [a] **FcM29 -** Cameroon highly CQR strain *P.f.;* [a] **Nigerian -** CQS stain *P.f.*

Fig. 23. Structures and antimalarial activities of chimeras **138–146**

Certain simplifications of structure lead to trioxaquine **140** that was very active against both CQS and CQR strains of *P. falciparum* [109]: ED_{50} = 5 and 18 mg kg^{-1} day^{-1} (*P. vinckei*), i.p. and *p.o.* administration, respectively. Parasitemia clearance, without recrudescence, was achieved after 18 mg kg^{-1} day^{-1} i.p. dose, and no toxic effects in mice were observed even at 120 mg kg^{-1} day^{-1} *p.o.* dose over four consecutive days. Coupling of the same trioxane ketone and primaquine produced trioxaquine **141**, which demonstrated significantly lower activity against all three examined strains, and thus limited this concept only to 4-aminoquinolines. Trioxaquine **142** also exhibited high *in vitro* and *in vivo* activity [110]. The compound completely cured mice infected with CQR strain *P. vinckei* and CQS strain *P. vinckei* in 30 mg kg^{-1}day^{-1} *p.o.* doses.

Other variations of trioxane structures produced derivatives **143** [111] and **144** [112], which expressed lower activities.

Although some very active trioxaquines were prepared, the concept of these drugs did not justify itself for several reasons. First, these compounds did not show expected synergism, since in many cases parent trioxane ketones [103, 112] were more active than hybrids – see ketone **139** *vs* chimera **138** for comparison. In addition, 4-aminoquinolines **145** itself are antimalarials. They suppressed parasitemia in mice (i.m., 87–97%), but as with trioxaquines **144**, no treated mice survived [112]. Even though some authors wish to establish these chimeras as therapeutics with dual activity [110, 113] all available evidence on the mechanism of antimalarial activity of trioxanes and 4-aminoquinolines points out that they have the same target [110]. Lastly, many of these hybrid molecules were tested as inseparable mixtures of diastereomers. These circumstances deprive one of the possibilities to realize the actual scope of the antimalarial capacity of these compounds.

Based on the same concept and enhanced activities of trioxolanes with the basic side chain, chimeric trioxolane **146** was obtained (Figure 23) [114]. Although it is very active, *in vitro* against K1 and NF54 strains and *in vivo* against ANKA strain of *P. berghei*, **146** did not achieve the synergic effect of two pharmacophores, especially when compared to trioxolanes **76** and **80**.

Another variations of trioxane hybrid antimalarials were made with covalently bonded DHA and C(18)-quinine acid **147** (Figure 24) [115]. Two segments were bonded with a hydrolytically labile ester bond, and it is possible that under physiological conditions, hybrid **148**
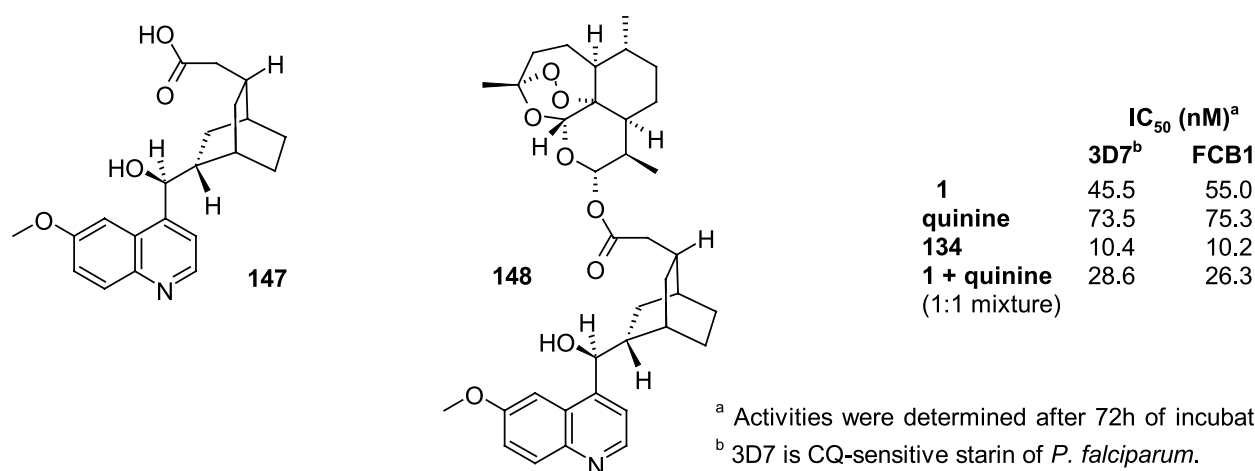

	IC$_{50}$ (nM)[a]	
	3D7[b]	FCB1
1	45.5	55.0
quinine	73.5	75.3
134	10.4	10.2
1 + quinine (1:1 mixture)	28.6	26.3

[a] Activities were determined after 72h of incubation;
[b] 3D7 is CQ-sensitive starin of *P. falciparum*.

Fig. 24. Structure and antimalarial activity of derivatives **147** and **148**

actually delivers two active agents. This approach seems to be logical since there is evidence that ARTs and quinine are active during the same stage of the *P. falciparum* life cycle [116], but differ in mechanism of action [115]. Obtained results showed that hybrid **148** had significantly higher antimalarial activity than ART and quinine alone, or when it is compared to an equimolar mixture of ART and quinine. However, the observed results would be more significant if the authors compared hybrid **148** to DHA rather than ART or/and to acid **147**.

Based on data obtained from research on the mechanism of tetraoxane action **[101]** (*vide infra*) chimeric tetraoxaquines were designed with the aim to exert effects of the presence of two pharmacophores within the same molecule

(Figure 25) [117]. Three of them were as active as ART or MFQ against tested strains of *P. falciparum*, and all of them showed higher activity than CQ against CQR strains. Although a synergic effect was not achieved, it should be noted that the derivatives exhibited higher activity than non-chimeric derivatives having related structure (compare **149** to **85**, **150** to **90** and **91**, **151** and **152** to **133**) [89c]. *In vivo* experiments (*p.o.*, *P. berghei* KBG 173 strain) revealed that derivatives **149** and **150** cured all tested mice at 320 mg kg^{-1}day^{-1} doses. At lower doses of 80 mg kg^{-1}day^{-1} the derivatives were less effective, however, they still cured 3/5 of examined mice. Both compounds have minimum active doses (MAD) of 20 mg kg^{-1}day^{-1} with no toxic effects even at the highest applied dose of 960 mg kg^{-1}.

IC$_{50}$ (nM)		
	D6	**W2**	**TM91C235**
149	2.33	2.00	3.70
150	9.05	5.76	9.90
151	6.53	4.47	8.54
152	33.45	22.95	30.20
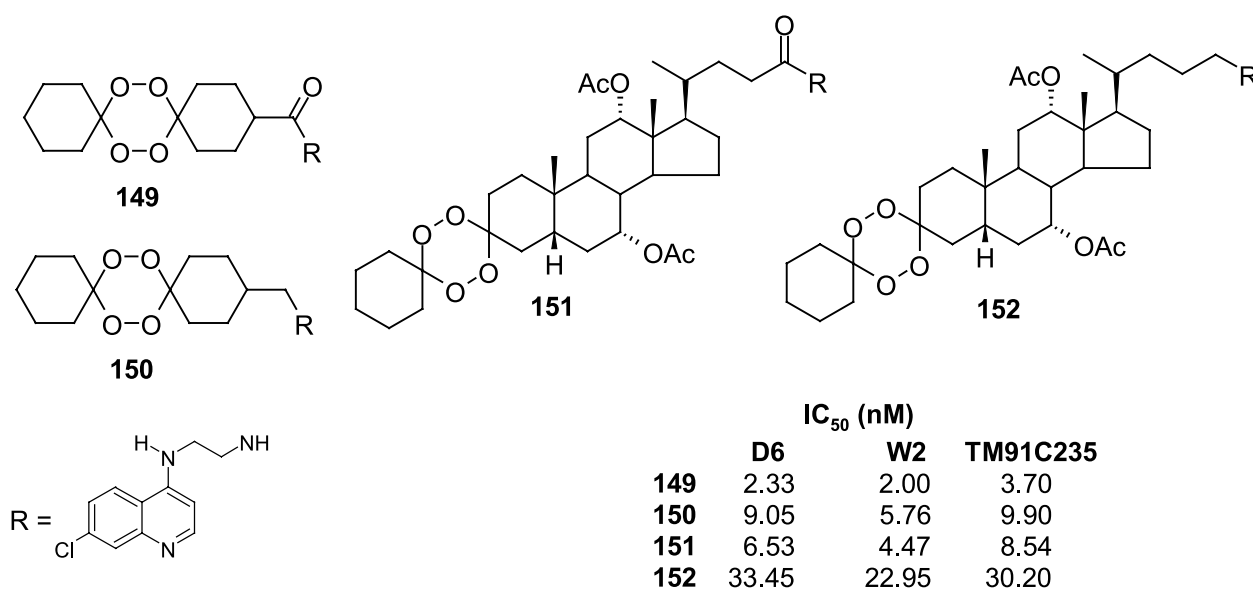
Fig. 25. Structures and antimalarial activities of derivatives **149–152**

9. METABOLISM AND DRUG COMBINATION ASSAYS

Although significant numbers of 1,2,4,5-tetraoxane antimalarials have been reported and some exhibit extraordinary antimalarial activity, their pharmacological properties have been much less explored than those of

1,2,4-trioxanes. Vennerstrom and co-workers were the first to give detailed metabolic and pharmacological profiles of tetraoxanes [118]. It was shown that the compound **153** (Figure 26) is synergistic with MFQ and quinine against both CQS D6 and CQR W2 *P. f.* strains. Tetraoxane **153** is also synergistic with CQ, unlike ART that shows an additive effect with CQ and

| | IC$_{50}$ (nM) | |
	D6	W2
153	6.90	3.40
154	14.76	6.80
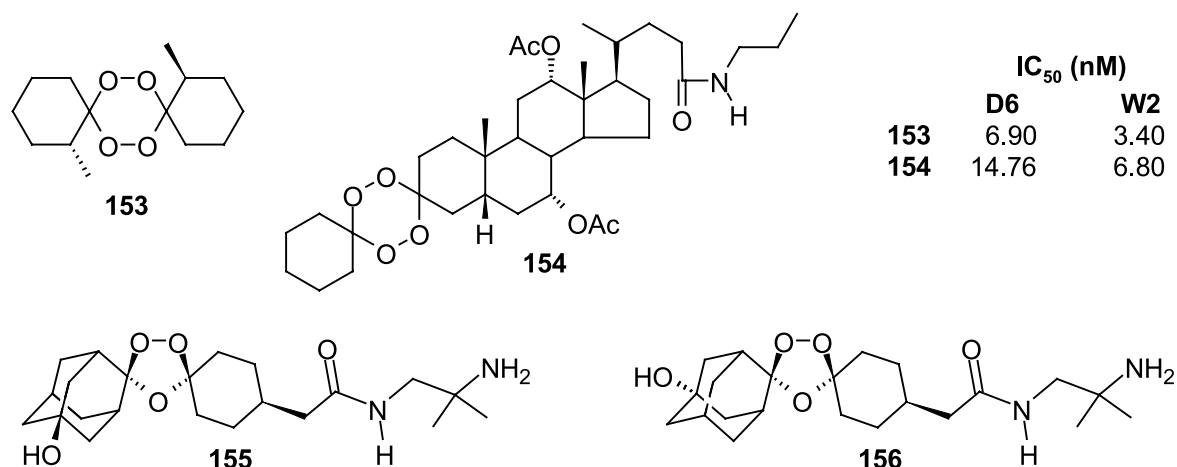
Fig. 26. Structures and antimalarial activities of derivatives **153–156**

synergism only at high concentrations [119]. In addition, **153** is synergistic with ART in contrast to other semisynthetic derivatives which have uniformly additive effects. Derivative **153** is a modest inhibitor of human CYP1A2 activity and has a different metabolic pathway versus ART. Artemisinin induces its own metabolism [118, 120] and is metabolized by CYP2B6, CYP2C19 and to a less extent by CYP3A4. Tetraoxane **153** is most likely metabolized by CYP1A2 and unlike artemisinin, **153** has prophylactic activity protecting 4/7 mice against *P. berghei*. These results suggest that tetraoxanes have different metabolic pathways with regard to ART and may have a somewhat different mechanism of action. Šolaja et al. reported the results of metabolic stability assays and metabolic identification for many cyclohexylidene and steroidal mixed tetraoxanes [89b, 89c, 99, 101, 102, 117]. Incubation of human, mouse, rat and rhesus monkey liver microsomes with examined compounds showed dissimilar half-lives and various mono- and di-hydroxylated products, and the products of dehydration and deacetylation were detected as well. Unfortunately, correlation of metabolic stability assays and proposed metabolite structures with the *in vitro* and *in vivo* antimalarial activities of these derivatives could not be carried out. It is important to note that no products of peroxide bond

cleavage were detected. Drug combination assays of tetraoxane **154** (Figure 26) showed that this derivative is additive with ART, DHA and artesunate **8c** against both CQS (D6) and CQR (W2 and TM91C235) *P. falciparum* strains, however, tetraoxane **154** showed synergism with artelinic acid **10a** against all three strains [121]. Tetraoxane **154** is additive with MFQ under high concentrations against D6 and TM91C235, but at low concentrations it exhibits an antagonistic effect. With chloroquine, tetraoxane **154** showed antagonism against all three tested strains.

After incubation of ozonide **60 OZ277** (Figure 14) with human liver microsomes *in vitro*, three mono-hydroxylated metabolites were detected [122]. Two major metabolites **155** and **156** (Figure 26) appeared after hydroxylation of the adamantane substructure. Both metabolites had low antimalarial activity with IC$_{50}$ values > 245 nM against the CQR K1 *P. falciparum* strain.

Earlier in this text was mentioned that the incubation of isotopically labelled artemisone **28e*** afforded metabolites **29–33**, with *syn*hydroxyl and peroxide groups (Scheme 3) [46]. Incubation with microsomes and 14 recombinant CYP isoforms together with selective inhibitors showed that only recombinant CYP3A4 significantly metabolized **28**, indicat-

ing that artemisone and ART, in spite of being structurally similar, have different metabolic profiles in *P. falciparum*. Therefore, it is possible that they can exert their antimalarial activity through similar but not identical mechanisms.

