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Review

REVIEW

Antimalarial peroxides

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Abstract: The problem of endemic malaria continues unabated globally. Malaria affects 40 % of the global population, causing an estimated annual mortality of 1.5–2.7 million people. The World Health Organization (WHO) estimates that 90 % of these deaths occur in sub-Saharan Africa among infants under the age of five. While a vaccine against malaria continues to be elusive, chemotherapy remains the most viable alternative towards treatment of the disease. During last years, the situation has become urgent in many ways, but mainly because of the development of chloroquine-resistant (CQR) strains of *Plasmodium falciparum* (*Pf*). The discovery that artemisinin (ART, **1**), an active principle of *Artemisia annua* L., expresses a significant antimalarial activity, especially against CQR strains, opened new approaches for combating malaria. Since the early 1980s, hundreds of semi-synthetic and synthetic peroxides have been developed and tested for their antimalarial activity, the results of which were extensively reviewed. In addition, in therapeutic practice, there is no reported case of drug resistance to these antimalarial peroxides. This review summarizes recent achievements in the area of peroxide drug development for malaria chemotherapy.

Keywords: antimalarial; peroxides; trioxanes; trioxolanes; tetraoxanes; chimeras.

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1. INTRODUCTION

The problem of endemic malaria continues unabated globally. Malaria affects 40 % of the global population, causing an estimated annual mortality of 1.5–2.7 million people.[1] The World Health Organization (WHO) estimates that 90 % of these deaths occur in sub-Saharan Africa among infants under the age of five.[2] While a vaccine against malaria continues to be elusive,[3] chemotherapy remains the most viable alternative towards treatment of the disease. During the last years, the situation has become urgent in many ways, but mainly because the malaria parasite has developed multiple drug resistance to clinically established drugs (Fig. 1). This resistance is most serious with chloroquine (CQ), the most widely employed and cheapest drug used to treat malaria, and CQ-resistant (CQR) strains of *Plasmodium falciparum* (*Pf*) – Indochina W2, Brazil IEC-306, FCR3 and K1. The problem of combating malaria is even more complex because of the contemporary development of resistance of the mosquito vector to currently employed insecticides.

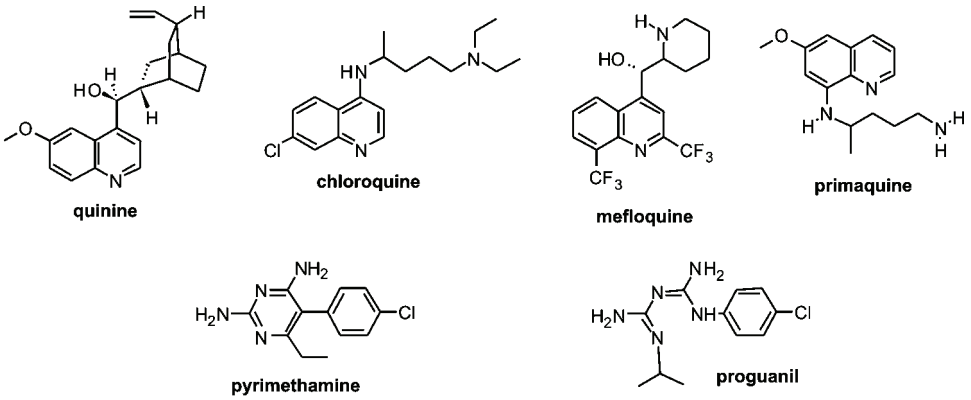


Fig. 1. Antimalarial drugs against which the malaria parasite has developed resistance.

The discovery that artemisinin (ART, **1**), an active principle of *Artemisia annua* L., expresses a significant antimalarial activity, especially against CQ-resistant (CQR) strains,[4] opened new approaches for combating malaria. Since the early 80s, hundreds of semi-synthetic and synthetic peroxides have been developed and tested for their antimalarial activity, the results of which were exten-

sively reviewed.[5] In addition, in therapeutic practice, there is no reported case of drug resistance to these antimalarial peroxides. The only evidence for *in vitro* resistance was found in French Guiana where certain ART derivatives were used in uncontrolled and illegal self-medication.[6]

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* are responsible for 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, which is responsible for the majority of fatal outcomes.

The pathogenesis[7] and life cycle of the malaria parasite are complex,[8] consisting of two stages: a sexual stage (sporogony), which occurs within the mosquito, and an asexual stage (schizogony), which occurs in the host.[5c,9] The illness is started when an infected female mosquito of the genus *Anopheles* feeds on the blood of an uninfected vertebrate host. Mosquitoes inject parasites (sporozoites) into the subcutaneous tissue, less-frequently directly into the bloodstream. In less than 1 hour, the sporozoites travel to the liver, invade the hepatocites and undergo exoerythrocitic schizogony. After some time, depending on the plasmodium species, the schizonts are transformed into merozoites, which after release into the blood stream invade erythrocytes. After significant reorganization of the membrane proteins of an occupied erythrocyte,[8e] the merozoites undergo erythrocytic schizogony, which comprises young rings (12 h after erythrocyte infection), mature rings (18 h), early trophozoites (24 h), mature trophozoites (30 h), early schizonts (36 h) and mature schizonts (42 h). At the end of erythrocytic schizogony, the parasites return into the merozoite form but enormously multiplied causing splattering of the erythrocyte. The released merozoites invade new red blood cells and start a new erythrocytic schizogony cycle. Erythrocytic schizogony occurs every 2–3 days, depending on the plasmodium species. Each cycle is accompanied by typical malaria symptoms, such as fever, chills, headache and exhausttion. After several cycles, some of the merozoites undergo sexual development and are transformed into gametocytes.

The gametocytes remain in the erythrocytes and are consumed by an anopheles mosquito. In the mosquito, female and male gametocytes join and form zygote. Within 18 to 24 h, the zygote transforms into a slowly motile ookinete. Between 7 and 15 days, depending on the plasmodium species and the 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 infected, the mosquito bites a susceptible vertebrate host; a new parasite cycle commences. The length of the *P. falciparum* life cycle is presented in Table I.

TABLE I. Length of the various stages of the life cycle of *P. falciparum*

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

The malaria parasite has a limited capacity for *de novo* amino acid synthesis and its survival is dependent on haemoglobin proteolysis. Parasite digests haemoglobin in the food vacuole (FV), supplies itself with 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 inhibiting enzymes, by peroxidation of membranes, and by producing oxygen radicals in the acidic environment of the FV.[10] In order to protect itself, the parasite eliminates haematin by polymerizing it into hemozoin. Hemozoin is a non-covalent aggregate of several units of haematin linked *via* coordinate bonds formed between Fe(III) of one haematin and the carboxylate side chain of the adjacent one.[11] Hemozoin is insoluble, and it is accumulated in the lymphatic tissue, liver, bone marrow and the brain. It was found that haem [Fe(II)PPIX] can not polymerize to hemozoin and is an effective inhibitor of Fe(III)PPIX polymerization, even better than CQ.[12]

3. ARTEMISININ AND ITS DERIVATIVES

The herb *Artemisia annua L.* has been used in Chinese traditional medicine for centuries. The usage of plants extracts for the treatment of malaria fever is well documented.[5a] The active ingredient of the potion was identified as artemisinin **1** (ART, qinghaosu – QHS, Fig. 2), a sesquiterpene lactone with an endoperoxide function.[4] The very same compound was also isolated by the Belgrade group in the early 1970s; however, the wrong structure of **1** was proposed (Fig.

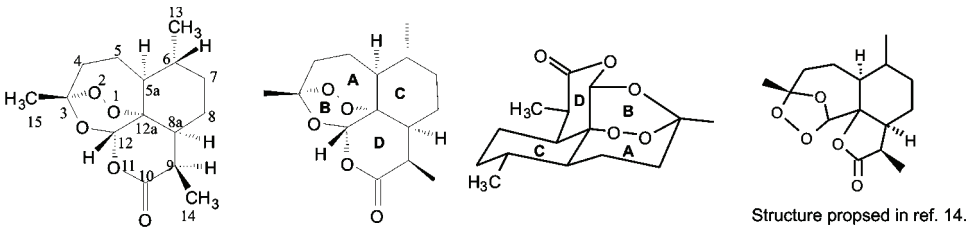
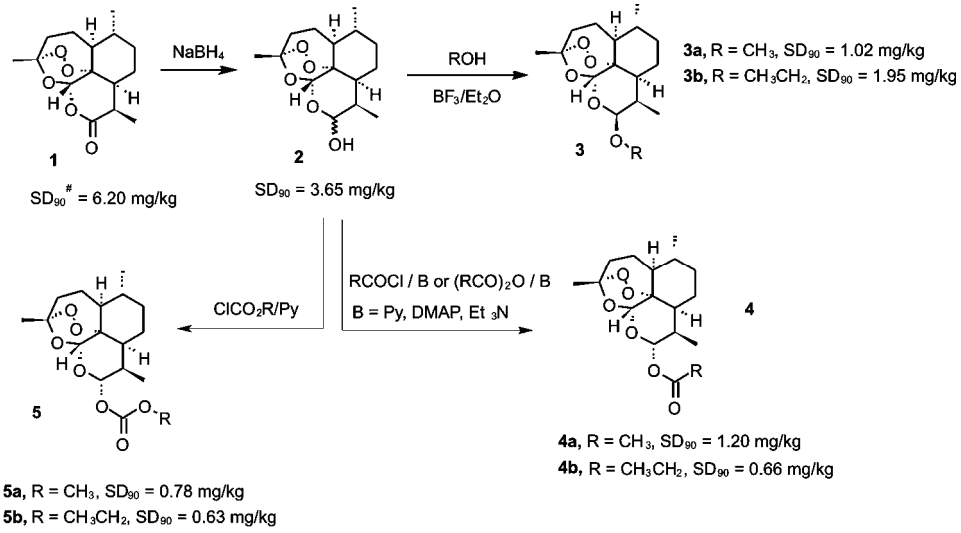


Fig. 2. Structure of artemisinin.

2).[13] The structure of ART was elucidated in 1979 by X-ray analysis, which was supported by total synthesis.[14] Since then, many total syntheses have been achieved,[15] including the newest one.[16] ART is an erythrocytic schizonticide which exhibits rapid activity against all types of human and most animal malaria. It is effective against both CQ-sensitive (CQS) and CQR strains of *P. falciparum* ((IC_{50}) D6 = 9.0 nM, W2 = 6.7 nM, TM91C235 = 13.0 nM)[17] and it has been successfully used for the treatment of severe cerebral malaria.[5c] 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. Metabolites isolated after p.o. administration were devoid of peroxide function and had no antimalarial activity, strongly suggesting that the peroxide function is a critical part of the pharmacophore. It was assumed that the good activity of **1** was due, at least partially, to its amphiphilic structure that facilitates cell membrane permeability. The shortcoming of the usage of **1** is the high recrudescence rates related to its pharmacokinetic profile, which is characterized by a short half-life, low oral bioavailability and auto-induction of metabolism.