It is especially interesting, and should be underlined, that a common denominator in metabolic transformation is the iron species in the haem prosthetic group of CYPs, which could take part in Fenton chemistry with organic peroxide. However, during the described interaction of all these structurally diverse compounds with CYP enzymes, peroxide bond scission never occurred.

10. MECHANISM OF ACTION

Many research groups were involved in solving the mechanism of action of antimalarial peroxides, and plenty of reports can be found on this subject. However, the scientific literature is largely controversial, and many authors disagree about the site of action, putative target and lethal reaction species, and the actual killers of the parasite.

Several groups investigated the mechanism of action for more than fifteen years [123–127]. In general, it was found that the process starts with the formation of oxygen radicals **157** and **158**, which arise upon homolytic peroxide bond scission in the presence of ferrous ions (Scheme 5). It is assumed that ions **157** and **158** undergo intramolecular rearrangements (1,5-hydrogen shift and β–scission) to form C-radicals **159** and **160**. Both O- and C-centred radicals are highly reactive species and are lethal to parasites. During these processes, one or more intermediates probably reacts with vital biomolecules, inhibits their activity and causes the parasites to die. It was proved that one of the side products is high-valent iron-oxo species Fe(IV)=O, which could also be toxic to the parasite [128, 129]. In the absence of a suitable target that would be alkylated the products **161** and **162** were formed [127b]. The adducts **163–167** (Figure 27) were found to be indicative of primary and

secondary radicals and the ability of ART and its derivatives to act as alkylating agents [123c, 126, 127]. The results obtained with artesunic acid and trioxaquine **140** additionally confirmed the capability of peroxides or chimeras to alkylate haem [130].

After the incubation of erythrocytes infected with D6 or FCR3 *P.f.* strains and isotope-labelled [10-^{3}H]-**6** or [15-^{3}H]-**7b**, radioactivity was detected in protein fractions originated from the parasite [131]. After treatment with EtSH or 8 M urea it was concluded that proteins and isotopically labelled fragments of **6** and **7b** are covalently bonded. No radioactivity was detected after the incubation with nonfected erythrocytes or with radiolebeled deoxo-ART. Some of alkylated proteins were *P. falciparum* membrane proteins MSA-1, MSA-2, CRA.5.1, TCTP (Translationally Controlled Tumour Protein) and histidine-enriched protein (42 kDa) [132, 133]. TCTP possesses bonded haem and to some extent is present in the food vacuole (FV) membrane. Compound **6** reacted *in vitro* with recombind TCTP in the presence of in situ generated haem and formed covalent adduct in a 1:1 ratio. The amount of covalent adduct decreased to 60% when one of the cysteines was chemically blocked.

A second proposed target was raised upon results of very important research [134] that ART is a very effective inhibitor of sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) orthologous (*P. falciparum* ATP6) and that a catalytic amount of Fe(II) enhanced the inhibitory activity of ART. It was evidenced that the possible site of action of ART could be outside the FV, and that the trigger for ART toxicity towards the parasite could be Fe(II) situated in the cytosol and not necessarily the free haem within the FV. *In vitro* resistance of 530 *P. falciparum* isolates from three countries (Cambodia, French Guiana and Senegal) towards ART and its closest derivatives were investigated [10]. It was found that resistance was positively correlated only with mutation of SERCA *P. falciparum* ATPase6 genes and that *P. falciparum* ATPase6 is the target of ART

antimalarials. All resistant isolates came from areas with uncontrolled use of ART derivatives.

ART and arteether **7b** effectively inhibit FV proteolytic activity of enzymes that degrade haemoglobin, specifically cysteine-protease [135]. Compound **E-64** (for the structure see ref. 136) a specific inhibitor of cysteine-prote-ase is both an ART and **7b** antagonist. These
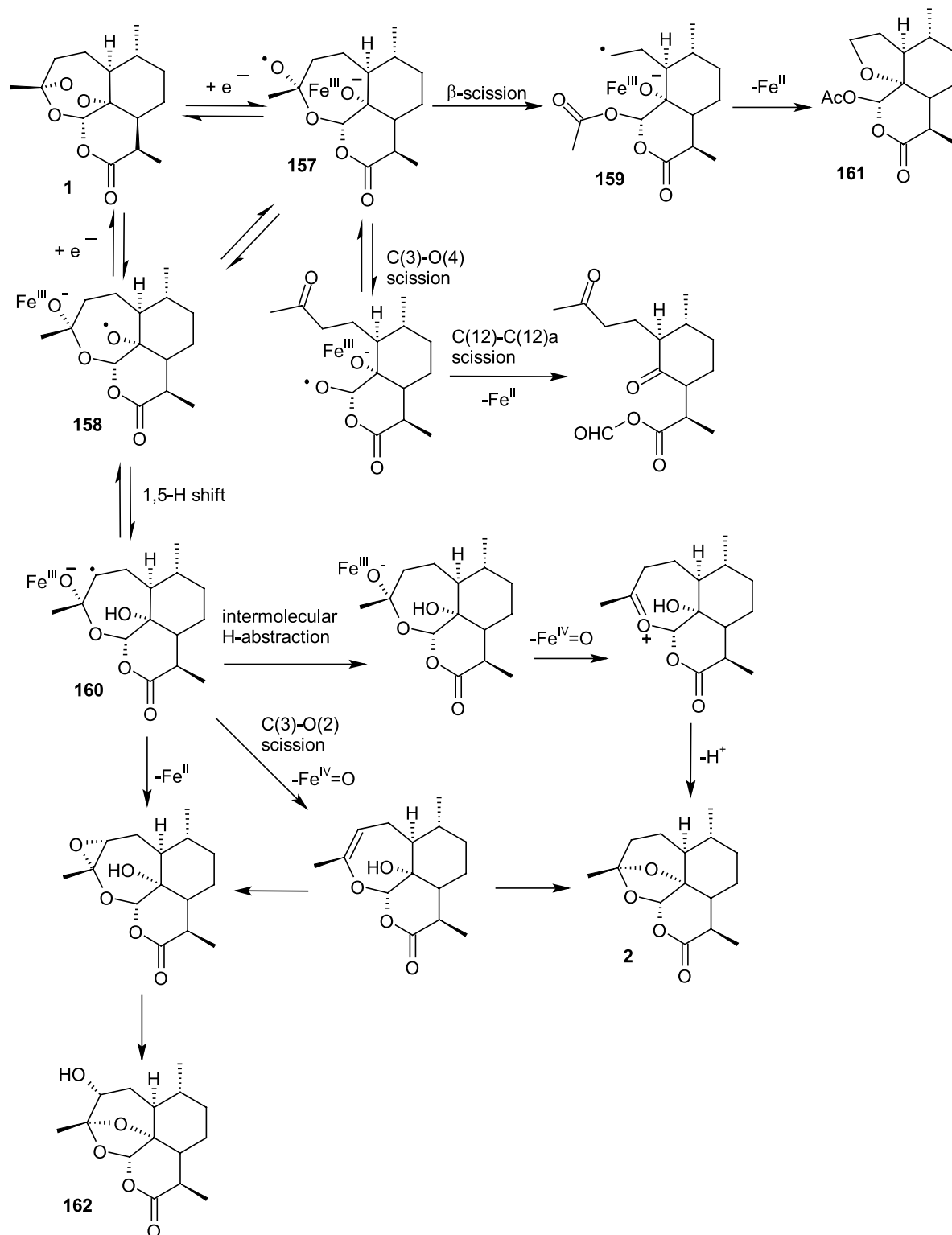
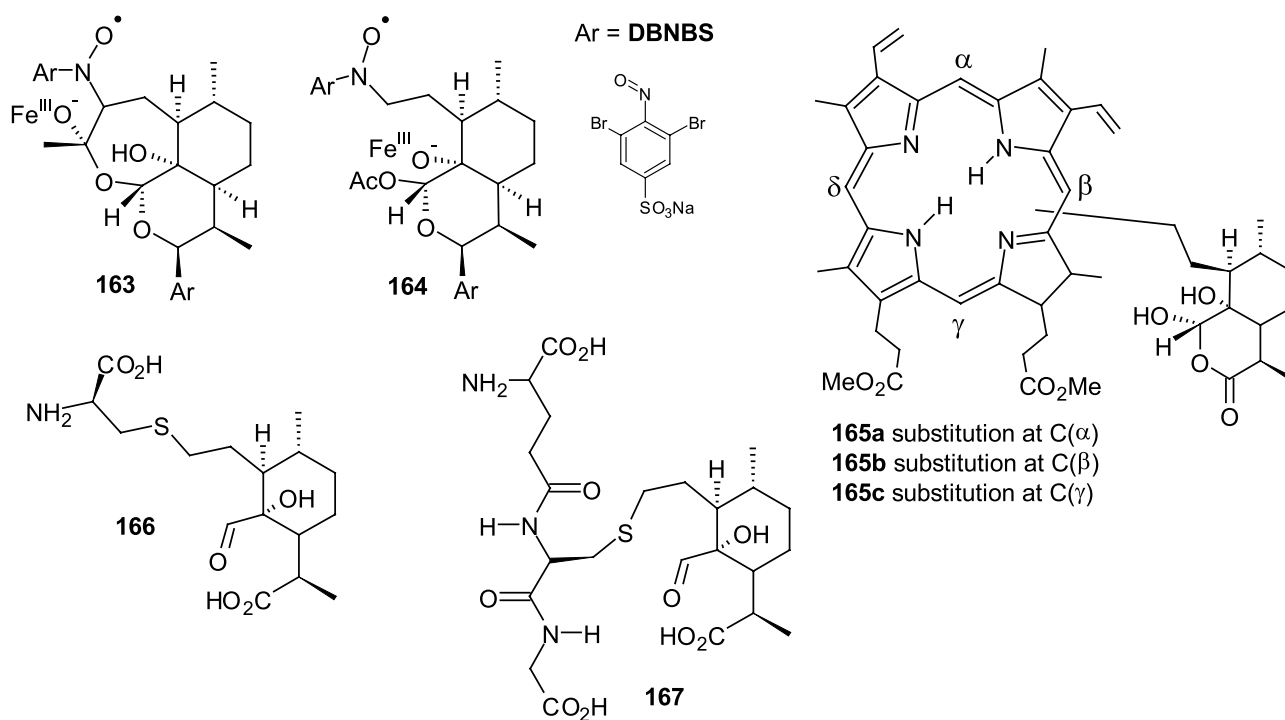**Scheme 5.** Proposed mechanism of Fe(II) induced decomposition of artemisinin and the generation of lethal O- and C-radical species

Fig. 27. Structures of covalent adducts **163–167** derived from C-centered radicals

observations further confirmed by *ex vivo* experiments show accumulation of haemoglobin in parasites treated with ART, suggesting the inhibition of haemoglobin degradation. According to the above findings, it is not clear how artemisins and other peroxides would exert their antimalarial activity after reaction with free haem in the FV, where they inhibit catabolism of haemoglobin and the liberation of haem.

Recently [137], it was found that artemisinin, sodium artesunate and dihydroartemisinin react with haemoglobin (ferrous haem), but not with methaemoglobin (ferric haem) under standard solution conditions (50 mM phosphate buffer, pH 7, 37 ºC). The authors claim that the reaction selectively occurs at the haem sites and consists of the progressive, slow decay of the Soret band because of haem alkylation and subsequent loss of π-electron delocalization. This finding further complicates the elucidation the mechanism of action of artemisinins.

Antimalarial activity does not necessarily correlate with chemical reactivity. Amino artemisinins **28a** and **28b** react readily with haem to give the expected products, but derivatives **3** and

168 (Figure 28) did not [45,138]. On the contrary, derivative **3** reacted with aqueous Fe(II) but **168**, **28a** and **28b** were inert [45, 139]. Very interesting information came from the discovery that compounds such as **169** – **172**, which cannot generate either primary or secondary C-radicals, exhibit pronounced antimalarial activity [125]. Similar to **3**, artemisone **28e** readily reacted with aqueous Fe(II) affording corresponding products [139]. Both compounds reacted with Fe(OAc)$_2$, and in the presence of radical trapper 4-oxo-TEMPO, gave corresponding covalent adducts (3% for **3**, 10% for **28e** and 73% for **173**). DFO, an iron chelator, antagonized the antimalarial activity of aqueous Fe(II)-susceptible artesunate and trioxane **3**, but had no observable effect on aqueous Fe(II)-resistant derivative **168**, nor on artemisone **28e** [139]. It was found that **28e** efficiently alkylates haem [140].