3.1. First generation of artemisinin derivatives

The first semi-synthetic derivatives of ART were simple ethers (**3**), esters (**4**) and carbonates (**5**) of dihydroartemisinin (DHA, **2**) (Scheme 1).[4b,5a] DHA is a lactol easily obtained from **1** by $NaBH_4$ reduction. It is twice as active as ART but exhibits a relatively high degree of neurotoxicity. In spite of poor oral bio-



Scheme 1. Transformations of artemisinin into first generation derivatives (SD_{90} – the dose required for 90% suppression of parasitemia; data taken from ref. 4b. Compounds were administred i.m. as oil suspension to mice infected with *P. berghei*).

availability, high recrudescence and noteworthy reports on its neurotoxicity, DHA is as effective against severe cerebral malaria as ART.

Artemether **3a** and arteether **3b** are β-alkyl ethers of DHA designed to increase lipid solubility, pharmacokinetic profile and antimalarial activity as compared to **1** and **2**. Both derivatives are fast acting blood schizonticides and are especially active against CQR strains. Some studies indicated significant neurotoxicity when the drugs were administered in high doses, probably due to their metabolism to DHA. Later studies showed that neurotoxicity occurred when the compounds were administered at least five times higher doses than is recommended. Today, artemether **3a** is the most widely used derivative and is applied as an oil solution for i.m. injection (Artenam® and Artemos®), or recently in combination with lumefantrine (Coartem®).

Although carbonates **5** exhibited higher *in vivo* activity than **1**, **2** and **3**, there is no reported clinical application, probably because of their low stability under physiological conditions, due to their rapid hydrolysis to **2**.

For treatment of severe forms of malaria, water-soluble derivatives of ART, such as sodium artesunate **4c**[4b] and artelinic acid **6**,[18] are indispensable. Both compounds can be administered intravenously (i.v.) and thus can be delivered much faster and be more efficacious than less polar ones which are administered i.m. as an oil suspension.

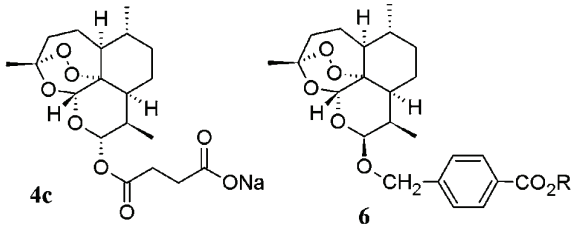
Artesunate **4c** (Fig. 3) rapidly diminishes parasitemia and is very efficacious in the restoration to consciousness of comatose cerebral malaria patients.[19] Its shortcoming is high recrudescence of the disease and **4c** is normally used in combination therapies with mefloquine[5e] and amodiaquine (Arsucam®). Na artesunate is used as a freshly prepared solution in dextrose or saline because of its rapid hydrolysis to **2**.

Artelinic acids **6a** (Fig. 3) possess a C(10) β-ether linkage and are thus hydrolytically more stable than artesunate **4c**. The acid **6a** expresses *in vitro* activity comparable to **1** and **4c** against D6 and W2* strains of *P. falciparum*, but showed superior *in vivo* activities against *P. berghei* as compared to both compounds.[18] Moreover, the acid **6a** has a longer plasma-life,[18] a higher plasma concentration, higher binding capacities and the lower toxicity among the first generation of semi-synthetic derivatives of ART **1**.[5c] Although the methyl ester **6c** exhibits higher *in vitro* activity, it is less suitable because of its low solubility in water.

The described derivatives suffer from serious disadvantages − short plasma life and CNS toxicity as consequence of their rapid metabolism into dihydroART **2**.[20] Ethers **3** are metabolized by cytochrome P-450, forming the C(α)-hydroxyl derivative **7**, which is later transformed into **2** (Scheme 2). Esters **4** simply hyd-

*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.

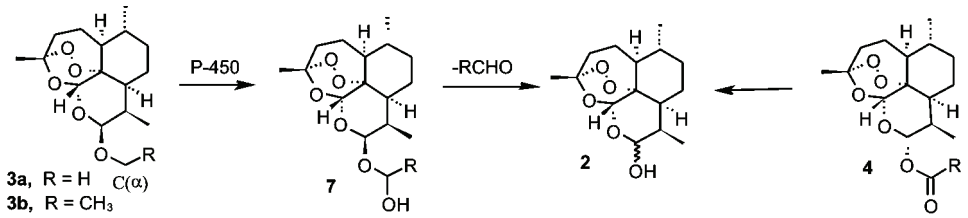
rolyze to **2**. In addition, all these derivatives possessing acetal or hemi-acetal group in the D-ring readily hydrolyze under acidic conditions after p.o. administration. The longer plasma half-life of artelinic acid **6a** is probably the result of steric hindrance and poorer accessibility to P-450. The second generation of semi-synthetic derivatives of ART was designed to overcome these disadvantages.



	IC_{50} (nM)[a]	
	D6	W2
1	10.38	2.34
2	1.44	2.43
3a[b]	2.29	1.01
6a, R = H	9.73	3.30
6b, R = K	1.74	0.92
6c, R = CH_3	1.78	0.86

[a] Data taken from reference 18; [b] Data taken from ref. 20.

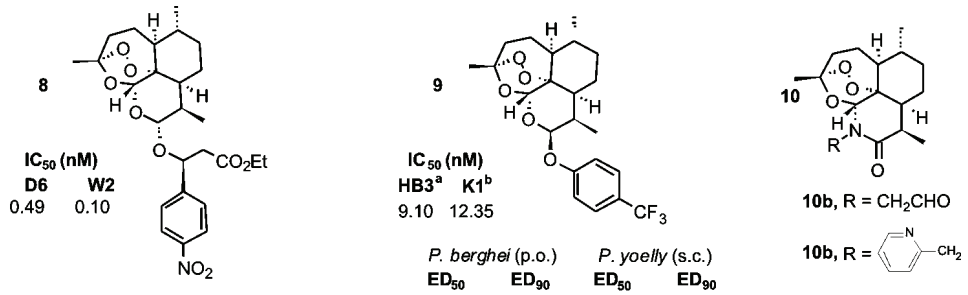
Fig. 3. Structures and antimalarial activities of derivatives **4a** and **6**.



Scheme 2. Metabolic transformations of derivatives **3**, **4** and **7**.

3.2. Second generation of artemisinin derivatives

The first efforts towards metabolically stable compounds included a modification of artelinic acid **6**.[20] The new compounds (Fig. 4) exhibited higher activity against the W2 than against the D6 clone. It was found that electronic effects, the



	P. berghei (p.o.)		*P. yoelii* (s.c.)	
	ED_{50} (mg/kg)	ED_{90} (mg/kg)	ED_{50} (mg/kg)	ED_{90} (mg/kg)
9	2.7	5.4	2.2	3.1
4c	4.0	13.0	3.6	13.5

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

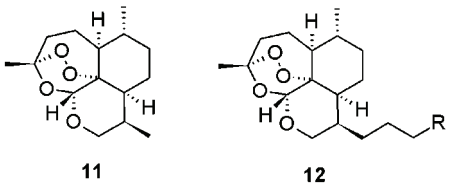
Fig. 4. Structures and antimalarial activities of derivatives **8**–**10** (ED_{50} – effective dose, the dose that decreases parasitemia by 50 %).

configuration at C(α) (the (*S*)-isomers were more active than the (*R*) ones), lipophilicity and steric factors have a considerable influence on the antimalarial activity. The most active compound **8** was 10, 20 and 40 times more active than compounds **3b**, **1** and **6a**, respectively.

Replacing the *O*-alkyl group with an *O*-phenyl group should prevent oxidative dealkylation with P-450 and the formation of dihydroartemisinin **2**. Consequently, a series of C(10)-aryloxy derivatives **9** and lactams **10** were synthesized and their antimalarial activity assessed (Fig. 4).[21] Derivative **9** was *in vitro* as active as artemether but had outstanding *in vivo* antimalarial activity, which was higher than the clinically used sodium artesunate. Moreover, derivative **9** was metabolically more stable than the parent compound (H instead of CF_3).

Many 11-azaartemisinins **10** (Fig. 4) were described as being more stable under physiological conditions.[22] In addition, the possibility of changing the substituents on the nitrogen enabled the fine-tuning of the activity. New compounds **10a** and **10b** were, respectively, 26 and 22 times more active then ART.[22b]

The superior activity of deoxoartemisinin **11**[23] encouraged the synthesis of a series of C(9)-substituted derivatives **12** (Fig. 5).[24,25] Derivatives **12a**–**b** were 21–33 times more active than ART against the W2 clone and 50–70 times more active against the D6 clone, clearly demonstrating that the removal of the lactone carbonyl provides excellent potency enhancement.[25] These and some new derivatives[25] were tested *in vivo* both s.c. and p.o. The activity of derivative **12a** was superior to that of ART, curing all mice at an 8 mg/kg/day s.c. dose.



12a, R = CH_3
12b, R = C_6H_5
12c, R = *p*-ClC_6H_4
12d, R = *p*-FC_6H_4
12e, R = *p*-$MeOIC_6H_4$
12f, R = 3,4-$Cl_2C_6H_3$
12g, R = 3,4-$CF_3C_6H_3$
12h, R = 3,4-$F_2C_6H_3$

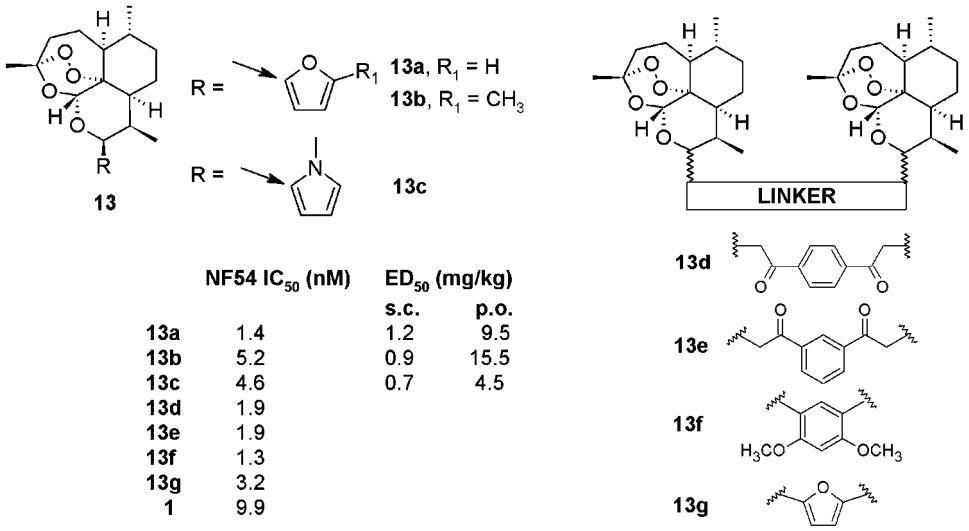
	IC_{50} (nM)[a]	
	D6	W2
1	8.25[b]	4.29[b]
11	2.16[b]	0.56[b]
12b	2.42	3.62
12c	4.67	6.39
12g	1.53	13.98
12h	2.01	15.91

[a] Data taken from ref. 24
[b] Data taken from ref. 22

Fig. 5. Structures and antimalarial activities of derivatives **11** and **12**.

A series of C(10) carbon heterocyclic substituted derivatives (Fig. 6) of deoxyartemisinin were synthesized using a short and efficacious synthetic procedure.[26] The compounds were tested *in vitro* against the CQ-sensitive (CQS) NF54 *P.f.* strain and demonstrated activity at least that of ART. Derivative **13a** was the most active in the series with IC_{50} = 1.4 nM. A special group of these derivatives were of dimeric structure. The compounds were more stable under physiological conditions than the parent compound **1** and simultaneously retained

very good to excellent activity.[27] Some of these derivatives were significantly more active (IC_{50} = 1.3–3.2 nM) than ART (IC_{50} = 9.9 nM) against the CQS NF54 *P.f.* strain.



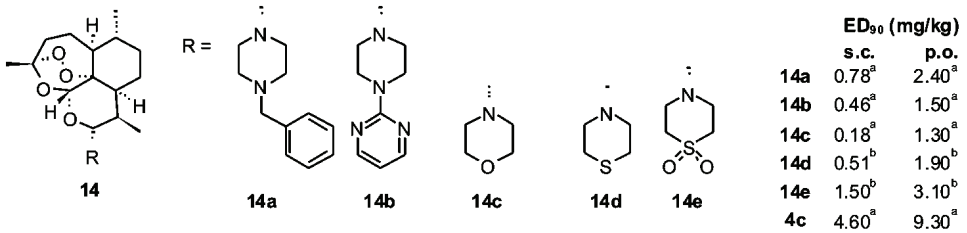
	NF54 IC_{50} (nM)	ED_{50} (mg/kg) s.c.	ED_{50} (mg/kg) p.o.
13a	1.4	1.2	9.5
13b	5.2	0.9	15.5
13c	4.6	0.7	4.5
13d	1.9		
13e	1.9		
13f	1.3		
13g	3.2		
1	9.9		

Fig. 6. Structures and antimalarial activities of derivatives **13**.

3.3. New artemisinin derivatives

With the aim of preventing metabolic transformation of artemisins to dihydroartemisinin **2** and thus avoiding neurotoxicity, several 10-(alkylamino)artemisinins **14** were designed (Fig. 7).[28,29] All the tested compounds showed excellent *in vivo* activities against *P. berghei*, with **14c**, as the most active derivative, being almost 25 times (s.c.) and 7 times (p.o.) more active than artesunate **4c**. Unfortunately, **14c** suffers from being seriously neurotoxic even at low doses, thus indicating once again that the more lipophilic compounds are more toxic.[29] However, derivative **14e** (artemisone) showed no toxicity and possesses tractable physicochemical properties. *In vivo* experiments revealed that artemisone has a greatly enhanced bioavailability, as reflected in the greater and significantly more sustained activity in plasma as compared to other artemisins. Moreover, in 3-day combinations with mefloquine (5 mg/kg) or amodiaquine (20 mg/kg), a single oral dose of artemisone (10 mg/kg) completely cured infected monkeys.[29]

A series of C(10)-ether derivatives **15** (Fig. 8) possessing voluminous lipophilic groups were tested p.o. against multi-drug resistant *P. yoelii nigeriensis* and several of them were 2 to 4 times more active than β-arteether.[30] Most active were the **15a** and **15b** derivatives that afforded 100 % protection at a 12 mg/kg×4 day dose. The least active derivative was the corresponding β-isomer of **15a**.



	ED_{90} (mg/kg) s.c.	p.o.
14a	0.78[a]	2.40[a]
14b	0.46[a]	1.50[a]
14c	0.18[a]	1.30[a]
14d	0.51[b]	1.90[b]
14e	1.50[b]	3.10[b]
4c	4.60[a]	9.30[a]

[a] Data taken from ref. 28; [b] Data taken from ref. 29

Fig. 7. Structures and antimalarial activities of derivatives **14**.

Similar relative relationships between α- and β-epimers were observed with other derivatives described in this study, which is in sharp contrast to the activity profile of arteether, where the β-isomer showed higher activity than the α-isomer. The corresponding C(10)-esters **16** (Fig. 8) were more active *in vivo* than β-arteether against *P. yoelii nigeriensis*, with **16c** being the most active derivative with 4/5 cured mice at 12 mg/kg×4 day p.o. dose.[31] Comparing the members of the two series, the structurally similar esters were less active than the corresponding ethers (**16a** *vs*. **15a**, and **16b** *vs*. **15b**).

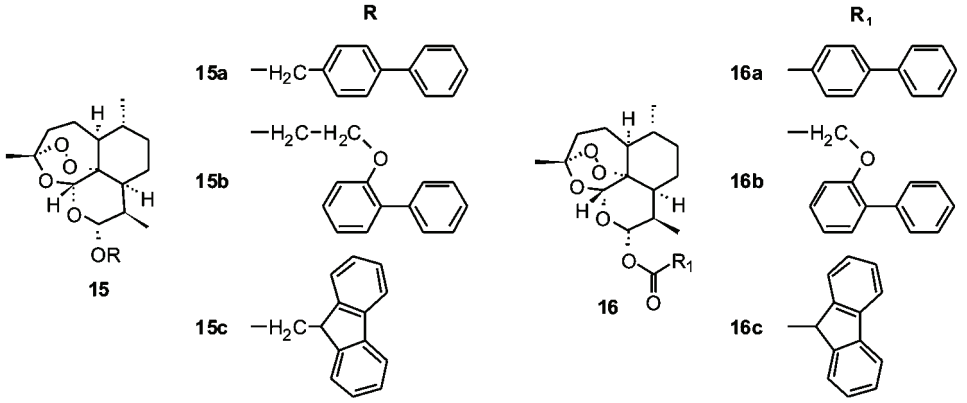
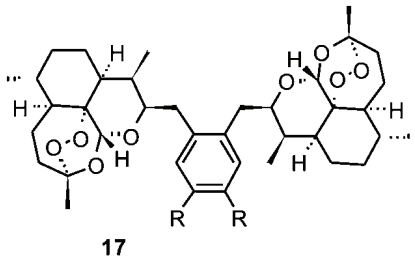


Fig. 8. Structures of derivatives **15** and **16**.

Hydrolytically stable C(10)-nonacetal artemisinin dimers **17** (Fig. 9), which possess phthalate derivatives as linker, showed higher *in vitro* antimalarial activities against the CQS NF54 *P. falciparum* strain than ART **1**, with **17a** and **17b** being the most active.[32] These two dimers were, respectively, 3 times and 37 times more efficacious than artesunate **4c** when administered s.c. and **17b** was 1.5 times more efficacious than **4c** (p.o.).

A new generation of ART dimers with excellent *in vivo* activity in *P. berghei* infected mice was developed.[33,34] Eleven new derivatives **18**–**24** (Fig. 10) showed curative activity at a 3×30 mg/kg oral dose. At this dose, the average

mouse survival period was ≥ 3 times longer in comparison to artesunate **4c** (> 30 days for derivatives **18**–**24** *vs*. 7 days for **4c**).[34]



17

	NF54 IC_{50} (nM)	ED_{50} (mg/kg) s.c.	p.o.
17a R = CO_2Me	1.6	0.71	
17b R = CH_2OH	0.77	0.06	2.6
1	6.6		
4c		2.2	4.0

Fig. 9. Structures and antimalarial activities of dimers **17**.

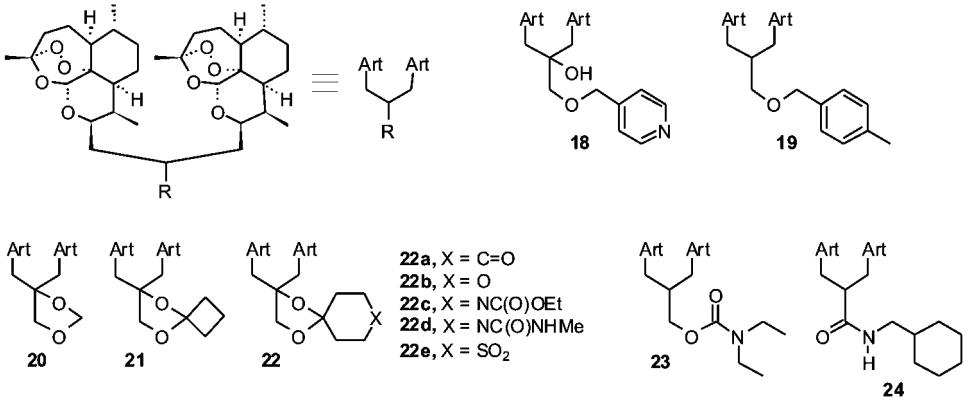


Fig. 10. Structures of derivatives **18**–**24**.