Due to high specificity of ARTs for malaria parasite SERCA ATPase, and the inhibition of SERCA orthologues of *P. falciparum* (*Pf*ATP6) and *P. vivax* (*Pv*SERCA) [134], it was found that artemisone **28e** is an even better inhibitor, having a K_i =1.7 ± 0.6 nM for *Pf*ATP6, and a K_i = 0.072
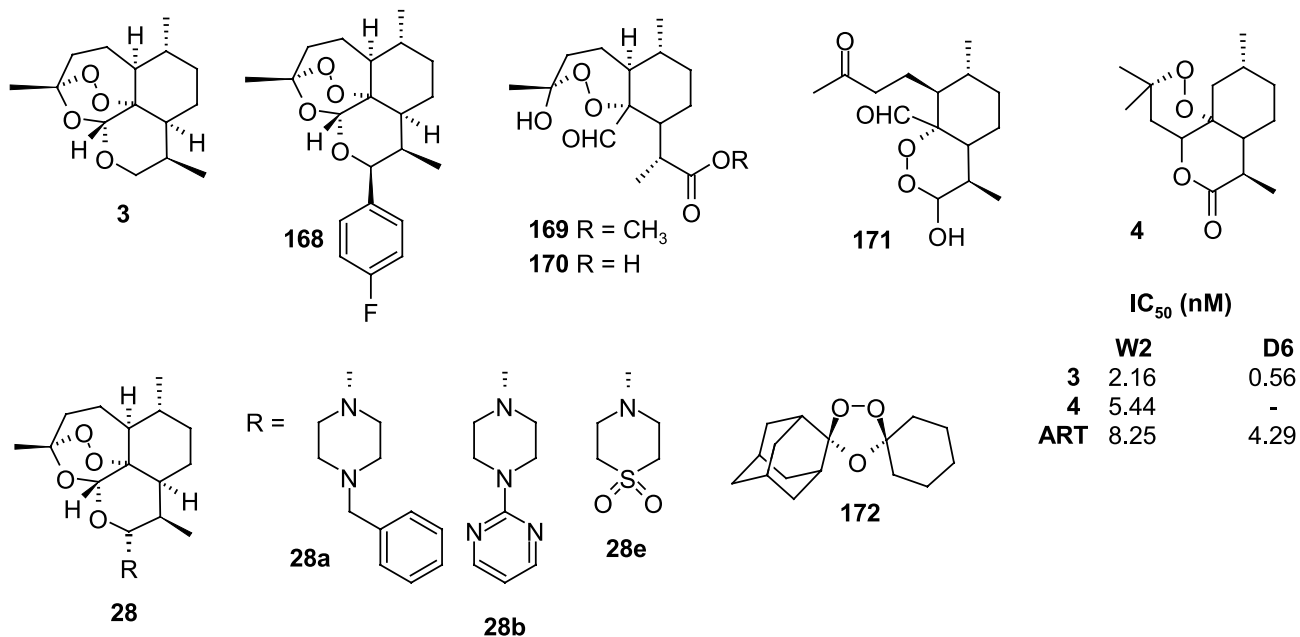
Fig. 28. Structures and antimalarial activities of derivatives **3**, **168–173**
and selected derivatives of **28**

± 0.012 nM for *Pv*SERCA. For comparison with ART: K_i = 169 ± 31 nM for *Pf*ATP6 and K_i = 7.7 ± 4.9 nM for *Pv*SERCA [46]. In contrast to these derivatives, compound **168** has low inhibitory activity against *Pf*ATP6 (K_i = 277 ± 39 nM), which is in contrast to its high *in vitro* potency (IC$_{50}$ 3D7 = 1.44 nM) [139].

Studies with conjugates **173**–**175** of ART derivatives, ozonides and 1,2,4,5-tetraoxanes with acridine and nitrobenzyldiazole (NBD) fluorochromes (Figure 29) showed that they

accumulate only in infected erythrocytes, both within the cytoplasm and the FV of the parasite [91, 141]. Formation of the stabile adducts of acridine and NBD tagged peroxides with biomolecules within parasite was inhibited by co-incubation with the iron chelator DFO. In addition, the investigated peroxides and their conjugates showed marked antagonism in combination with iron chelators DFO and DFP [141]. All investigated conjugates showed high *in vitro* antimalarial activity in the 5–13
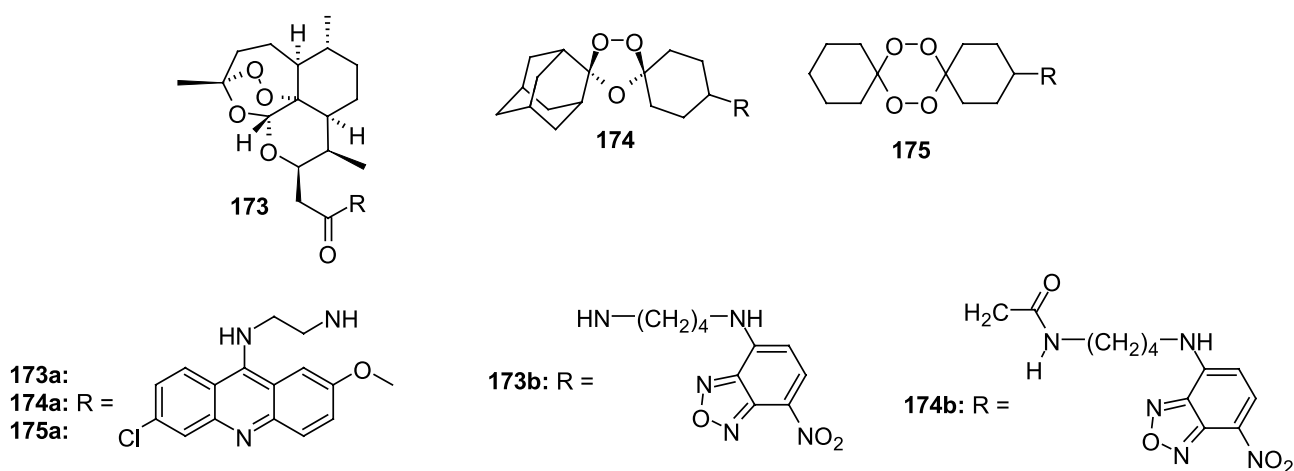
Fig. 29. Structures of conjugates **173–175**

nM range against the 3D7 *P. falciparum* strain. These results suggest that both the cytoplasm and FV of the parasite could be equally possible sites of action of antimalarial peroxides, and that the haem from FV or chelatable "free" iron from the cytoplasm both could trigger the scission of the peroxide bond.

Adamantyl peroxide-ozonides **OZ277**, **OZ439** and **177**, tetraoxane **176** and 1,2,4-trioxane **178** (Figure 30) were subjected to reaction with different ferrous salts. It was shown that **OZ439** [76] and tetraoxane **176** [91] showed significant stability under the applied reaction conditions, while **OZ277** [76] and trioxanes **177** and **178** readily reacted and formed secondary carbon radicals that were intercepted with TEMPO [142].

Ozonide **OZ277** inhibits *Pf*ATP6 with lower potency in comparison to ART – K_i (**OZ277**) = 7.7 mM *vs* K_i (ART) = 79 nM, and thus suggests that these two peroxides may have different mechanisms of action [143]. However, the two
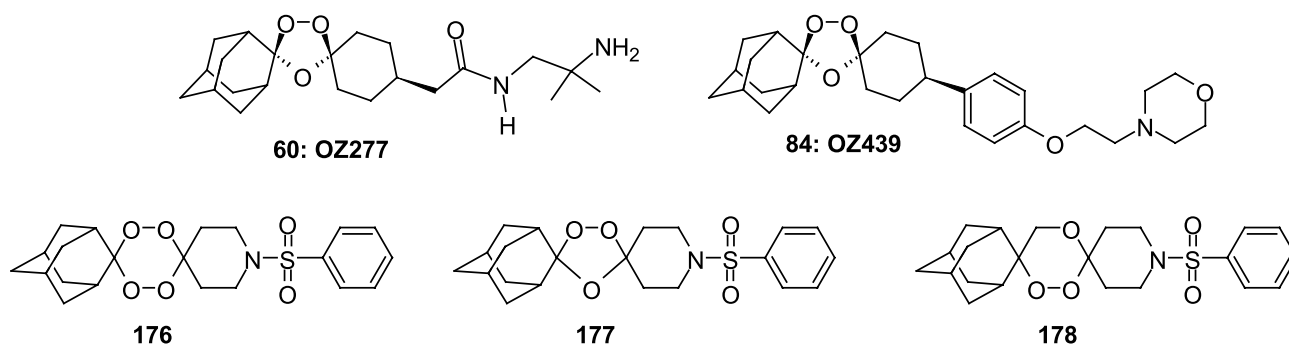
Fig. 30. Structures of peroxides **OZ277**, **OZ439** and **176–178**

compounds have certain similarities like abrogation of the activity of **OZ277** in the presence of DFO, antagonism in combination with DFO [143] and formation of C-centred radicals during reaction with Fe(II), and the formation of the corresponding adduct with TEMPO [65]. Fluorescent derivate of **OZ277** was localized in the parasite cytosol in one parasite and in the FV in the other. In the cytosol it was associated with the parasite endoplasmatic reticulum. In addition, the ozonide **OZ277** showed antagonism in combination with artesunate.

Based on the finding that the *in vitro* antimalarial activity of artemisinin was significantly increased under a 2 % carbon monoxide atmosphere (by 40–50 %) and under 20 % oxygen atmosphere (by 20–30 %) [144], a detailed study of the interaction of artemisone and related derivatives with various forms of haemoglobin (Hb), haem, as well as an analysis of their antimalarial activities was performed [145]. In contrast to ART, artemisone does not react with Hb-Fe(II) and oxyHb-Fe(II) to produce metHb-Fe(III); in addition, it was shown that both artemisinin and artemisone (and **28a**, **28b**, **168**) are inactive toward Hb-Fe(II) in a 2% carbon monoxide atmosphere. On the other hand, both compounds induced the oxidation of the haemoglobin catabolic product, haem-Fe(II), to produce haem-Fe(III). In addition, on exposure of haem-Fe(II) and Hb-Fe(II) to carbon monoxide, stable complexes were formed, which are also inactive for reactions with peroxide antimalarials. However, in a biologically relevant experiment, both compounds showed increased antimalarial activity against *P. falciparum* W2 strain under a 2% carbon monoxide atmosphere, while the activity of CQ remained unchanged. The authors suggested [145] that peroxide antimalarials behave as reactive oxygen species (ROS), or that they produce ROS via Haber-Weiss chemistry. Furthermore, it was concluded that passivation of haem-Fe(II) by its conversion into the CO complex results in decreased decomposition of artemisinins (artemisone included), thus making them more available for reaction with their actual target. The authors proposed that Fe(II), regardless

of its origin (Hb-Fe(II) or haem-Fe(II)), is not the sole initiator of O–O scission, and suggested that another one should be searched for [145].

A three-dimensional QSAR pharmacophore model of the antimalarial activity of *bis*-steroidal and mixed steroidal 1,2,4,5-tetraoxanes was developed [146]. The model contains two hydrogen bond acceptors (lipid) and one hydrophobic (aliphatic) feature, and maps well onto the potent analogues and many other active peroxide antimalarials such as ART, arteether, artesunic acid, and simpler tetraoxanes. It appears that the presence of at least one hydrogen bond acceptor in the trioxane or the tetraoxane moiety is necessary for good activity for this class of compounds. Docking calculations with haem suggested that the proximity of the Fe(II) and oxygen atom of the trioxane or the tetraoxane moiety favours potent activity of the compounds and that electron transfer from the peroxide oxygen is crucial for mechanism of action.

Similarly to artemisinins, results from investigation of tetraoxane's mechanism of action are also contradictory. Reaction of tetraoxane **179** with Fe^{2+} in the presence of spin-trap reagent

TEMPO (a C-radical trapper) gave covalent adducts **180** and **181** (Figure 31) which confirmed C-radicals as interemediers during the process [147] same as it happened with ART. The same results were obtained with lipophilic tetraoxane **184**, and with more polar and water soluble **106** (**RKA182**), independent from using ferrous salts or solvents. In addition, during reaction of these tetraoxanes with haem, covalent adduct **185** was isolated, confirming the alkylation capability of tetraoxanes. It is interesting that the same type of adduct was isolated no matter which tetraoxane was used, obviously due to more stable radicals as dominant intermediates. In the same study, during Fe^{2+} induced cleavage of tetraoxanes in the presence of 2-linoleoyl-1-palmitoyl-*sn*-phosphatidylcholine (PC), observed peroxidation of PC was observed, which confirmed an ROS generating characteristic for Fenton chemistry.

Differences between 1,2,4,5-tetraoxanes and the other peroxides were underlined with the studies of the mechanism of action of *bis*- and mixed steroidal tetraoxanes [101]. Performing experiments under the same conditions as for other antimalarial active peroxides, including
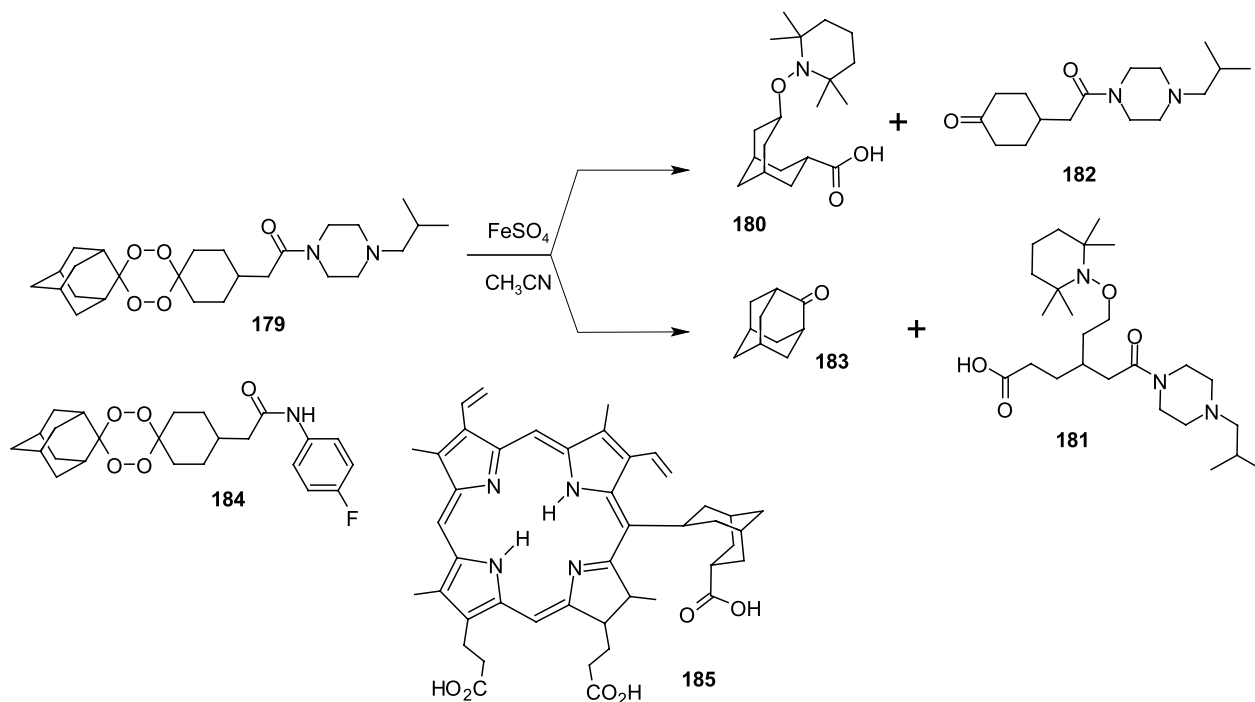

Fig. 31. Fe(II)-mediated degradation of tetraoxane **179**

those presented above [147], steroidal tetraoxanes unexpectedly generated only oxygen centred radicals that did not further rearrange to carbon centred radicals. When using DMPO and DEPMPO, both O- and C-radical traps, the EPR experiments revealed only DMPO-˙OR and DEPMPO-˙OR spin-trapped adducts. The corresponding ketones were isolated as the only organic products from the reaction mixture and no traces of rearranged products that would result from C-radical intermediates were detected. Indirect evidence of existing high valent Fe(IV)=O species was obtained from HMDB → HMB rearrangement. Based on the evidence two pathways (Scheme 6) were proposed suggesting that tetraoxane peroxides serve as the RO˙ radical source, as well as the source of the Fe(IV)=O species. RO˙ radical species are capable of membrane hydroperoxidation (RBC membrane, parasite membrane, cytosol, or FV) or possibly attack other vital biomolecules. Support for this observation was given in the study of Kumura et al. [148], who reported sharp differences in reactivity between tetraoxanes and ART during Fe^{2+} induced oxidative degradation of phospholipid PC. While ART were unreactive

under the applied conditions, tetraoxanes (both water soluble and lipophilic derivatives) were highly active, and yielded products of oxidation of PC even under inert reaction conditions. These results confirmed the existence of ROS, O-centred radicals and/or Fe(IV)=O, suggested in the study with steroidal tetraoxanes [101]. The same products were observed from reaction of PC with Fe^{2+} under O_2 atmosphere. Furthermore, O'Neill [147] and Kumura [148] observed the same product of peroxidation of PC. Since in both studies, experiments were performed under inert atmosphere, it is concluded that ROS, which reacts with PC, could originate only from tetraoxanes.