4. 1,2,4-TRIOXANES

Very important finding was the high antimalarial activity of trioxanes. These compounds have a much simpler structure than the ARTs, *e.g*., the trioxanes **25**––**29** (Fig. 11).[35–38] The activity data significantly contributed to the understanding of the minimum structural requirements for exhibition of good antimalarial activity. The results strongly indicate that ring D and the lactone ring are of no importance for good antimalarial activity, but all derivates confirmed the importance of the unique 1,2,4-trioxane structure. The results showed that small stereo or structural differences have a significant contribution to the activity, *e.g*., epimers **25** showed different *in vitro* activity[35] (particularly significant against the CQS strain D6), or in the case of derivatives **26**, in which the methyl substituents are replaced with the spirocyclopentyl group (**26a** *vs*. **26b**).[35] Contrary to the observed stereoselectivity, the enantiomers of **27a** and **27c** or **27b** and **27d** showed very similar activities, suggesting that *cis*-fusion significantly contributes to the activity.[36] Probably the most interesting example is represented by epimers **28**,

where the change of the configuration at one stereocentre dramatically changed the activity.[37] A similar structure-activity relationship, SAR, could be developed for **29** and **30**.[38]

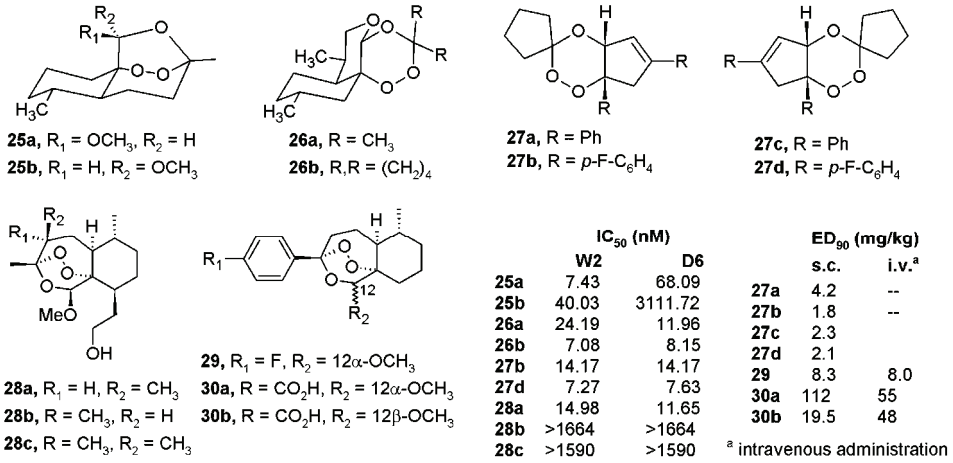


Fig. 11. Structures and antimalarial activities of derivatives **25**–**30**.

Structurally similar to the *cis*-fused trioxanes **27**, the *trans*-fused derivatives **31**–**34** (Fig. 12) also exhibited high activities.[39] They caused a 96–100 % suppression of parasitemia on day 4 after a 96 mg/kg/day p.o. dose, with the spirocycloheptane **33** as the most active. Although trioxanes **31**–**34** are somewhat less effective than *β*-arteether under the same test conditions (100 % of suppression at

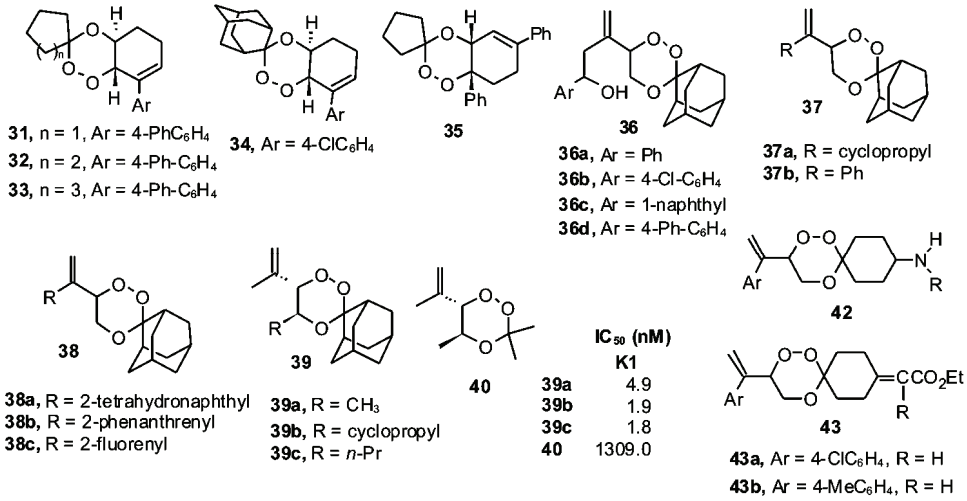


Fig. 12. Structures and antimalarial activities of derivatives **31**–**43**.

48 mg/kg/day), the obtained results suggested that *trans*-fusion may also provide good antimalarial activity and that some other structural aspects should additionally be taken into consideration. Accordingly, the *cis*-fused trioxane **35** (Fig. 10) showed IC_{50} and IC_{90} values of 893 nM and 1845 nM, respectively, against the W2 *P. falciparum* clone, while under the same screening conditions, the *cis*--fused trioxane **27a** showed corresponding values of 15 nM and 36 nM, respectively.[40]

Trioxanes **36**–**43** (Fig. 12) were obtained due to efforts aimed at creating peroxides with a simpler structure but still sufficiently active for treating malaria. Using different starting materials, such as geranyl acetate[41] (trioxanes **36**), cyclopropyl or cyclohexyl allylic alcohols[42] (trioxanes **37**), aryl substituted allyl alcohols (trioxanes **38**)[43] or methyl substituted allyl alcohols (trioxanes **39** and **40**)[44] for obtaining β-hydroxyhydroperoxides which were later coupled to different carbonyl compounds, very active trioxanes with diverse structures were obtained.[45,46] Of all the given examples, the most active were the 2-adamantyl derivatives. Derivatives **36**–**38** were tested against multidrug resistant *P. yoelli* in mice and exhibited 100 % suppression of parasitemia on day 4 at 96 and 48 mg/kg×4 days (Peter's test) doses p.o. The most active 2-fluorenyl derivate **38c** showed 100 % of suppression of parasitemia on day 4 even at 24 mg/kg×4 days, which appears to be the half effective dose of arteether.[43] Intramuscular injection decreased the activity of these derivatives and thus confirmed, once again, that hydrophobic compounds show better bioavailability on oral administration. Derivatives **39** and **40** with a C(5)-alkyl substituted 1,2,4-trioxane ring were tested against the CQR K1 *P.f.* strain and showed activities which strongly depended on the C(3)-substituent. While the spiroadamantane derivates **39** were as active as ART **1**, the *gem*-dimethyl derivative **40** was 270 times less active.[44] Replacing the spiroadamantyl with a spirocyclohexyl group bearing an ionisable arylamino moiety, as in **42**,[45] or other polar groups, as in **43**,[46] also resulted in loss of activity. Although some derivatives, such as **43a** and **43b**[46] that exhibit 100 % suppression of parasitemia on day 4 at 96, 48 and 24 mg/kg×4 days doses p.o., were active, in general, these compounds were less potent than the adamantyl derivatives. These results convincingly introduced the adamantyl-spiro-1,2,4-trioxane motif as a significant contributor to good antimalarial activity.

The antimalarial activity of a series of steroids possessing the 1,2,4-trioxane moiety **44**–**46** (Fig. 13) was also tested.[47] Only the pregnane-based trioxanes **45a**–**f** expressed good activity. They showed 100 % suppression of parasitemia on day 4 and 40–100 % protection at a 96 mg/kg×4 days dose, with **45b** being the most efficacious. Cholestane- and tigonenin-based derivatives were much less successful with 15–73 % suppression.

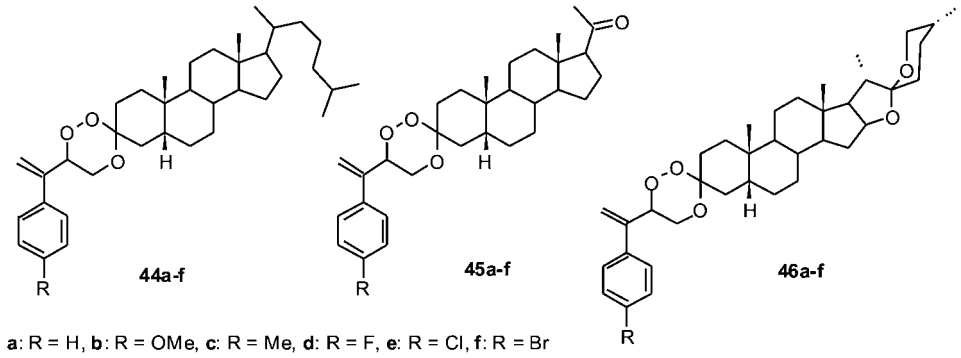


Fig. 13. Structures of steroid-based 1,2,4-trioxanes **44**–**46**.

5. 1,2,4-TRIOXOLANES

1,2,4-Trioxolanes, the ozonides, are a very well known class of organic compounds. They are intermediates in the transformation of olefins into carbonyls during ozonolysis. It was an unexpected and surprising discovery[48] that ozonides are stable enough and that some of them express excellent activity against the malaria parasite, as do the structurally similar 1,2,4-trioxanes. Moreover, these compounds were more active than artesunate **4c** and artemether **3a**, both *in vivo* and *in vitro*. The compounds **47**–**49** (Fig. 14) showed superior pharmacokinetic results, such as prolonged half-life and enhanced bioavailability after a single oral dose. The derivative **49** had inferior antimalarial results and a higher recrudescence level compared to **48**, however, it was chosen as the development candidate primarily because of its improved toxicological profile and reduced concentrations in brain tissue after oral dosing.[48] The other derivatives **50**–**53** (Fig. 14) afforded further insight into SAR in the context of the physicochemical, biopharmaceutical, and toxicological profile of trioxolanes.[49] Interestingly, it was

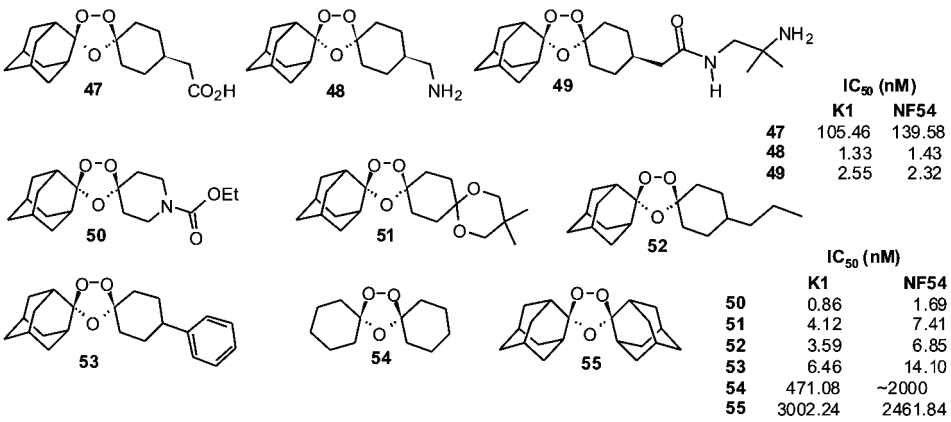


Fig. 14. Structures and antimalarial activities of derivatives **47**–**55**.

shown that symmetrically substituted derivatives **54** and **55** (Fig. 14) were significantly less active than their non-symmetrical counterparts,[49] thus confirming an earlier observation of higher activity of non-symmetrical substituted peroxides.[50]

The tolerance of the 1,2,4-trioxolane moiety to diverse synthetic conditions[51] enabled the synthesis of a significant number of derivatives and some of them showed very good *in vitro* and *in vivo* activities, *e.g.*, the derivatives **56**––**60**,[52] the piperidine derivatives **61**–**63**[53] and derivatives containing aliphatic and aromatic amino functional groups or azole heterocycles as substituents (**64**––**70**) (Fig. 15).[54] The lack of activity of **71** indicates the essential contribution of the spiro-adamantane system to the antimalarial properties of this class of compounds.[54] As the authors concluded from the obtained results, *in vitro* activities are not always a reliable predictor of *in vivo* potency.[52, 54] Many of the examined derivatives showed excellent *in vitro* results but failed during *in vivo* tests, toxi-

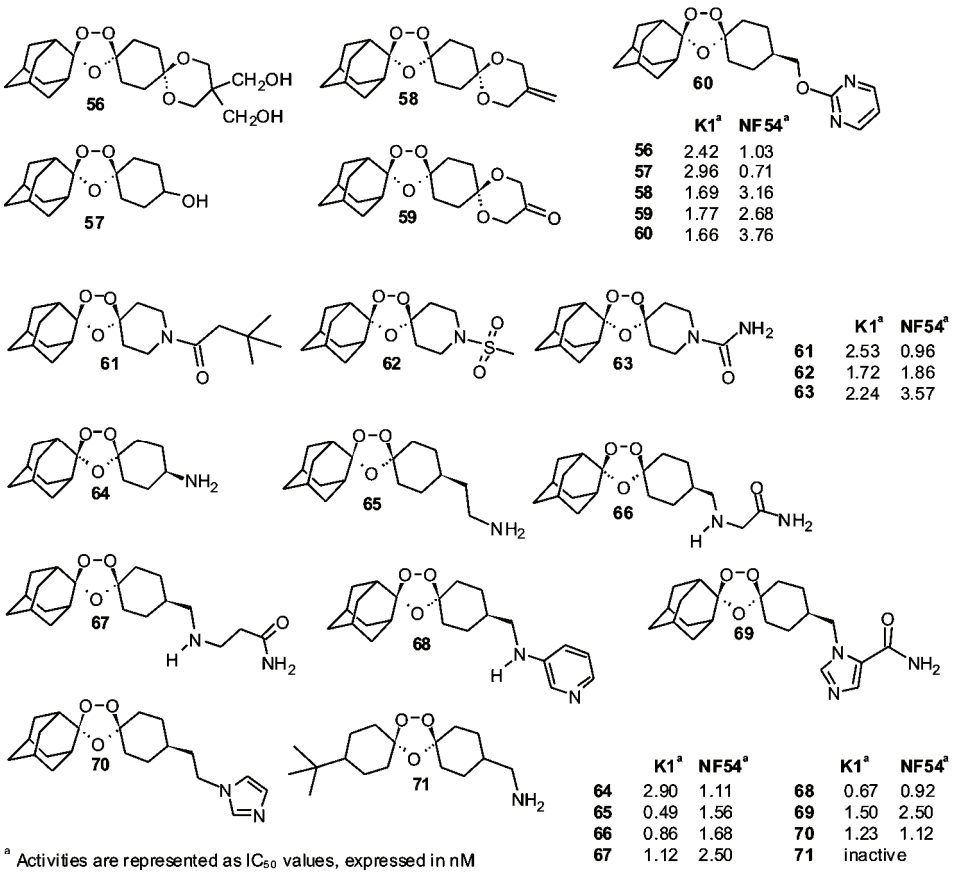


Fig. 15. Structures and antimalarial activities of derivatives **56**–**71**.

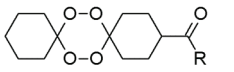
city trials or metabolic stability and bioavailability tests. The more lipophilic trioxolanes tend to have better oral activities but are metabolically less stable than their more polar counterparts are. Such behaviour is consistent with results obtained for other classes of synthetic peroxides. Trioxolanes with a wide range of neutral and basic groups had good antimalarial profiles, contrary to derivatives with acidic groups.

6. 1,2,4,5-TETRAOXANES

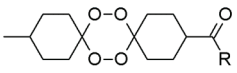
3,6-Substituted derivatives of 1,2,4,5-tetraoxacyclohexane (1,2,4,5-tetraoxane) have been known for many years and were used for different purposes.[55] They are readily formed by acid-catalyzed peroxyacetalization of carbonyl compounds with hydrogen peroxide or bis-trimethylsilylperoxide.[56–58] The discovery that inexpensive 3,6-dicyclohexylidene tetraoxanes exhibited pronounced antimalarial activity opened new possibilities in combating this pestilence.[59] Since then, many efforts have been made to find better procedures for synthesizing and designing new derivatives with improved activities.[60,61] Many of them were reviewed and their antimalarial activities analyzed.[5f,5g,62]

After the first report in which they were described,[50,63] mixed tetraoxanes* have acquired significant attention.[62] Since then, some new procedures for their synthesis,[64,65] or the synthesis of *gem*-dihydroperoxides as key precursors[66] have been developed with aim of improving the yields of tetraoxane compounds.

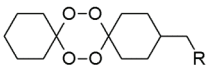
A new group of mixed dicyclohexylidene tetraoxanes, bearing polar neutral or basic groups, demonstrated high activities against both CQS and CQR *P.f.* strains (Fig. 16).[67] The compounds were designed with the aim of obtaining the simplest amphiphilic structures of the kind and to minimize the influence of steric effects on the antimalarial activity. In addition to this, a thorough examination



72: R = $NHCH_2CH_2N(CH_3)_2$



79: R = OCH_3
80: R = NH_2
81: R = $NHCH_2CH_2N(CH_3)_2$



73: R = OH
74: R = N_3
75: R = NH_2
76: R = NHC_6H_{11}
77: R = $NHPr^i$
78: R = $NHCH_2Ph$

	IC_{50} (nM)		
	D6	W2	TM91C235
72[a]	11.24	9.72	7.24
73[b]	15.18	6.54	12.62
74[b]	9.87	6.07	11.29
75[b]	12.84	7.79	19.75
76[b]	11.18	6.17	10.78
77[b]	9.40	7.54	9.27
78[b]	13.82	8.19	13.89
79[c]	12.90	6.10	16.0
80[c]	13.60	8.40	18.80
81[c]	14.90	6.10	22.60

[a] Data from ref. 67a; [b] Data from ref. 67b;
[c] Data from ref. 67c. Compounds were tested as mixture of diastereoisomers

Fig. 16. Structures and antimalarial activities of derivatives **72**–**81**.

* The term "mixed tetraoxanes"describes 1,2,4,5-tetraoxacyclohexanes differently substituted at positions 3 and 6.[50]

of the chemical stability under basic and acidic conditions, and under oxidative, reductive and reductive amination conditions revealed a significant stability of the tetraoxane moiety that enabled the synthesis of variety of derivatives.[67c] Interestingly, the most active compounds within the group of dicyclohexylidene tetraoxanes **72**–**81** have very similar activities irrespective of the presence of neutral (**74**, **79**), polar protic (**73**) or a basic ionisable group (**72**, **75**–**78**, **81**). Such behaviour impedes profound SAR analysis. In the present set of derivatives, amines **75** and **76** were the most active *in vivo*. They both cured 5/5 mice at 300 mg kg^{-1} day^{-1} doses s.c.* with 150 mg kg^{-1} day^{-1} doses s.c., **75** retained the same efficiency but **76** was less active with 4/5 cured mice.

A structurally similar group of cyclohexylidene mixed tetraoxanes was synthesized[68] using a procedure previously applied to this class of compounds (Fig. 17).[50,67a] The compounds were screened against CQS *P.f.* strain 3D7 and the most active derivatives **82**–**88** had activities within the same range as the ones described above. Monospiro tetraoxanes that exhibited a much lower antimalarial potency (**87** and **88**) confirmed the superiority of the 3,6-dispiro-1,2,4,5-tetraoxane structural motif. Adamantyl derivatives **85** and **86** showed 100 % inhibition on p.o. administration at 30 mg kg^{-1} doses and derivative **86** had better *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 **86**; ED_{50} = 5.88 mg kg^{-1} and ED_{90} = 10.57 mg kg^{-1} for artemether.

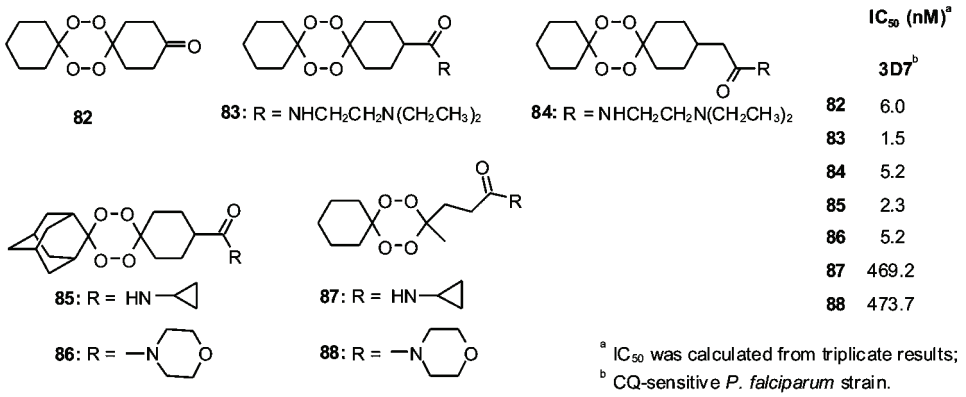


Fig. 17. Structures and antimalarial activities of derivatives **82**–**88**.

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 (Fig 18).[69] The derivatives that contain polar sulphonamide groups at one end and a highly lipophilic adamantyl

* Modifed Thompson test. "Cure" is defined as a mouse alive at day 31 with no parasitemia.

group at the other end exhibit activity in the 3–30 nM range, with **89**–**92** as the most active compounds against the 3D7 *P. f.* strain. In addition, derivative **89** was tested *versus* seven additional strains of *P. falciparum* and exhibited activity in the 1.9–3.8 nM range. Compounds **89** and **91** were noticeably active *in vivo* (p.o., *P. berghei* ANKA): ED_{50} = 6.61 mg kg^{-1} for **89** and ED_{50} = 7.93 mg kg^{-1} for **91**, ED_{50} = 8.42 mg kg^{-1} for ART.