Very recently, a non iron-mediated mechanism of bioactivation has been proposed by Haynes and Monti [149]. Methylene blue (MB) was the first synthetic drug ever used in humans, and it was Paul Erlich who cured two patients from malaria using MB in 1891 [150]. The discovery that ART and artemether exhibit synergic effects with methylene blue [151], unlike chloroaminoquinolines, initiated systematic examination of reactivity relationship between the drugs with the aim to possibly correlate their mechanism
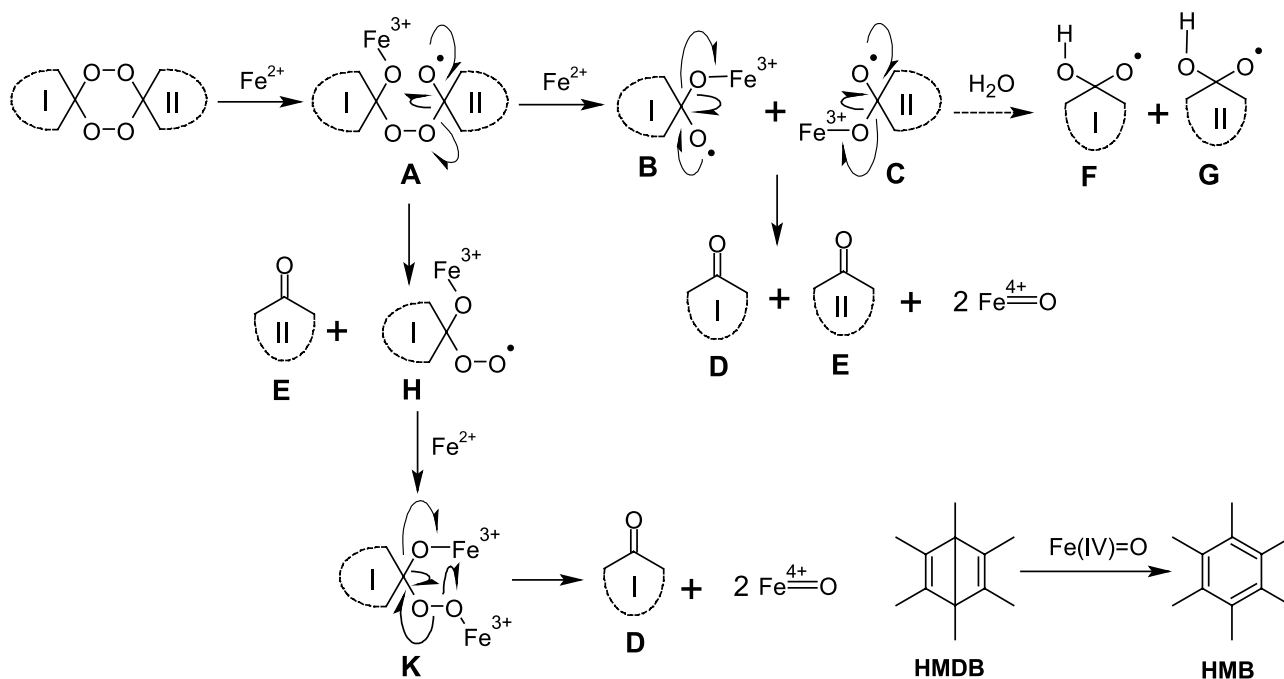
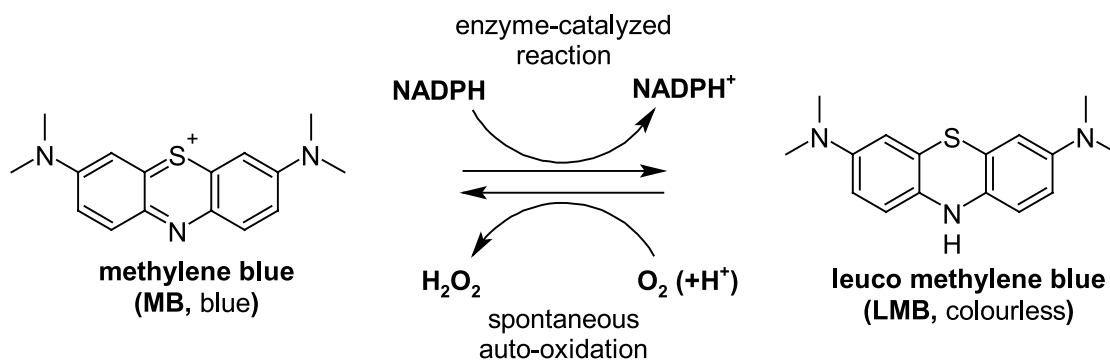**Scheme 6**. Proposed mechanism of action of tetraoxanes

Fig. 32. Redox-cycling interconversion between methylene blue (**MB**) and leucomethylene blue (**LMB**)

of antimalarial action. Subsequent research by the same group [152] revealed that MB is a redox-cycling agent that produces H_2O_2 at the expense of O_2 and of NADPH in each cycle (Figure 32). Thus, MB consumes the NADPH and O_2 needed for the pathogen's metabolism, very probably seriously affecting the NADPH/NADP ratio.

The results of subsequent research revealed that artemisone, other artemisinins [149] and other peroxide antimalarials like ozonides and tetraoxanes [153] are active in the presence of MB-ascorbic acid, MB-N-benzyl-1,4-dihydronicotinamide (BNAH), riboflavin–BNAH and riboflavin–NADPH systems, and yield identical products as those that were isolated from the reaction of same the antimalarials with Fe(II). According to this proposal, antimalarial peroxides act as oxidants, re-oxidizing LMB and $FADH_2$, and so contribute to the depletion of NADPH (Scheme 7).
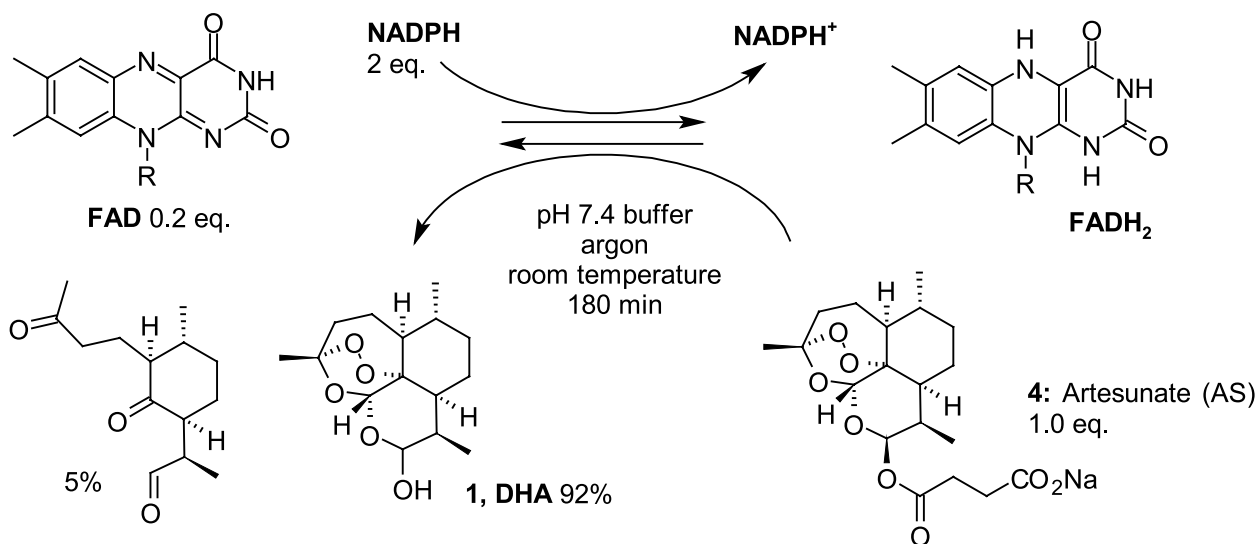
Scheme 7. Biomimetic catalytic system under physiological conditions: works with other flavins, all examined artemisinins, tetraoxane, and trioxolane antimalarials

11. CONCLUSION

Synthetic and semi-synthetic peroxides are effective drugs and are used with success in the treatment of severe malaria. They are especially efficient against CQR strains of *P. falciparum*, the cause of cerebral malaria. Accessibility, relatively inexpensive preparation, and the stability of the 1,2,4-trioxane and 1,2,4,5-tetraoxane groups to a broad spectrum of reaction conditions enables the syntheses of derivatives of diverse structures and makes possible the discovery of even more effective drug(s). All authors emphasized the low toxicity of these compounds with rare cases of unwanted side effects. To date, there are no major examples of appearance of resistance of *Plasmodium* species, except in cases when drugs were used without proper control. These facts, together with the possibility for combination with other non-peroxide drugs, chiefly aminoquinolines, opens unrestricted possibilities in combating malaria.

The reported results concerning the mechanism of antimalarial peroxide action are contradictory at first glance. However, having in mind that the compounds significantly differ in their structures that are responsible for different logP, bioavailability, passage through cellular membranes, and stereospecific interactions with assumed receptors or trigger species, the observed differences are logical. Our opinion is that antimalarial peroxides may themselves have different action pathways and that they may have different targets. The above-mentioned does not exclude certain common behaviours, such as the capability of acting as alkylating agents. In addition, this does not exclude the possibility that one compound is active against several different targets simultaneously. Perhaps it should not be expected that all peroxides fit into unique mechanism of action. It is more likely that they have complex, multi-targeted mechanisms, including the oxidative stress [139].

REFERENCES

[1] Malaria Foundation International, http://www.malaria.org/ (accessed May, 2012)

[2] C. Hay, A. Guerra, A. Tatem, R. Noor, R. Snow, The global distribution and population at risk of malaria: past, present and future, *Lancet Infect. Dis.*, **4**, 327-336 (2009).

[3] S. L. Hoffman, G. M. Subramanian, F. H. Collins, J. C. Venter, Plasmodium, human and Anopheles genomics and malaria, *Nature*, **415**, 702-709 (2002).

[4] A. Maxmen, Malaria surge feared (*The WHO releases action plan to tackle the spread of insecticide-resistant mosquitoes*), *Nature,* **485**, 293 (2012).

[5] World Health Organization, Global Malaria programme (GMP), http://www.who.int/malaria/ (accessed May, 2012)

[6] X.-D. Luo and C.-C. Shen, The Chemistry, Pharmacology and Clinical Applications of Qinghaosu (Artemisinin) and Its Derivatives, *Med. Res. Rev.*, **7,** 29-52 (1987).

[7] (a) D. L. Klayman, Quinghaosu (Artemisinin): An Antimalarial Drug from China, *Science*, **228,** 1049-1055 (1985); (b) K. Borstnik, I.-H. Paik, T. A. Shapiro, G. H. Posner, Antimalarial chemotherapeutic peroxides: artemisinin, yingzhaosu A and related compounds, *Int. J. Parasitology,* **32,** 1661-1667 (2002); (c) K. Borstnik, I.-H. Paik, T. A. Shapiro, G. H. Posner, Malaria: New Chemotherapeutic Peroxide Drugs, *Mini Rev. Med. Chem.*, **2,** 573-583 (2002); (d) Y. Lin, Y.-L. Wu, An Over Four Millennium Story Behind Qinghaosu (Artemisinin) - A Fantastic Antimalarial Drug from a Traditional Chinese Herb, *Current Med. Chem.*, **10,** 2197-2230 (2003); (e) P. M. O'Neill, G. H. Posner, A Medicinal Chemistry Perspec-

tive on Artemisinin and Related Endoperoxides, *J. Med. Chem.*, **47**, 2945-2964 (2004); (f) Y. Tang, Y. Dong, J. L. Vennerstrom, Synthetic Peroxides as Antimalarials, *Med. Res. Rev.*, **24**, 425-448 (2004); (g) A. Masuyama, J.-M. Wu, M. Nojima, H.-S. Kim, Y. Wataya, 1,2,4,5-Tetraoxacycloalkanes: Synthesis and Antimalarial Activity, *Mini Rev. Med. Chem.*, **5**, 1035-1043 (2005); (h) R. D. Slack, A. M. Jacobine, G. H. Posner, Antimalarial peroxides: advances in drug discovery and design, *Med. Chem. Commun.*, **3**, 281-297 (2012).

[8] D. M. Opsenica, B. A. Šolaja, Antimalarial Peroxides, *J. Serb. Chem. Soc.*, **74**, 1155-1193 (2009).

[9] Dejan M. Opsenica, Bogdan A. Šolaja, Second-Generation Peroxides: The OZs and Artemisone in Treatment and Prevention of Malaria: Antimalarial Drug Chemistry, Action and Use, Henry M. Staines, Sanjeev Krishna, (Eds.); Series: Milestones in Drug Therapy; Series Editors: Michael J. Parnham, Jacques Bruinvels. Springer, Basel, 2012, pp. 191-211.

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

[11] L. H. Miller, D. I. Baruch, K. Marsh, O. K. Doumbo, The pathogenic basis of malaria, *Nature*, **415**, 673-679 (2002).

[12] (a) C. E. Chitnis, M. J. Blackman, Host Cell Invasion by Malaria Parasites, *Parasitology Today,* **16**, 411-415 (2000); (b) L. H. Bannister, J. M. Hopkins, R. E. Fowler, S. Krishna, G.H. Mitchell, A Brief Illustrated Guide to the Ultrastructure of *Plasmodium falciparum* Asexual Blood Stages, *Parasitology Today,* **16**, 427-433 (2000); (c) P. Baldacci, R. Ménard, The elusive malaria sporozoite in the mammalian host, *Mol. Microbiology*, **54**, 298-306 (2004); (d) B. M. Cooke, K. Lingelbach, L. H. Bannister, L. Tilley, Protein trafficking in Plasmodium falciparum-infected red blood cells *Trends in Parasitology*, **20**, 581-589 (2004); (e) P. D. Parker, L. Tilley, Nectarios Klonis, *Plasmodium falciparum* induces reorganization of host membrane proteins during intraerythrocytic growth *Blood*, **103**, 2404-2406 (2004); (f) J. M. Przyborski, M. Lanzer, Protein transport and trafficking in Plasmodium falciparum-infected erythrocytes, *Parasitology*, **130**, 373-388 (2005).

[13] M. Frédérich, J.-M. Dogné, L. Angenot, P. De Mol, New Trends in Anti-Malarial Agents *Curr.*

Med. Chem., **9**, 1435-1456 (2002).

[14] S. E. Francis, D. J. Sullivan Jr., D. E. Goldberg, Hemoglobin metabolism in the malaria parasite **Plasmodium falciparum**. *Ann. Rev. Microbiol.* **51**, 97-123 (1997).

[15] (a) S. Pagola, P. W. Stephens, D. S. Bohle, A. D. Kosar, S. K. Madsen, The structure of malaria pigment β-haematin *Nature*, **404**, 307-310 (2000); (b) D. J. Sullivan, Theories on malarial pigment formation and quinoline action, *Int. J. Parasitol.*, **32**, 1645-1653 (2002).

[16] D. Monti, B. Vodopivec, N. Basilico, P. Olliaro and D. Taramelli, A Novel Endogenous Antimalarial: Fe(II)-Protoporphyrin IXα (Heme) Inhibits Hematin Polymerization to β-Hematin (Malaria Pigment) and Kills Malaria Parasites, *Biochemistry*, **38**, 8858-8863 (1999).

[17] D. Jeremić, A. Jokić, A. Behbud and M. Stefanović, "New Type of Sesquiterpene Lactones Isolated From Artemisia Annua L. – Ozonide of Dihydroarteannuin", *8th International Symposium on The Chemistry of Natural Products, New Delhi, 1972*, C-54, 221.

[18] G. Schmid, W. Hofheinz, Total Synthesis of Qinghaosu, *J. Am. Chem. Soc.*, **105**, 624-625 (1983).

[19] Examination of *Chemical Abstracts* database afforded 35 references related to synthetic efforts towards **1** and structurally close derivatives, including one that enables synthesis of isotopically labelled artemisinin, M. A. Avery, W. K. M. Chong, C. Jenning-White, *J. Am. Chem. Soc.*, **114**, 974-979 (1992)

[20] J. S. Yadava, R. S. Babu, G. Sabitha, Stereoselective total synthesis of (+)-artemisinin *Tetrahedron Lett.*, **44**, 387-389 (2003).

[21] H.-D. Hao, Y. Li, W.-B. Han, Y. Wu, A Hydrogen Peroxide Based Access to Qinghaosu (Artemisinin), *Org. Lett.*, **13**, 4212-4215 (2011).

[22] (a) F. Lévesque, P. H. Seeberger, Continuous-Flow Synthesis of the Anti-Malaria Drug Artemisinin, *Angew. Chem. Int. Ed.*, **51**, 1706 –1709 (2012).; (b) Kevin Booker-Milburn, A light touch to a deadly problem, *Nature Chemistry*, **4**, 433 (2012).

[23] Average results obtained from greater than eight replicates. Internal WRAIR data

[24] M. A. Fügi, S. Wittlin, Y. Dong, J. L. Vennerstrom, Probing the Antimalarial Mechanism of Artemisinin and OZ277 (Arterolane) with Nonperoxidic Isosteres and Nitroxyl Radicals, *Antimicrob. Agen. Chemother.*, **54**, 1042–1046 (2010).

[25] M. Jung, X. Li, D. A. Bustos, H. N. ElSohly, J. D. McChesney, W. K. Milhous, Synthesis and Antimalarial Activity of (+)-Deoxoartemisinin, *J. Med. Chem.*, **33,** 1516-1518 (1990).

[26] M. A. Avery, P. Fan, J. M. Karle, J. D. Bonk, R. Miller, D. K. Goins, Structure-Activity Relationships of the Antimalarial Agent Artemisinin. 3. Total Synthesis of (+)-13-Carbaartemisinin and Related Tetra- and Tricyclic Structures *J. Med. Chem,* **39***,* 1885-1897 (1996).

[27] (a) S. L. Smitha, C. J. Sadler, C. C. Dodd, G. Edwards, S. A. Ward, B. K. Park, W. G. McLeana, The role of glutathione in the neurotoxicity of artemisinin derivatives *in vitro, Biochem. Pharmacol.*, **61**, 409–416 (2001). (b) S. L. Smith, J. Fishwick, W. G. McLean, G. Edwards, S. A. Ward, Enhanced In Virgo Neurotoxicity of Artemisinin Derivatives in the Presence of Haemin, *Biochem. Pharmacol.*, **53**, 5–10 (1997).

[28] A. Nontprasert, S. Pukrittayakamee, S. Prakongpan, W. Supanaranond, S. Looareesuwan, N. J. White, Assessment of the neurotoxicity of oral dihydroartemisinin in mice, *Transcaction of the Royal Society of Tropical Medicine and Hygiene*, **96**, 99-101 (2002).

[29] A. J. Lin, D. L. Klayman and W. K. Milhous, Antimalarial Activity of New Water-Soluble Dihydroartemisinin Derivatives, *J. Med. Chem.*, **30,** 2147-2150 (1987).

[30] A. J. Lin, M. Lee, D. L. Klayman, Antimalarial Activity of New Water-Soluble Dihydroartemisinin Derivatives. 2. Stereospecificity of the Ether Side Chain, *J. Med. Chem.*, **32,** 1249-1252 (1989).

[31] Q. Li, L. H. Xie, T. O. Johnson, Y. Si, A. S. Haeberle, P. J. Weina, Toxicity evaluation of artesunate and artelinate in *Plasmodium berghei*-infected and uninfected rats, *Trans. R. Soc. Trop. Med. Hyg.*, **101***,* 104-112 (2007).

[32] R. F. Genovese, D. B. Newman, T. G. Brewer, Behavioral and neural toxicity of the artemisinin antimalarial, arteether, but not artesunate and artelinate, in rats, *Pharmacol. Biochem. Behav.*, **67**, 37-44 (2000).

[33] Y. Si, Q.-G. Li, L. Xie, K. Bennett, P. J. Weina, S. Mog, T. O. Johnson, Neurotoxicity and Toxicokinetics of Artelinic Acid Following Repeated Oral Administration in Rats, *Int. J. Toxicol.*, **26**, 401-410 (2007).

[34] C. Singh, R. Kanchan, S. Chaudhary, S. K. Puri, Linker-Based Hemisuccinate Derivatives of Artemisinin: Synthesis and Antimalarial Assessment against Multidrug-Resistant *Plasmodium yoelii nigeriensis* in Mice1, *J. Med. Chem.*, **55**, 1117−1126 (2012).

[35] A. J. Lin, R. E. Miller, Antimalarial Activity of New Dihydroartemisinin Derivatives. 6. α-Alkylbenzylic Ethers, *J. Med. Chem.*, **38,** 764-770 (1995).

[36] P. M. O'Neill, A. Miller, L. P. D. Bishop, S. Hindley, J. L. Maggs, S. A. Ward, S. M. Roberts, F. Scheinmann, A. V. Stachulski, G. H. Posner, B. K. Park, Synthesis, Antimalarial Activity, Biomimetic Iron(II) Chemistry, and in Vivo Metabolism of Novel, Potent *C*-10-Phenoxy Derivatives of Dihydroartemisinin, *J. Med. Chem.*, **44,** 58-68 (2001).

[37] (a) M. A. Avery, J. D. Bonk, W. K. M. Chong, S. Mehrotra, R. Miller, W. Milhous, D. K. Goins, S. Venkatesan, C. Wyandt, I. Khan and B. A. Avery, Structure-Activity Relationships of the Antimalarial Agent Artemisinin. 2. Effect of Heteroatom Substitution at O-11: Synthesis and Bioassay of N-Alkyl- 1 l-aza-9-desmethylartemisinin, *J. Med. Chem.*, **38,** 5038-5044 (1995); (b) D. S. Torok, H. Ziffer, S. R. Meshnick, X. Q. Pan and A. Ager, Syntheses and Antimalarial Activities of N-Substituted 11-Azaartemisinins *J. Med. Chem.*, **38,** 5045-5050 (1995); (c) B. Mekonnen, E. Weiss, E. Katz, J. Ma, H. Ziffer and D. E. Kyle, Synthesis and Antimalarial Activities of Base-Catalyzed Adducts of 11-Azaartemisinin, *Bioorg. Med. Chem.*, **8,** 1111-1116 (2000).

[38] M. A. Avery, S. Mehrotra, T. L. Johnson, J. D. Bonk, J. A. Vroman, R. Miller, Structure-Activity Relationships of the Antimalarial Agent Artemisinin. 5. Analogs of 10-Deoxoartemisinin Substituted at C-3 and C-9 *J. Med. Chem.,* **39,** 4149-4155 (1996).

[39] M. A. Avery, M. Alvim-Gaston, J. A. Vroman, B. Wu, A. Ager, W. Peters, B. L. Robinson, William Charman, Structure-Activity Relationships of the Antimalarial Agent Artemisinin. 7. Direct Modification of (+)-Artemisinin and In Vivo Antimalarial Screening of New, Potential Preclinical Antimalarial Candidates, *J. Med. Chem.,* **45,** 4321-4335 (2002).

[40] G. H. Posner, M. H. Parker, J. Northorp, J. S. Elias, P. Ploypradith, S. Xie, T. A. Shapiro, Orally Active, Hydrolytically Stable, Semisynthetic, Antimalarial Trioxanes in the Artemisinin Family *J. Med. Chem.*, **42,** 300-304 (1999).

[41] G. H. Posner, P. Ploypradith, M. H. Parker, H. O'Dowd, S.-H. Woo, J. Northrop, M. Krasavin, P. Dolan, T. W. Kensler, S. Xie, T. A. Shapiro, Antimalarial, Antiproliferative, and Antitumor Activities of Artemisinin-Derived, Chemically Robust,

Trioxane Dimers *J. Med. Chem.*, **42,** 4275-4280 (1999).

[42] I.-H. Paik, S. Xie, T. A. Shapiro, T. Labonte, A. A. N. Sarjeant, A. C. Baege, G. H. Posner, Second Generation, Orally Active, Antimalarial, Artemisinin-Derived Trioxane Dimers with High Stability, Efficacy, and Anticancer Activity *J. Med. Chem.*, **49,** 2731-2734 (2006).

[43] G. H. Posner, I.-H. Paik, W. Chang, K. Borstnik, S. Sinishtaj, A. S. Rosenthal, T. A. Shapiro, Malaria-Infected Mice Are Cured by a Single Dose of Novel Artemisinin Derivatives *J. Med. Chem.* **50,** 2516-2519 (2007).

[44] G. H. Posner, W. Chang, L. Hess, L. Woodard, S. Sinishtaj, A. R. Usera, W. Maio, A. S. Rosenthal, A. S. Kalinda, J. G. D'Angelo, K. S. Petersen, R. Stohler, J. Chollet, J. Santo-Tomas, C. Snyder, M. Rottmann, S. Wittlin, R. Brun, T. A. Shapiro, Malaria-Infected Mice Are Cured by Oral Administration of New Artemisinin Derivatives, *J. Med. Chem.* **51** 1035-1042 (2008).

[45] R. K. Haynes, W.-Y. Ho, H.-W. Chan, B. Fugmann, J. Stetter, S. L. Croft, L. Vivas, W. Peters, L. Robinson, Highly Antimalaria-Active Artemisinin Derivatives: Biological Activity Does Not Correlate with Chemical Reactivity, *Angew. Chem. Int. Ed.*, **43,** 1381-1385 (2004).

[46] R. K. Haynes, B. Fugmann, J. Stetter, K. Rieckmann, H.-D. Heilmann, H.-W. Chan, M.-K. Cheung, W.-L. Lam, H.-N. Wong, S. L. Croft, L. Vivas, L. Rattray, L. Stewart, W. Peters, B. L. Robinson, M. D. Edstein, B. Kotecka, D. E. Kyle, B. Beckermann, M. Gerisch, M. Radtke, G. Schmuck, W. Steinke, U. Wollborn, K. Schmeer, A. Römer, Artemisone – a highly active antimalarial drug of the artemisinin class. *Angew. Chem. Int. Ed.*, **45,** 2082-2088 (2006).

[47] S. D'Alessandro, M. Gelati, N. Basilico, E. A. Parati, R. K. Haynes, D. Taramelli, Differential effects on angiogenesis of two antimalarial compounds, dihydroartemisinin and artemisone: Implications for embryotoxicity, *Toxicology*, **241**, 66–74 (2007).

[48] L. Vivas, L. Rattray, L. B. Stewart, B. L. Robinson, B. Fugmann, R. K. Haynes, W. Peters, S. L. Croft, Antimalarial efficacy and drug interactions of the novel semi-synthetic endoperoxide artemisone in vitro and in vivo, *J. Antimicrob. Chemoth.,* **59,** 658–665 (2007).