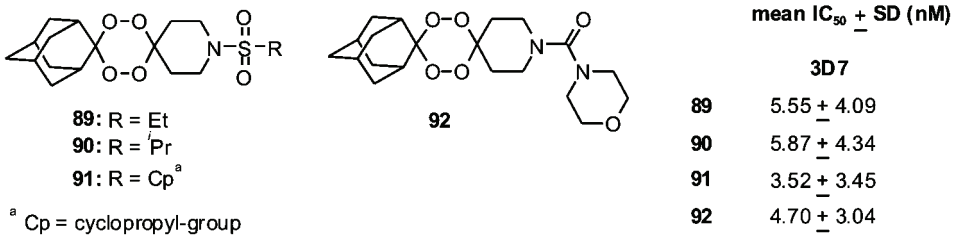


Fig. 18. Structures and antimalarial activities of derivatives **89**–**92**.

Some other types of tetraoxanes were less successful as antimalarials. Such examples are symmetrically substituted 3,3,6,6-tetraalkyl-1,2,4,5-tetraoxanes derived from acyclic ketones[70] and symmetric and non-symmetric 1,2,4,5-tetraoxane derivatives of substituted benzaldehydes (Fig. 19).[71] The compounds were tested as mixtures of the 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 **93**–**95** were the most active but still they were 1.6–2.2 times less active than the corresponding tetraoxanes **96** derived from cyclohexane, and 40–50 times less active than artemether.[70] Within the benzaldehyde series, compound **97** was the most active, while the other members were significantly less active with IC_{50} values in the range 1.4–17 μM,[71] which is far less than the corresponding 1,2,4,5-tetraoxanes or 1,2,4-trioxanes considered as active. Nevertheless, these results offer valuable basic information for the correlation between structure and antimalarial activity of the simple peroxides.

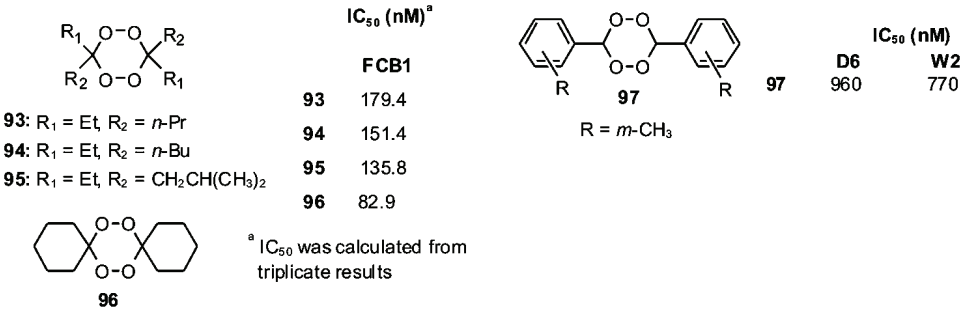


Fig. 19. Structures and antimalarial activities of derivatives **93**–**97**.

1,2,4,5,7,8-Hexaoxonanes are customary side products of the synthesis of 1,2,4,5-tetraoxacyclohexanes (Fig. 20). Their activities against K1 and NF54 *P. f.* strains showed that hexaoxonanes **98**–**103** are convincingly less active than the corresponding tetraoxanes, with 3–4 orders of magnitude lower IC_{50} values.[72] Since peroxide bonds are essential for antimalarial activity, such poor efficiency was attributed to steric hindrance of the peroxide bonds. An analogous behaviour was also noticed with the asymmetric 1,2,4,5,7,8-hexaoxonane **104**.[67c]

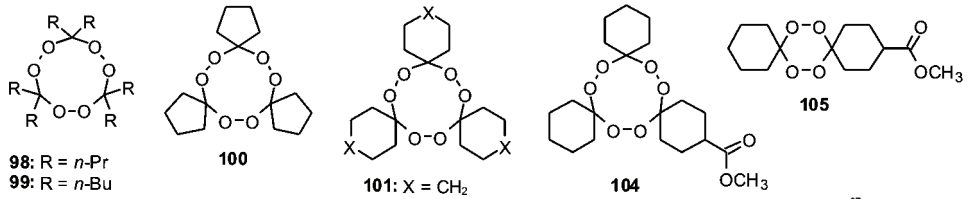


Fig. 20. Structures and antimalarial activities of derivatives **98**–**105**.

The first described steroidal 1,2,4,5-tetraoxanes **106** and **107** were derivatives from 5α-cholestane-3-one (Fig. 21).[58] Although tetraoxane **106** was less active in comparison to ART (IC_{50} (D6) = 155 nM), the results 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 the antimalarial activity (Fig. 21).[57,73] The compounds obtained as series of diastereomers, named as *cis*-C(2)C(2a) and *trans*-C(2)C(2a), showed moderate to high activity against both CQS and CQR strains. The most active were the primary amide **108** (IC_{50} (W2) = 18.79 nM) and the *n*-propyl amides **109** and **110** (IC_{50} (D6) = 9.29 nM and 20.08 nM, respectively).

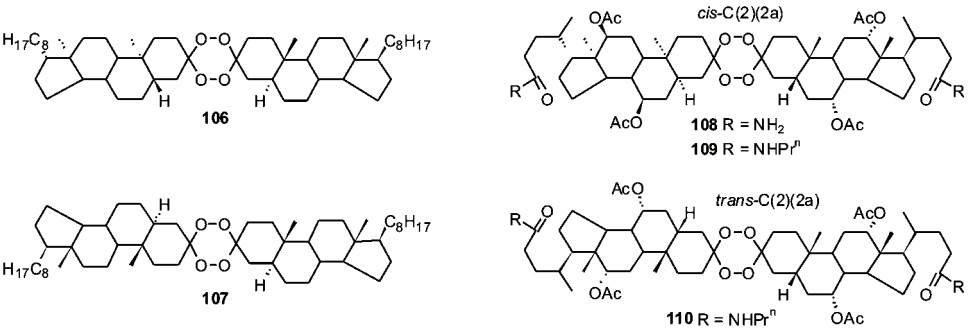
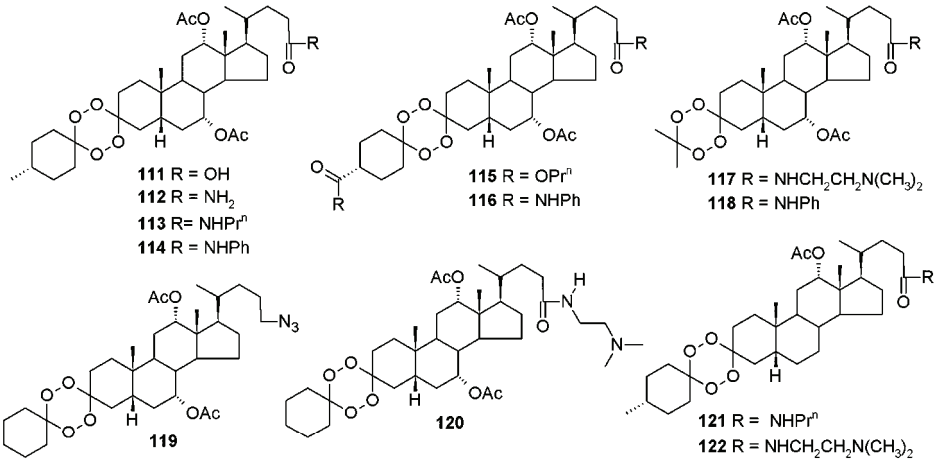


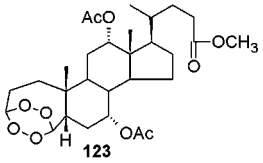
Fig. 21. Structures of derivatives **106**–**110**.

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,[50,74] 4-aryl,[75] 4-carboxy substituted cyclohexanones[76] and the acetone[77] afforded the synthesis of a numbers of various derivatives (Fig. 22). The stability of the tetraoxane moiety under a range of reaction conditions enabled the synthesis of diverse derivatives.[67c] Some of the shown mixed steroidal tetraoxanes exhibited impressive *in vitro* and *in vivo* antimalarial activity. The contribution of the cholic acid moiety as carrier is clearly emphasized by the pronounced activity of derivative **118;** bis-isopropylidene tetraoxane was completely inactive.[78]



	IC50 (nM)		
	D6	W2	TM91C235
111[a]	1.53	0.98	-
112[a]	1.17	0.58	-
113[a]	3.22	3.82	-
114[b]	5.48	7.86	6.24
115[c]	6.70	9.00	8.80
116[c]	6.20	11.40	8.10
117[d]	11.60	9.79	7.16
118[d]	6.97	5.02	15.75
119[e]	8.60	6.30	13.93
120[b]	28.18	14.49	19.65
121[f]	9.81	10.10	12.35
122[f]	12.83	16.83	14.72

	IC90 (nM)		
	D6	W2	TM91C235
121[f]	20.21	30.49	23.37
122[f]	18.66	19.77	24.25



[a] Data taken from ref. 49; [b] Data taken from ref. 73; [c] Data take from ref. 75; [d] data taken from ref. 76; [e] data taken from ref. 66c; f data taken from ref. 74

Fig. 22. Structures and antimalarial activities of derivatives **111**–**123**.

When administered to mice (p.o., *P. berghei* KBG 173 strain), tetraoxane **112** cured 4/5 mice at 200 mg kg^{-1} day^{-1} doses,* while being moderately active

* Modifed Thompson test. "Cure" is defined as a mouse alive at day 31 with no parasitemia.

when applied at 50 mg kg^{-1} day^{-1} (cured 2/5).[50] The administration of tetraoxane **117** (p.o.) produced 2/5 cured 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.[77] All the cured mice had negative blood smears during all the test days (6–31). Tetraoxane **120** cured 5/5 mice at 320 mg kg^{-1} day^{-1} and demonstrated moderate activity using lower doses (80 mg kg^{-1} day^{-1}) with 3/5 cured mice.[74] It should be emphasized that all these compounds showed no toxic effects towards the tested animals at any applied concentration. In addition, they have low activity against the Vero cell line, exhibiting a cytotoxicity/antimalarial potency ratio 1/(1400 – 9500).[50] Furthermore, the *n*-propyl amide **113** revealed no healthy erythrocyte (RBC) membrane lysis,[79] suggesting that the antimalarial activity of these compounds was the consequence of interaction specific to infected RBC, and was not the result of uncontrolled RBC membrane lysis.