[49] J. Nagelschmitz, B. Voith, G. Wensing, A. Roemer, B. Fugmann, R. K. Haynes, B. M. Kotecka, K. H. Rieckmann, M. D. Edstein, First assessment in humans of the safety, tolerability, pharmacokinetics, and ex vivo pharmacodynamic antimalarial activity of the new artemisinin derivative artemisone. *Antimicrob. Agents. Ch.,* **52**, 3085–3091 (2008).

[50] T. G. Brewer, S. J. Grate, J. O. Peggins, P. J. Weina, J. M. Petras, B. S. Levine, M. H. Heiffer, B. G. Schuster, Fatal neurotoxicity of arteether and artemether, *Am. J. Trop. Med. Hyg.,* **51,** 251–259 (1994).

[51] N. III Obaldia, B. M. Kotecka, M. D. Edstein, R. K. Haynes, B. Fugmann, D. E. Kyle, K. H. Rieckmann, Evaluation of artemisone combinations in *aotus* monkeys infected with *Plasmodium falciparum, Antimicrob. Agents. Ch.,* **53,** 3592–3594 (2009).

[52] C. W. Jefford, J. A. Velarde, G. Bernardinelli, D. H. Bray, D. C. Warhurst, W. K. Milhous, Synthesis, Structure and Antimalarial Activity of Tricyclic 1,2,4-Trioxanes Related to Artemisinin, *Helv. Chim. Acta*, **76,** 2775- 2788 (1993).

[53] C. W. Jefford, S. Kohmoto, D. Jaggi, G. Timari, J.-C. Rossier, M. Rudaz, O. Barbuzzi, D. Gerard, U. Burger, P. Kamalaprija, J. Mareda, G. Bernardinelli, Synthesis, Structure and antimalarial Activity of some Enantiomerically Pure *cis*-Fused Cyclopenteno-1,2,4-Trioxanes, *Helv. Chim. Acta,* **78,** 647-662 (1995).

[54] G. H. Posner, C. H. Oh, D. Wang, L. Gerena, W. K. Milhous, S. R. Meshnick, W. Asawamahasadka, 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.,* **37,** 1256- 1258 (1994).

[55] G. H. Posner, H. B. Jeon, M. H. Parker, M. Krasavin, I.-H. Paik, T. A. Shapiro, Antimalarial Simplified 3-Aryltrioxanes: Synthesis and Preclinical Efficacy/Toxicity Testing in Rodents, *J. Med. Chem.,* **44,** 3054-3058 (2001).

[56] C. Singh, N. Gupta, S. K. Puri, Photooxygenation of 3-aryl-2-cyclohexenols: synthesis of a new series of antimalarial 1,2,4-trioxanes, *Tetrahedron Lett.*, **46,** 205-207 (2005).

[57] C. W. Jefford, New developments in synthetic peroxidic drugs as artemisinin mimics *Drug Discovery Today*, **12,** 487-495 (2007).

[58] C. Singh, N. Gupta, S. K. Puri, Geraniol-Derived 1,2,4-Trioxanes with Potent In-Vivo Antimalarial

Activity, *Bioorg. Med. Chem. Lett.*, **13,** 3447-3450 (2003).

[59] C. Singh, N. C. Srivastav, S. K. Puri, Synthesis and antimalarial activity of 6-cycloalkylvinyl substituted 1,2,4-trioxanes, *Bioorg. Med. Chem.*, **12,** 5745-5752 (2004).

[60] C. Singh, R. Kanchan, U. Sharma, S. K. Puri, New Adamantane-Based Spiro 1,2,4-Trioxanes Orally Effective against Rodent and Simian Malaria, *J. Med. Chem.,* **50,** 521-527 (2007).

[61] A. G. Griesbeck, T. T. El-Idreesy, L.-O. Höinck, J. Lex, R. Brun, Novel spiroanellated 1,2,4-trioxanes with high in vitro antimalarial activities, *Bioorg. Med. Chem. Lett.*, **15,** 595-597 (2005).

[62] C. Singh, H. Malik, S. K. Puri, Orally active amino functionalized antimalarial 1,2,4-trioxanes *Bioorg. Med. Chem. Lett.*, **14,** 459-462 (2004).

[63] C. Singh, H. Malik, S. K. Puri, Orally Active 1,2,4-Trioxanes: Synthesis and Antimalarial Assessment of a New Series of 9-Functionalized 3-(1-Arylvinyl)-1,2,5-trioxaspiro[5.5]undecanes against Multi-Drug-Resistant *Plasmodium yoelii nigeriensis* in Mice *J. Med. Chem.*, **49,** 2794-2803 (2006).

[64] C. Singh, U. Sharma, G. Saxena, S. K. Puri, Orally active antimalarials: Synthesis and bioevaluation of a new series of steroid-based 1,2,4-trioxanes against multi-drug resistant malaria in mice, *Bioorg. Med. Chem. Lett.*, **17,** 4097-4101 (2007).

[65] J. L. Vennerstrom, S. Arbe-Barnes, R. Brun, S. A. Charman, F. C. K. Chiu, J. Chollet, Y. Dong, A. Dorn, D. Hunziker, H. Matile, K. McIntosh, M. Padmanilayam, J. S. Tomas, C. Scheurer, B. Scorneaux, Y. Tang, H. Urwyler, S. Wittlin, W. N. Charman, Identification of an antimalarial synthetic trioxolane drug development candidate, *Nature,* **430,** 900-904 (2004).

[66] (a) J. L. Vennerstrom, Y. Dong, J. Chollet, H. Matile, Spiro and Dispiro 1,2,4-trioxolane antimalarials. U.S. 6,486,199 (2002); (b) J. L. Vennerstrom, Y. Tang, Y. Dong, J. Chollet, H. Matile, M. Padmanilayam, W. N. Charman, Spiro and Dispiro 1,2,4-trioxolane antimalarials, U.S. 6,825,230 (2004).

[67] Y. Tang, Y. Dong, J. M. Karle, C. A. DiTusa, J. L. Vennerstrom, Synthesis of Tetrasubstituted Ozonides by the Griesbaum Coozonolysis Reaction: Diastereoselectivity and Functional Group Transformations by Post-Ozonolysis Reactions, *J. Org. Chem.,* **69,** 6470-6473 (2004).

[68] J. L. Vennerstrom, Y. Dong, S. A. Charman, S. Wittlin, J. Chollet, X. Wang, K. Sriraghavan, L. Zhou, H. Matile, W. N. Charman, Spiro and Dispiro 1,2,4-trioxolane antimalarials, US 2008/0125441 A1 (2008).

[69] J. L. Vennerstrom, Y. Dong, J. Chollet, H. Matile, X. Wang, K. Sriraghavan, W. N. Charman, Spiro and Dispiro 1,2,4-trioxolane antimalarials, U.S. 7,371,778 (2008).

[70] Y. Dong, J. Chollet, H. Matile, S. A. Charman, F. C. K. Chiu, W. N. Charman, B. Scorneaux, H. Urwyler, T. J. Santo, C. Scheurer, C. Snyder, A. Dorn, X. Wang, J. M. Karle, Y. Tang, S. Wittlin, R. Brun, J. L. Vennerstrom, Spiro and dispiro-1,2,4-trioxolanes as antimalarial peroxides: charting a workable structure-activity relationship using simple prototypes, *J. Med. Chem.,* **48,** 4953-4961 (2005).

[71] Y. Tang, Y. Dong, S. Wittlin, S. A. Charman, J. Chollet, F. C. K. Chiu, W. N. Charman, H. Matile, H. Urwyler, A. Dorn, S. Bajpai, X. Wang, M. Padmanilayam, J. M. Karle, R. Brun, J. L. Vennerstrom, Weak base dispiro-1,2,4-trioxolanes: Potent antimalarial ozonides, *Bioorg. Med. Chem. Lett.,* **17,** 1260–1265 (2007).

[72] Y. Dong, S. Wittlin, K. Sriraghavan, J. Chollet, S. A. Charman, W. N. Charman, C. Scheurer, H. Urwyler, J. S. Tomas, C. Snyder, D. J. Creek, J. Morizzi, M. Koltun, H. Matile, X. Wang, M. Padmanilayam, Y. Tang, A. Dorn, R. Brun, J. L. Vennerstrom, The structure-activity relationship of the antimalarial ozonide arterolane (OZ277), *J. Med. Chem.,* **53,** 481–491 (2010).

[73] Y. Dong, Y. Tang, J. Chollet, H. Matile, S. Wittlin, S. A. Charman, W. N. Charman, J. S. Tomas, C. Scheurer, C. Snyder, B. Scorneaux, S. Bajpai, S. A. Alexander, X. Wang, M. Padmanilayam, S. R. Cheruku, R. Brun, J. L. Vennerstrom, Effect of functional group polarity on the antimalarial activity of spiro and dispiro-1,2,4-trioxolanes, *Bioorg. Med. Chem.,* **14**, 6368-6382 (2006).

[74] M. Padmanilayam, B. Scorneaux, Y. Dong, J. Chollet, H. Matile, S. A. Charman, D. J. Creek, William N. Charman, J. S. Tomas, C. Scheurer, S. Wittlin, R. Brun, J. L. Vennerstrom, Antimalarial activity of N-alkyl amine, carboxamide, sulfonamide, and urea derivatives of a dispiro-1,2,4-trioxolane piperidine, *Bioorg. Med. Chem. Lett.*, **16,** 5542-5545 (2006)

[75] Anti-Malaria Project, Ranbaxy, http://www.ranbaxy.com/socialresposbility/mm.aspx (accessed, May 13 2012).

[76] S. A. Charman, S. Arbe-Barnes, I. C. Bathurst, R. Brun, M. Campbell, W. N. Charman, C. K. Francis, F. C. K. Chiu, J. Chollet, J. C. Craft, D. J. Creek, Y. Dong, H. Matile, M. Maurer, J. Morizzi, T. Nguyen, P. Papastogiannidis, C. Scheurer, M. David, D. M. Shackleford, K. Sriraghavann, L. Stingelin, Y. Tang, H. Urwyler, X. Wang, K. L. White, S. Wittlin, L. Zhou, J. L. Vennerstrom, Synthetic ozonide drug candidate OZ439 offers new hope for a single-dose cure of uncomplicated malaria, *Proc. Natl. Acad. Sci. USA,* **108,** 4400 – 4405 (2011).

[77] (a) T. Ledaal, A Simple Laboratory Method for the Preparation of (Dimeric) Ketone Peroxides, *Acta Chem. Scand.*, **21,** 1656-1657 (1967); (b) P. R. Story, B. Lee, C. E. Bishop, D. D. Denson, P. Busch, Macrocyclic Synthesis. II. Cyclohexanone Peroxides, *J. Org. Chem.*, **35,** 3059-3062 (1970); (c) J. R. Sanderson, K. Paul, P. R. Story, D. D. Denson, J. A. Alford, Macrocycles. The Synthesis and Thermal Decomposition of Some Disubstituted Dicyclohexylidene Diperoxides, *Synthesis*, 159–161 (1975); (d) J. R. Sanderson, A. G. Zeiler, A Simple Synthesis of Dicyclohexylidene Diperoxide and Tricyclohexylidene Triperoxide, *Synthesis*, 125-127 (1975); (e) J. H. Sanderson, A. G. Zeile, R. J. Wilterdink, An Improved Synthesis of Dicyclohexylidene diperoxide, *J. Org. Chem.*, **40,** 2239-2241 (1975); (f) J. R. Sanderson, R. J. Wilterdink, A. G. Zeler, The preparation of Dicycloalkylidene Diperoxides by a Two-Step Procedure, *Synthesis*, 479-481 (1976).

[78] J. L. Vennerstrom, H.-N. Fu, W. Y. Ellis, A. L. Ager, Jr., J. K. Wood, S. L. Andersen, L. Gerena and W. K. Milhous, Dispiro- 1,2,4,5-tetraoxanes: A New Class of Antimalarial Peroxides *J. Med. Chem.*, **35,** 3023-3027 (1992).

[79] C. W. Jefford, A. Jaber, J. Boukouvalas, Efficient preparation of 1,2,4,5-Tetraoxanes from Bis(trimethylsilyl) Peroxide and Carbonyl Compounds, *Synthesis*, 391-393 (1988).

[80] D. Opsenica, G. Pocsfalvi, Z. Juranić, B. Tinant, J. P. Declercq, D. E. Kyle, W. K. Milhous, B.A. Šolaja, Cholic Acid Derivatives as 1,2,4,5-Tetraoxane Carriers: Structure and Antimalarial and Antiproliferative Activity, *J. Med. Chem.*, **43,** 3274–3282 (2000).

[81] N. M. Todorović, M. Stefanović, B. Tinant, J.-P. Declercq, M. T. Makler and B. A. Šolaja, Steroidal geminal Dihydroperoxides and 1,2,4,5-tetraoxanes: Structure Determination and Their Antimalarial Activity, *Steroids*, **61,** 688-696 (1996).

[82] (a) Y. Dong and J. L. Vennerstrom, Dispiro-1,2,4,5-tetraoxanes via Ozonolysis of Cycloalkanone O-

Methyl Oximes: A Comparison with the Peroxidation of Cycloalkanones in Acetonitrile-Sulfuric Acid Media, *J. Org. Chem.*, **63,** 8582-8585 (1998); (b)Y. Dong, H. Matile, J. Chollet, R. Kaminsky, J. K. Wood, J. L. Vennerstrom, Synthesis and Antimalarial Activity of 11 Dispiro-1,2,4,5-tetraoxane Analogues of WR 148999. 7,8,15,16-Tetraoxadispiro[5.2.5.2]hexadecanes Substituted at the 1 and 10 Positions with Unsaturated and Polar Functional Groups, *J. Med. Chem.,* **42,** 1477-1480 (1999); (c) H.-S. Kim, Y. Shibata, Y. Wataya, K. Tsuchiya, A. Masuyama, M. Nojima, Synthesis and Antimalarial Activity of Cyclic Peroxides, 1,2,4,5,7-Pentoxocanes and 1,2,4,5-Tetroxanes *J. Med. Chem.*, **42,** 2604-2609 (1999); (d) P. H. Dussault, J. M. Raible, Ozonolysis in the Presence of Lewis Acids: Directed Addition to Carbonyl Oxides, *Organic Lett.*, **2,** 3377-3379 (2000).

[83] K. Žmitek, S. Stavber, M. Zupan, D. Bonnet-Delpon, J. Iskra, Fluorinated alcohol directed formation of dispiro-1,2,4,5-tetraoxanes by hydrogen peroxide under acid conditions, *Tetrahedron*, **62,** 1479-1484 (2006).

[84] Y. Dong, Synthesis and Antimalarial Activity of 1,2,4,5-Tetraoxanes *Mini Rev. Med. Chem.*, **2,** 113-123 (2002).

[85] H. S. Kim, K. Tsuchiya, Y. Shibata, Y. Wataya, Y. Ushigoe, A. Masuyama, M. Nojima, K. J. McCullough, Synthetic methods for unsymmetrically-substituted 1,2,4,5-tetraoxanes and of 1,2,4,5,7-pentoxocanes, *J. Chem. Soc., Perkin Trans. 1*, 1867-1870 (1999).

[86] J. Iskra, D. Bonnet-Delponb, J.-P. Bégué, One-pot synthesis of non-symmetric tetraoxanes with the H₂O₂/MTO/fluorous alcohol system *Tetrahedron Lett.*, **44,** 6309-6312 (2003).

[87] A. O. Terent'ev, A. V. Kutkin, Z. A. Starikova, M. Yu. Antipin, Yu. N. Ogibin, G. I. Nikishin, New Preparation of 1,2,4,5-Tetraoxanes, *Synthesis*, 2356-2366 (2004).

[88] (a) K. Žmitek, M. Zupan, S. Stavber, J. Iskra, Iodine as a Catalyst for Efficient Conversion of Ketones to gem-Dihydroperoxides by Aqueous Hydrogen Peroxide, *Org. Lett.*, **8,** 2491-2494 (2006); (b) K. Žmitek, M. Zupan, S. Stavber, J. Iskra, The Effect of Iodine on the Peroxidation of Carbonyl Compounds, *J. Org. Chem.,* **72,** 6534-6540 (2007); (c) K. Žmitek, M. Zupan, J. Iskra, α-Substituted organic peroxides: synthetic strategies for a biologically important class of *gem*-dihydroperoxide and perketal derivatives, *Org. Biomol. Chem.*, **5,** 3895-3908 (2007); (d) B. Das, M. Krishnaiah, B. Veeranjaneyulu, B. Ravikanth, A simple and ef-

ficient synthesis of gem-dihydroperoxides from ketones using aqueous hydrogen peroxide and catalytic ceric ammonium nitrate, *Tetrahedron Lett.*, **48,** 6286-6289 (2007); (e) B. Das, B. Veeranjaneyulu, M. Krishnaiah, P. Balasubramanyam, Synthesis of *gem*-dihydroperoxides from ketones using silica-supported sodium hydrogen sulfate as a heterogeneous catalyst, *J. Mol. Catal. A: Chem.*, **284,** 116-119 (2008).

[89]   (a) I. Opsenica, N. Terzić, D. Opsenica, W. K. Milhous, B. Šolaja, 7,8,15,16-tetraoxa-dispiro[5.2.5.2] hexadecane-3-carboxylic acid derivatives and their antimalarial activity, *J. Serb. Chem. Soc.*, **69,** 919-922 (2004); (b) I. Opsenica, D. Opsenica, M. Jadranin, K. Smith, W. K. Milhous M. Stratakis, B. Šolaja, On peroxide antimalarials, *J. Serb. Chem. Soc.*, **72,** 1181-1190 (2007); (c) I. Opsenica, D. Opsenica, K. S. Smith, W. K. Milhous, B. A. Šolaja, Chemical Stability of the Peroxide Bond Enables Diversified Synthesis of Potent Tetraoxane Antimalarials, *J. Med. Chem.,* **51,** 2261-2266 (2008).

[90]   R. Amewu, A. V. Stachulski, S. A. Ward, N. G. Berry, P. G. Bray, J. Davies, G. Labat, L. Vivas, P. M. O'Neill, Design and synthesis of orally active dispiro 1,2,4,5-tetraoxanes; synthetic antimalarials with superior activity to artemisinin, *Org. Biomol. Chem.*, **4,** 4431-4436 (2006).

[91]   G. L. Ellis, R. Amewu, S. Sabbani, P. A. Stocks, A. Shone, D. Stanford, P. Gibbons, J. Davies, L. Vivas, S. Charnaud, E. Bongard, C. Hall, K. Rimmer, S. Lozanom, M. Jesús, D. Gargallo, S. A. Ward, P. M. O'Neill, Two-Step Synthesis of Achiral Dispiro-1,2,4,5-tetraoxanes with Outstanding Antimalarial Activity, Low Toxicity, and High-Stability Profiles *J. Med. Chem.,* **51,** 2170-2177 (2008).

[92]   P. M. O'Neill, R. K. Amewu, G. L. Nixon, F. Bousejr, E. Garah, M. Mungthin, J. Chadwick, A. E. Shone, L. Vivas, H. Lander, V. Barton, S. Muangnoicharoen, P. G. Bray, J. Davies, B. K. Park, S. Wittlin, R. Brun, M. Preschel, K. Zhang, S. A. Ward, Identification of a 1,2,4,5-Tetraoxane Antimalarial Drug-Development Candidate (RKA182) with Superior Properties to the Semisynthetic Artemisinins, *Angew. Chem. Int. Ed.*, **49,** 5693 –5697 (2010).

[93]   P. Ghorai, P. H. Dussault, Broadly Applicable Synthesis of 1,2,4,5-Tetraoxanes, *Org. Lett.,* **11,** 213-216 (2009).

[94]   K. Žmitek, S. Stavber, M. Zupan, D. Bonnet-Delpon, S. Charneau, P. Grellier, J. Iskra, Synthesis and antimalarial activities of novel 3,3,6,6-tetraalkyl-1,2,4,5-tetraoxanes, *Bioorg. Med. Chem.*, **14,** 7790-7795 (2006).

[95]   H. Atheaya, S. I. Khan, R. Mamgaina, D. S. Rawata, Synthesis, thermal stability, antimalarial activity of symmetrically and asymmetrically substituted tetraoxanes, *Bioorg. Med. Chem. Lett.*, **18,** 1446-1449 (2008).

[96]   Y. Dong, D. Creek, J. Chollet, H. Matile, S. A. Charman, S. Wittlin, J. K. Wood, J. L. Vennerstrom, Comparative Antimalarial Activities of Six Pairs of 1,2,4,5-Tetraoxanes (Peroxide Dimers) and 1,2,4,5,7,8-Hexaoxonanes (Peroxide Trimers), *Antimicrob. Agents Ch.*, **51,** 3033-3035 (2007).

[97]   D. Opsenica, G. Angelovski, G. Pocsfalvi, Z. Juranić, Ž. Žižak, D. Kyle, W. K. Milhous, B. A. Šolaja, Antimalarial and Antiproliferative Evaluation of Bis-Steroidal Tetraoxanes, *Bioorg. Med. Chem.* **11,** 2761-2768 (2003).

[98]   B. A. Šolaja, N. Terzić, G. Pocsfalvi, L. Gerena, B. Tinant, D. Opsenica, W. K. Milhous, Mixed Steroidal 1,2,4,5-Tetraoxanes: Antimalarial and Antimycobacterial Activity *J. Med. Chem.* **45,** 3331-3336 (2002).

[99]   N. Terzić, D. Opsenica, D. Milić, B. Tinant, K. S. Smith, W. K. Milhous, B. A. Šolaja, Deoxycholic Acid-Derived Tetraoxane Antimalarials and Antiproliferatives, *J. Med. Chem.*, **50,** 5118-5127 (2007).

[100] D. Opsenica, D. E. Kyle, W. K. Milhous, B. A. Šolaja, Antimalarial, antimycobacterial and antiproliferative activity of phenyl substituted mixed tetraoxanes, *J. Serb. Chem. Soc.*, **68,** 291-302 (2003).

[101] I. Opsenica, N. Terzić, D. Opsenica, G. Angelovski, M. Lehnig, P. Eilbracht, B. Tinant, Z. Juranić, K. S. Smith, Y. S. Yang, D. S. Diaz, P. L. Smith, W. K. Milhous, D. Đoković, B. A. Šolaja, Tetraoxane Antimalarials and Their Reaction with Fe(II), *J. Med. Chem.,* **49,** 3790-3799 (2006).

[102] D. M. Opsenica, N. Terzić, P. L. Smith, Y. Yang, L. Anova, K. S. Smith, B. A. Šolaja, Mixed tetraoxanes containing the acetone subunit as antimalarials, *Bioorg. Med. Chem.* **16,** 7039-7045 (2008).

[103] C. W. Jefford, J.-C. Rossier, W. K.Milhous, The Structure and Antimalarial Activity of Some 1,2,4-Trioxanes, 1,2,4,5-Tetraoxanes and Bicyclic Endoperoxide. Implication for the Mode of Action, *Heterocycles*, **52,** 1345-1352 (2000).

[104] D. Opsenica, Z. Juranić, B. A. Šolaja, unpublished results. In brief, the whole blood from a healthy person, with K EDTA added as an anticoagulant, was incubated for 48 h at 37 ºC with test compound **6** within concentration range of 60 mM – 6 nM. No increase of hemoglobin level in plasma was detected, as com-

pared to blank probe. Blood diluted with physiological solution was used as blank, while blood diluted with de-ionized water was used as positive control probe.

[105] D. Opsenica, G. Pocsfalvi, W. K. Milhous and B. A. Šolaja, Antimalarial peroxides: the first intramolecular 1,2,4,5-tetraoxane, *J. Serb. Chem. Soc.*, **67,** 465-471 (2002).

[106] A. O. Terent'ev, D. A. Borisov, V. V. Chernyshev and G. I. Nikishin, Facile and Selective Procedure for the Synthesis of Bridged 1,2,4,5-Tetraoxanes; Strong Acids As Cosolvents and Catalysts for Addition of Hydrogen Peroxide to Diketones, *J. Org. Chem.*, **74**, 3335–3340 (2009).

[107] O. Dechey-Cabaret, F. Benoit-Vical, A. Robert, B. Meunier, Preparation and Antimalarial Activities of "Trioxaquines", New Modular Molecules with a Trioxane Skeleton Linked to a 4-Aminoquinolines, *ChemBioChem*, 281-283 (2000).

[108] L. K. Basco, O. Dechey-Cabaret, M. Ndounga, F. S. Meche, A. Robert, B. Meunier, In Vitro *Activities of DU-1102, a New Trioxaquine Derivative, against* Plasmodium falciparum *Isolates, Antimicrob. Agents Chemother.*, **45,** 1886-1888 (2001).

[109] O. Dechey-Cabaret, F. Benoit-Vical, C. Loup, A. Robert, H. Gornitzka, A. Bonhoure, H. Vial, J.-F. Magnaval, J.-P. Séguéla, B. Meunier, Synthesis and Antimalarial Activity of Trioxaquine Derivatives, *Chem. Eur. J.*, **10,** 1625-1636 (2004).

[110] F. Coslédan, L. Fraisse, A. Pellet, F. Guillou, B. Mordmüller, P. G. Kremsner, A. Moreno, D. Mazier, J.-P. Maffrand, B. Meunier, Selection of a trioxaquine as an antimalarial drug candidate, *P. Natl. Acad. Sci. USA*, **105,** 17579-17584 (2008).

[111] O. Dechey-Cabaret, F. Benoit-Vical, A. Robert, J.-F. Magnaval, J.-P. Séguéla, B. Meunier, Synthesis and biological evaluation of a new trioxaquine containing a trioxane moiety obtained by halogenocyclisation of a hemiperoxyacetal, *C. R. Chim.*, **6,** 153-160 (2003).

[112] C. Singh, H. Malik, S. K. Puri, Synthesis and antimalarial activity of a new series of trioxaquines, *Bioorg. Med. Chem.* **12,** 1177-1182 (2004).

[113] B. Meunier, Hybrid Molecules with a Dual Mode of Action: Dream or Reality? *Acc. Chem. Res.*, **41,** 69-77 (2008).

[114] J. L. Vennerstrom, Y. Dong, J. Chollet, H. Matile, M. Padmanilayam, Y. Tang, W. N. Charman, Spiro and Dispiro 1,2,4-Trioxolane Antimalarials, US 20040039008 A1 (2004).

[115] J. J. Walsh, D. Coughlan, N. Heneghan, C. Gaynora, A. Bellb, A novel artemisinin–quinine hybrid with potent antimalarial activity, *Bioorg. Med. Chem. Lett.*, **17,** 3599-3602 (2007).

[116] T. S. Skinner, L. S. Manning, W. A. Johnston, T. M. E. Davis, *In Vitro* Stage-specific Sensitivity of *Plasmodium falciparum* to Quinine and Artemisinin Drugs, *Int. J. Parasitology*, **26,** 519-525 (1996).

[117] I. Opsenica, D. Opsenica, C. A. Lanteri, L. Anova, W. K. Milhous, K. S. Smith, B. A. Šolaja, New Chimeric Antimalarials with 4-Aminoquinoline Moiety Linked to a Tetraoxane Skeleton, *J. Med. Chem.*, **51,** 6216-6219 (2008).

[118] J. L. Vennerstrom, A. L. Ager, S. L. Andersen, J. M. Grace, V. Wongpanich, C. K. Angerhofer, J. K. Hu, D. L. Wesche, Assessment of the Antimalarial Potential of tetraoxane WR148999, *Am. J. Trop. Med. Hyg.*, **62,** 573-578 (2000).

[119] S. Gupta, M. M. Thapar, S. T. Mariga, W. H. Wernsdorfer, A. Björkman, *Plasmodium falciparum: in vitro* Interactions of Artemisinin with Amodiaquine, Pyronaridine, and Chloroquine, *Exp. Parasitol.*, **100,** 28–35 (2002).