A series of tetraoxanes based on deoxycholic derivatives were prepared with the aim of comparing them to cholic acid-derived tetraoxanes (Fig. 22).[74] In general, these compounds follow the same trends as the cholic acid derivatives: higher activity of the 4”-methylcyclohexyl derivatives than the non-substituted cyclohexyl ones and higher activity of 4”*R*- over 4”*S*-epimers. However, tetraoxanes with deoxycholic acid-derived carriers are less active than the corresponding cholic acid derivatives suggesting that the C(7) acetyloxy group appreciably contributes to their antimalarial activity. Within this group of tetraoxanes, the derivatives **121** and **122** were the most active exhibiting activities similar to ART and mefloquine. *In vivo*, compound **122** cured 3/5 mice at 160 mg kg^{-1} day^{-1} doses (p.o., *P. berghei* KBG 173 strain), however, at lower doses of 40 mg kg^{-1} day^{-1}, the activity declined sharply with no cured mice.

The only intramolecular steroidal 1,2,4,5-tetraoxane **123** (Fig. 22) hitherto tested* exhibited moderate *in vitro* antimalarial activity against *P. falciparum* strains (IC_{50} (D6) = 0.63 μM; IC_{50} (W2) = 0.52 μM).[80a]

7. CHIMERIC PEROXIDE–QUINOLINE COMPOUNDS

A new concept for treating malaria was introduced with the chimeric peroxides, compounds that possess covalently bonded two well-known pharmacophores – the quinoline functionality and an appropriate peroxide moiety. The compounds were designed with the aim of overcoming resistance of the parasite to CQ-based drugs and to take advantage of the pharmacokinetic properties of trioxanes, ozonides and tetraoxanes. The initial set of derivatives were more active than chloroquine against CQR strains, with compound **124** being the most active on both laboratory strains[81] and on human isolates (Fig 23).[82]

* After submission of the manuscript, the authors became aware of new intramolecular tetraoxanes prepared by the Russian group: Alexander O. Terent’ev, Dmitry A. Borisov, Vladimir V. Chernyshev, Gennady I. Nikishin, *J. Org. Chem.* **74** (2009), doi jo900226b.

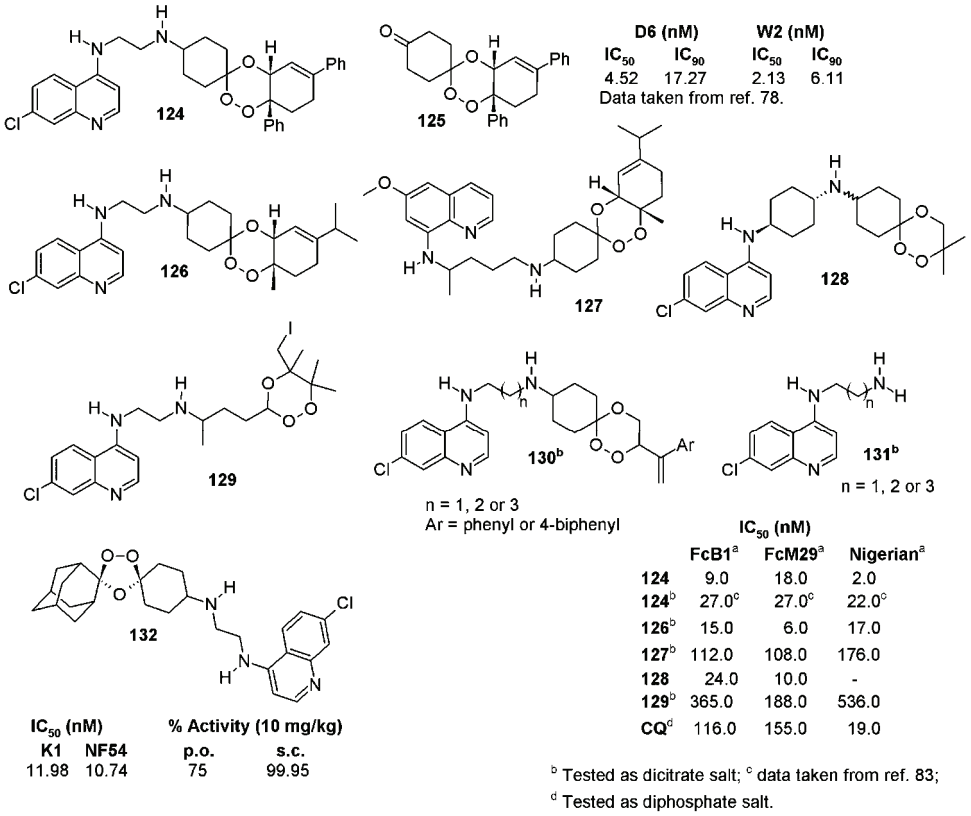


Fig. 23. Structures and antimalarial activities of derivatives **124**–**132**.

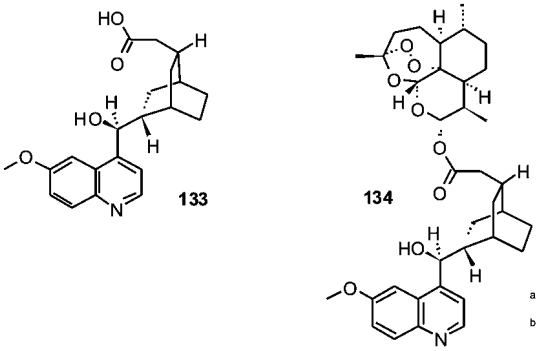
Certain simplifications of structure lead to trioxaquine **126** that was very active against both CQS and CQR strains of *P. falciparum*:[83] ED_{50} = 5 and 18 mg kg^{-1} day^{-1} (*P. vinckei*), i.p. and p.o. administration, respectively. Parasitemia clearance, without recrudescence, was achieved after an 18 mg kg^{-1} day^{-1} i.p. dose and no toxic effect in mice was 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 **127,** which demonstrated significantly lower activity against all three examined strains, and thus limited this concept to only 4-aminoquinolines. Trioxaquine **128** also exhibited high *in vitro* and *in vivo* activity.[84] The compound completely cured mice infected with CQR strain *P. vinckei* and CQS strain *P. vinckei* at 30 mg kg^{-1} day^{-1} p.o. doses.

Other variations in the trioxane structure produced derivatives **129**[85] and **130**,[86] which expressed lower activities than the previously described derivatives.

Although some very active trioxaquines were prepared, the concept of these drugs did not justify itself for several of reasons. First, these compounds did not show the expected synergism since in many cases the parent trioxane ketones[78,86] were more active than the hybrids, see ketone **125** *vs*. chimera **124** for comparison. In addition, 4-aminoquinolines **131** are themselves antimalarials. They suppressed parasitemia in mice (i.m., 87–97 %) but, as with trioxaquines **130**, no treated mice survived.[86] Even though some authors wish to establish these chimeras as therapeutics with dual activity,[84,87] all the available evidence on the mechanism of antimalarial activity of trioxanes and 4-aminoquinolines indicate that they have the same target.[84] Lastly, many of these hybrid molecules were tested as inseparable mixture of diastereomers. These circumstances deprived one of the possibilities to realize the actual scope of the antimalarial capacity of these compounds.

Based on the concept that the compounds have two integrated pharmacophores and enhanced activities of trioxolanes with the basic side chain, chimeric trioxolane **132** was obtained (Fig. 23).[88] Although it is very active *in vitro* against the K1 and NF54 strains and *in vivo* against the ANKA strain of *P. berghei*, **132** did not achieve a synergic effect of the two pharmacophores, especially when compared to trioxolanes **64** and **68**.

Another variation of trioxane hybrid antimalarials was made with covalently bonded dihydroartemisinin **2** and C(18)-quinine acid **133** (Fig 24).[89] The two segments were bonded with a hydrolytically labile ester bond and it is possible that under physiological conditions, hybrid **134** actually delivers two active agents.



	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 derivative **134**.

This approach seems to be logical since there is evidence that ARTs and quinine are active in the same stage of the *P. falciparum* life cycle,[90] but differ in the mechanism of their action.[89] The obtained results showed that hybrid **134** had a significantly higher antimalarial activity than ART and quinine alone, or when it is compared to an equimolar mixture of ART and quinine. However, the ob-

served results would be more significant if the authors had compared hybrid **134** to dihydroART **2** rather than ART and/or to acid **133**.

Based on data obtained from research into the mechanism of tetraoxane action[76] (*vide infra*), chimeric tetraoxaquines were designed with the aim of examining the effects of the presence of two pharmacophores within the same molecule (Fig. 25).[91] Three of them were as active as ART or mefloquine against the tested strains of *P. falciparum* and all of them showed higher activity than chloroquine against CQR strains. Although a synergic effect was not achieved, it could be noted that derivatives exhibited higher activity than the corresponding non-chimeric derivatives (compare **135** to **72**, **136** to **77** and **78**, **137** and **138** to **120**).[67c] *In vivo* experiments (p.o., *P. berghei* KBG 173 strain) revealed that derivatives **135** and **136** cured all tested mice at 320 mg kg^{-1} day^{-1} doses. At the lower dose of 80 mg kg^{-1} day^{-1}, the derivatives were less effective, however, but still cured 3/5 of the examined mice. Both compounds have a minimum active dose (MAD) of 20 mg kg^{-1} day^{-1} with no toxic effects even at the highest applied dose of 960 mg kg^{-1} day^{-1}.

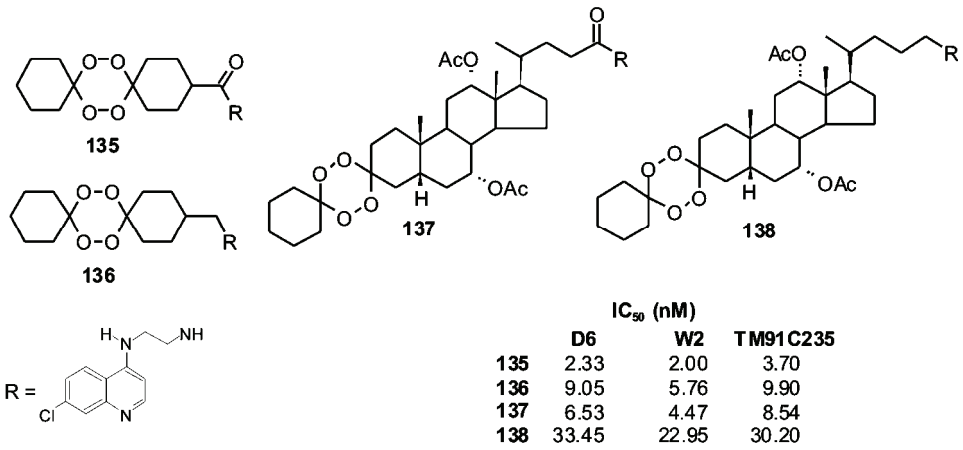
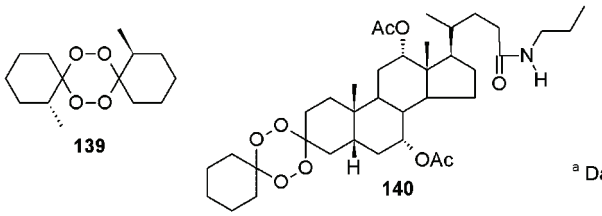


Fig. 25. Structures and antimalarial activities of derivatives **135**–**138**.