[120] M. Ashton, T. N. Hai, N. D. Sy, D. X. Huong, N. V. Huong, N. T. Niêu, L. D. Công, Artemisinin Pharmacokinetics Is Time-Dependent during Repeated Oral Administration in Healthy Male Adults. *Drug Metab. Dispos.,* **26,** 25-27 (1998).

[121] W. K. Milhous, L. Gerena, D. Opsenica, B. A. Šolaja, unpublished results.

[122] L. Zhou, A. Alker, A. Ruf, X. Wang, F. C. K. Chiu, J. Morizzi, S. A. Charman, W. N. Charman, C. Scheurer, S. Wittlin, Y. Dong, D. Hunziker, J. L. Vennerstrom, Characterization of the two major CYP450 metabolites of ozonide (1,2,4-trioxolane) OZ277, *Bioorg. Med. Chem. Lett.*, **18,** 1555-1558 (2008).

[123] (a) G. H. Posner and C. H. Oh, A Regiospecifically Oxygen-18 Labeled 1,2,4-Trioxane: A Simple Chemical Model System to Probe the Mechanism(s) for the Antimalarial Activity of Artemisinin (Qinghaosu), *J. Am. Chem. Soc.*, **114,** 8328-8329 (1992); (b) G. H. Posner, S. B. Park, L. Gonzalez, D. Wang, J. N. Cumming, D. Klinedinst, T. A. Shapiro, M. D. Bachi, Evidence for the Importance of High-Valent Fe=O and of a Diketone in the Molecular Mechanism of Action of Antimalarial Trioxane Analogs of Artemisinin, *J. Am. Chem. Soc.*, **118,** 3537-3538 (1996).

[124] (a) C. W. Jefford, F. Favarger, M. G. H. Vicente, Y. Jacquier, The Decomposition of Cis-Fused

Cyclopenteno-1,2,4-trioxanes Induced by Ferrous Salts and Some Oxophilic Reagents, *Helv. Chim. Acta*, **78,** 452-458 (1995); (b) C. W. Jefford, M. G. H. Vicente, Y. Jacquier, F. Favarger, J. Mareda, P. Millason-Schmidt, G. Brunner and U. Burger, The Deoxygenation and Isomerization of Artemisinin and Artemether and Their Relevance to Antimalarial Action, *Helv. Chim. Acta.*, **79,** 1475-1487 (1996); (c) C. W. Jefford, U. Burger, P. Millasson-Schmidt, G. Bernardinelli, B. L. Robinson and W. Peters, Epiartemisinin, a Remarkably Poor Antimalarial: Implication for the Mode of Action, *Helv. Chim. Acta*, **83,** 1239-1246 (2000).

[125] (a) R. K. Haynes and S. C. Vonwiller, The Behaviour of Qinghaosu (Artemisinin) in the Presence of Heme Iron(II) and (III), *Tetrahedron Lett.*, **37,** 253-256 (1996); (b) R. K. Hynes and S. C. Vonwiller, The Behaviour of Qinghaosu (Artemisinin) in the Presence of Non-Heme Iron(II) and (III), *Tetrahedron Lett.*, **37,** 257-260 (1996).

[126] (a) A. Robert, B. Meunier, Characterization of the First Covalent Adduct between Artemisinin and a Heme Model, *J. Am. Chem. Soc.*, **119** 5968-5969 (1997); (b) A. Robert, B. Meunier, Alkylating Properties of Antimalarial Artemisinin Derivatives and Synthetic Trioxanes when Activated by Reduced Heme Model, *Chem. Eur. J.*, **4**, 1287-1296 (1998); (c) J. A. Robert, J. Cazelles, B. Meunier, Characterization of the Alkylation Product of Heme by the Antimalarial Drug Artemisinin, *Angew. Chem. Int. Ed.*, **40** 1954-1957 (2001); (d) J. Cazelles, J. A. Robert, B. Meunier, Alkylating Capacity and Reaction Products of Antimalarial Trioxanes after Activation by a Heme Model *J. Org. Chem.*, **67,** 609-619 (2002).

[127] (a) W.-M. Wu, Z.-J. Yao, Y.-L. Wu, K. Jiang, Y.-F. Wang, H.-B. Chen, F. Shan, Y. Li, Ferrous ion induced cleavage of the peroxy bond in quinghaosu and its derivatives and the DNA damage associated with this process, *Chem. Commun.,* 2213-2214 (1996); (b) W.-M. Wu, Y. Wu, Y.-L. Wu, Z.-J. Yao, C.-M. Zhou, Y. Li, F. Shan, Unified Mechanistic Framework for the Fe(II)-Induced Cleavage of Qinghaosu and Derivatives/Analogues. The First Spin-Trapping Evidence for the Previously Postulated Secondary C-4 radical, *J. Am. Chem. Soc.*, **120,** 3316-3325 (1998); c) Y. Wu, Z.-Y. Yue, Y.-L. Wu, Interaction of Qinghaosu (Artemisinin) with Cysteine Sulfhydryl Mediated by Traces of Non-Heme Iron, *Angew.Chem. Int. Ed.*, **38,** 2580-2582 (1999); (d) D.-Y. Wang, Y.-L. Wu, A possible antimalarial action mode qinghaosu (artemisinin) series compounds. Alkylation of reduced glutathione by C-centered primary radicals produced

from antimalarial compound quinghaosu and 12-(2,4-dimethoxyphenyl)-12-deoxoqinghaosu, *Chem. Commun.*, 2193-2194 (2000).

[128] (a) G. H. Posner, J. N. Cumming, P. Ploypradith, C.-H. Oh, Evidence for Fe(IV)=O in the Molecular Mechanism of Action of the Trioxane Antimalarial Artemisinin*, J. Am. Chem.,* **117,** 5885-5886 (1995); (b) T. G. Traylor, A. R. Miksztal, Mechanisms of hemin-catalyzed epoxidations: electron transfer from alkenes, *J. Am. Chem. Soc.*, **109,** 2770-2774 (1987).

[129] (a) S. Kapetanaki, C. Varotsis, Ferryl-oxo heme intermediate in the antimalarial mode of action of artemisinin *FEBS Lett.*, **474,** 238-241 (2000); (b) S. Kapetanaki, C. Varotsis, Fourier Transform Infrared Investigation of Non-Heme Fe(III) and Fe(II) Decompostion of Artemisinin and of a Simplified Trioxane Alcohol, *J. Med. Chem.*, **44,** 3150-3156 (2001).

[130] S. A.-L. Laurent, C. Loup, S. Mourgues, A. Robert, B. Meunier, Heme Alkylation by Artesunic Acid and Trioxaquine DU1301, Two Antimalarial Trioxanes, *ChemBioChem*, **6,** 653-658 (2005).

[131] W. Asawamahasakda, I. Ittarat, Y.-M. Pu, H. Ziffer and S. R. Meshnick, Reaction of Antimalarial Endoperoxides with Specific Parasite Proteins, *Antimicrobial Agents and Chemotherapy*, **38,** 1854-1858 (1993).

[132] J. Bhisutthibhan, X.-Q. Pan, P. A. Hossler, D. J. Walker, C. A. Yowell, J. Carlton, J. B. Dame and S. R. Meshnick, The Plasmodium falciparum Translationally Controlled Tumor Protein Homolog and its Reaction with the Antimalarial Drug Artemisinin *J. Biol. Chem.*, **273,** 16192-16198 (1998).

[133] S. R. Meshnick, Artemisinin: mechanisms of action, resistance and toxicity, *Int. J. Parasitology*, **32,** 1655-1660 (2002).

[134] U. Eckstein-Ludwig, R. J. Webb, I. D. A. A. Van Goethem, J. M. East, A. G. Lee, M. Kimura, P. M. O'Neill, P. G. Bray, S. A. Ward, S. Krishna, Artemisinins target the SERCA of *Plasmodium falciparum, Nature*, **424,** 957-959 (2003).

[135] A. V. Pamdey, B. L. Tekwani, R. L. Singh, V. S. Chauhan, Artemisinin, an Endoperoxide Antimalarial, Disrupts the Hemoglobin Catabolism and Heme Detoxification Systems in Malarial Parasite, *J. Bio. Chemistry*, **274,** 19383-19388 (1999).

[136] (a) A. Coppi, C. Pinzon-Ortiz, C. Hutter, P. Sinnis, The **Plasmodium** circumsporozoite protein is proteolytically processed during cell invasion*, J. Exp. Med.*, **201,** 27-33 (2005); (b) F. Sarabia, A. Sánchez-Ruiz, S. Chammaa, Stereoselective synthesis

of E-64 and related cysteine proteases inhibitors from 2,3-epoxyamides *Bioorg. Med. Chem.*, **13**, 1691-1705 (2005).

[137] L. Messori, C. Gabbiani, A. Casini, M. Siragusa, F. F. Vincieri, A. R. Bilia, The reaction of artemisinins with hemoglobin: A unified picture, *Bioorg. Med. Chem.*, **14**, 2972-2977 (2006).

[138] R. K. Hynes, D. Monti, D. Taramelli, N. Basilico, S. Parapini, P. Olliaro, Artemisinin Antimalarials Do Not Inhibit Hemozoin Formation, *Antimicrobial Agents and Chemoterapy*, **47**, 1175 (2003).

[139] R. K. Haynes, W. C. Chan, C.-M. Lung, A.-C. Uhlemann, U. Eckstein, D. Taramelli, S. Parapini, D. Monti, S. Krishna, The Fe^{2+}-mediated decomposition, pfatp6 binding, and antimalarial activities of artemisone and other artemisinins: the unlikelihood of c-centered radicals as bioactive intermediates *ChemMedChem*, **2,** 1480-1497 (2007).

[140] F. Bousejra-El Garah, B. Meunier, A. Robert, The Antimalarial Artemisone Is an Efficient Heme Alkylating Agent *Eur. J. Inorg. Chem.*, 2133-2135 (2008).

[141] P. A. Stocks, P. G. Bray, V. E. Barton, M. Al-Helal, M. Jones, N. C. Araujo, P. Gibbons, S. A. Ward, R. H. Hughes, G. A. Biagini, J. Davies, R. Amewu, A. E. Mercer, G. Ellis, P. M. O'Neill, Evidence for a Common Non-Heme Chelatable-Iron-Dependent Activation Mechanism for Semisynthetic and Synthetic Endoperoxide Antimalarial Drugs, *Angew. Chem. Int. Ed.*, **46,** 6278-6283 (2007).

[142] S. Sabbani, P. A. Stocks, G. L. Ellis, J. Davies, E. Hedenstrom, S. A. Ward, P. M. O'Neill, Piperidine dispiro-1,2,4-trioxane analogues, *Bioorg. Med. Chem. Lett.*, **18**, 5804-5808 (2008).

[143] A.-C. Uhlemann, S. Wittlin, H. Matile, L. Y. Bustamante, S. Krishna, Mechanism of Antimalarial Action of the Synthetic Trioxolane RBX11160 (OZ277), *Antimicrob. Agents Chemother.*, **51,** 667-672 (2007).

[144] S. Parapini, N. Basilico, M. Mondania, P. Olliaroc, D. Taramelli, D. Montie, Evidence that haem iron in the malaria parasite is not needed for the antimalarial effects of artemisinin. *FEBS Lett.* **575,** 91–94 (2004).

[145] P. Coghi P, N. Basilico, D. Taramelli, W. C. Chan, R. K. Haynes, D. Monti, Interaction 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,* **4**, 2045 – 2053 (2009).

[146] A. K. Bhattacharjee, K. A. Carvalho, D. Opsenica, B. A. Šolaja, Structure-activity relationship study of steroidal 1,2,4,5-tetraoxane animalarials using computational procedures, *J. Serb. Chem. Soc.*, **70,** 329-345 (2005).

[147] F. B.-E. Garah, M. H.-L. Wong, R. K. Amewu, S. Muangnoicharoen, J. L. Maggs, J.-L. Stigliani, B. K. Park, J. Chadwick, S. A. Ward, P. M. O'Neill, Comparison of the Reactivity of Antimalarial 1,2,4,5-Tetraoxanes with 1,2,4-Trioxolanes in the Presence of Ferrous Iron Salts, Heme, and Ferrous Iron Salts/Phosphatidylcholine, *J. Med. Chem.*, **54**, 6443–6455 (2011).

[148] N. Kumura, H. Furukawa, A. N. Onyango, M. Izumi, S. Nakajima, H. Ito, T. Hatano, H. S. Kim, Y. Wataya, N. Baba, Different behavior of artemisinin and tetraoxane in the oxidative degradation of phospholipid, *Chem. Phys. Lipids*, **160**, 114–120 (2009).

[149] R. K. Haynes, W. C. Chan, H. N. Wong, K. Y. Li, W. K. Wu, K. M. Fan, H. H. Y. Sung, I. D. Williams, D. Prosperi, S. Melato, P. Coghi, D. Monti, Facile Oxidation of Leucomethylene Blue and Dihydroflavins by Artemisinins: Relationship with Flavoenzyme Function and Antimalarial Mechanism of Action, *ChemMedChem.,* **5**, 1282-1299 (2010).

[150] Rosenthal MD PJ (eds), *Antimalarial Chemotherapy: Mechanisms of Action, Resistance, and New Directions in Drug Discovery.* Humana Press, Totowa, New Jersey (2001).

[151] M. Akoachere, K. Buchholz, E. Fischer, J. Burhenne, W. E. Haefeli, R. H. Schirmer, K. Becker, In Vitro Assessment of Methylene Blue on Chloroquine-Sensitive and -Resistant *Plasmodium falciparum* Strains Reveals Synergistic Action with Artemisinins, *Antimicrob. Agents Chemother.,* **49,** 4592–4597 (2005).

[152] K. Buchholz, R. H. Schirmer, J. K. Eubel, M. B. Akoachere, T. Dandekar, K. Becker, S. Gromer, Interactions of Methylene Blue with Human Disulfide Reductases and Their Orthologues from *Plasmodium falciparum. Antimicrob., Agents Chemother.,* **52,** 183–191 (2008).

[153] R. K. Haynes, K. W. Cheu, M. M. K. Tang, M. J. Chen, Z. H. Guo, P. Coghi, D. Monti, Reactions of Antimalarial Peroxides with Each of Leucomethylene Blue and Dihydroflavins: Flavin Reductase and the Cofactor Model Exemplified, *ChemMedChem.,* **6,** 279-291 (2011).