8. METABOLISM AND DRUG COMBINATION ASSAYS

Although a significant numbers of 1,2,4,5-tetraoxane antimalarials have been reported and some of them exhibited extraordinary antimalarial activity, their pharmacological properties have been much less explored than those of the 1,2,4--trioxanes. Vennerstrom was the first to give a detailed metabolic and pharmacological profile of the tetraoxanes.[92] It was shown that compound **139** (Fig. 26) is synergistic with mefloquine and quinine against both CQS D6 and CQR W2 *P. f.* strains. Tetraoxane **139** is also synergistic with chloroquine, unlike ART that shows an additive interaction with chloroquine and synergism only at high con-

centrations.[93] In addition, **139** is synergistic with ART in contrast to other semi-synthetic derivatives which have a uniformly additive effect. Derivative **139** is a modest inhibitor of human CYP1A2 activity and has a different metabolic pathway to ART. Artemisinin induces its own metabolism[92] and is metabolized by CYP2B6, CYP2C19 and, to the less extent, by CYP3A4. Tetraoxane **139** is most likely metabolized by CYP1A2 and, unlike artemisinin, **139**, 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 a metabolic stability assay and metabolic identification for many cyclohexylidene and steroidal mixed tetraoxanes.[67b,67c,74,76,77,91] Incubation of human, mouse, rat and rhesus monkey liver microsomes with the examined compounds showed dissimilar half-lives and various mono- and dihydroxylated products, as well as the products of dehydration and deacetylation, were also detected. Unfortunately, the results of the metabolic stability assays and proposed metabolite structures could not be correlated with the *in vitro* and *in vivo* antimalarial activities of these derivatives. It is important to note that no products of peroxide bond cleavage were detected. Drug combination assays of tetraoxane **140** (Fig. 26) showed that this derivative is additive with ART **1**, dihydroART **2** and artesunate **4a** against both CQS (D6) and CQR (W2 and TM91C235) *P. falciparum* strains; however, tetraoxane **140** showed synergism with artelinic acid **6a** against all three strains.[94] Tetraoxane **140** is additive with mefloquine under high concentrations against D6 and TM91C235, but at low concentrations it exhibits an antagonistic effect. With chloroquine, tetraoxane **140** showed antagonism against all three tested strains.



	IC_{50} (nM)	
	D6	W2
138[a]	6.90	3.40
139[b]	14.76	6.80

[a] Data given in ref. 59; [b] data given in ref. 50

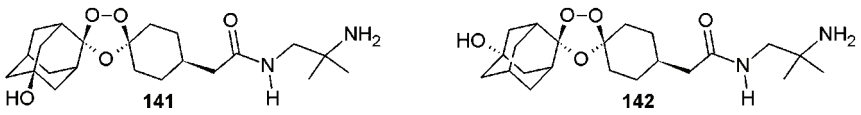
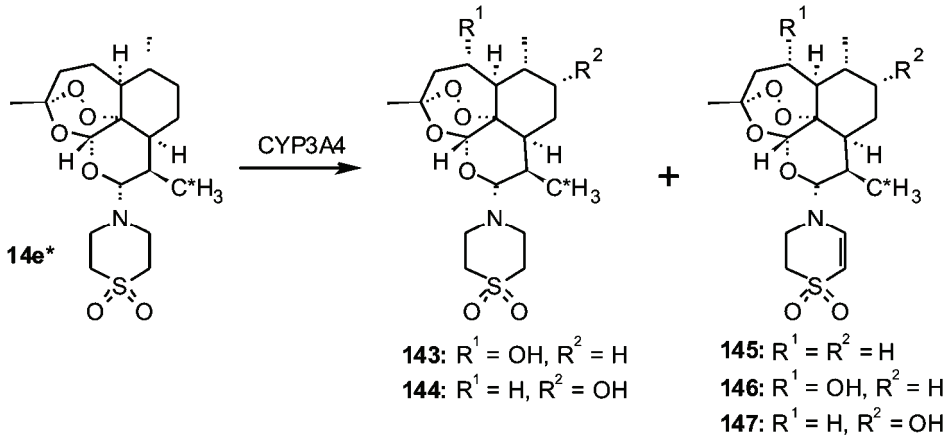


Fig. 26. Structures and antimalarial activities of derivatives **139**–**142**.

After incubation of ozonide **49** with human liver microsomes *in vitro*, three mono-hydroxylated metabolites were detected.[95] Two major metabolites **141** and **142** (Fig. 26) appeared after hydroxylation of the adamantane substructure. Both

141 and **142** had low antimalarial activity with IC_{50} values > 245 nM against the CQR K1 *P. falciparum* strain.

Incubation of isotopically labelled artemisone **14e*** with human liver microsomes after 30 min gave mono- and di-hydroxylated metabolites **143**–**147**, with *syn* hydroxyl and peroxide groups (Scheme 3).[29] Incubation with microsomes and 14 recombinant CYP isoforms together with selective inhibitors showed that only the recombinant CYP3A4 significantly metabolized artemisone, indicating that artemisone **14e** and ART, in spite of being structurally similar, have different metabolic profiles in *P. falciparum*. The isolated metabolites were tested against the *P. falciparum* K1 strain and the results showed that metabolites **144** and **145** exhibited similar activities to **14e** (**144**: IC_{50} = 5.51 nM and **145:** IC_{50} = 4.26 nM; **14e:** IC_{50} = 1.99 nM), but **143** was less active with IC_{50} = 61.55 nM.



Scheme 3. Metabolic transformations of artemisone.

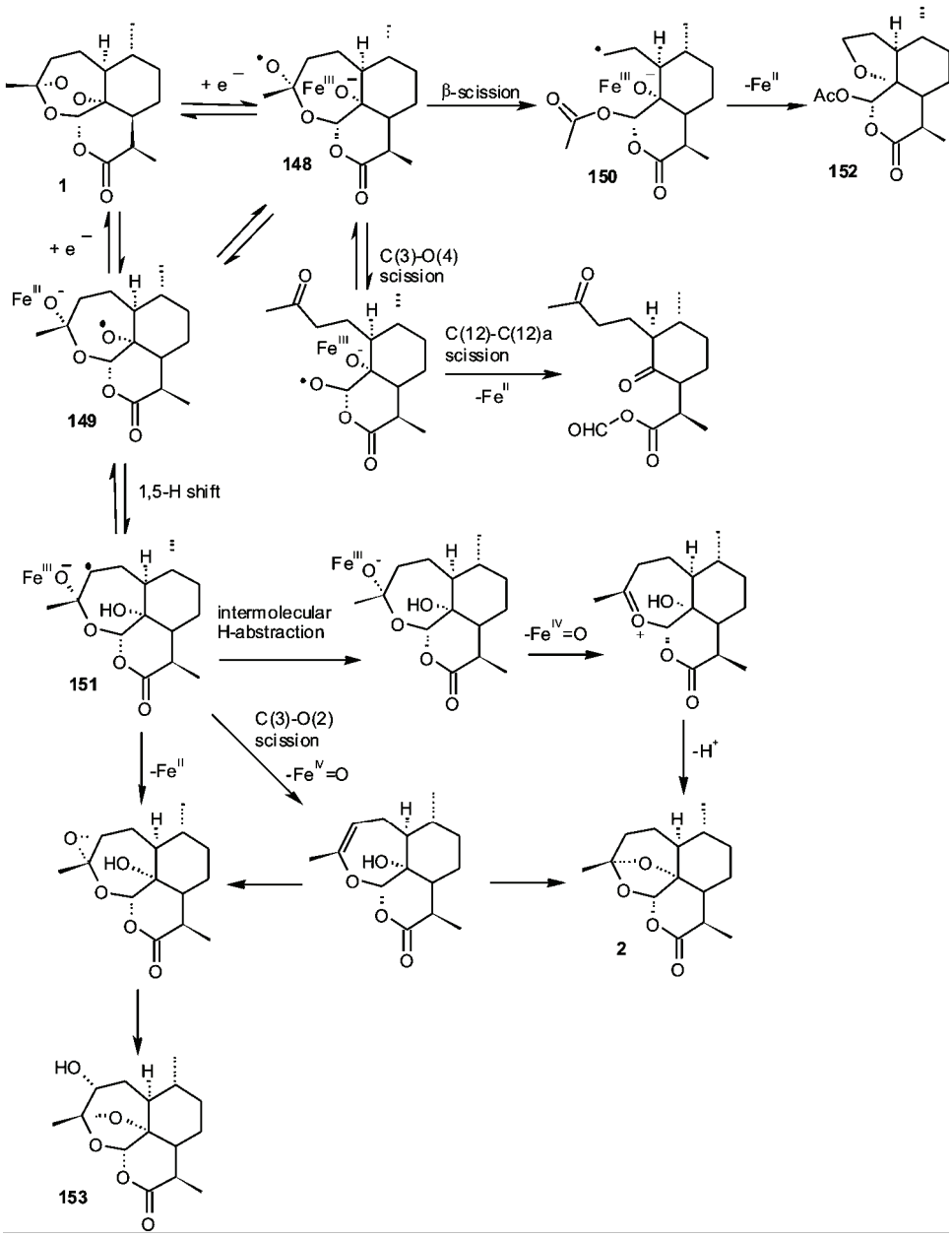
It can be seen that a common denominator is an iron species in the haem prosthetic group, and during interaction of all these structurally diverse compounds with CYP enzymes, scission of the peroxide bond never occurred.

9. MECHANISM OF ACTION

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

Several groups investigated the mechanism of action for more than fifteen years. [36,37,96–100] In general, it was found that the process commences with the formation of oxygen radicals **148** and **149,** which arise upon homolytic peroxide bond scission in the presence of ferrous ions (Scheme 4). It is assumed that ions

148 and **149** undergo intramolecular rearrangements (1,5-hydrogen shift and *β*-scission) to form C-radicals **150** and **151**. Both the O- and C-centred radicals



Scheme 4. Proposed mechanism of Fe(II)-induced decomposition of artemisinin and generation of lethal O- and C-radical species.

are highly reactive species and are lethal to the parasite. During these processes, one or more intermediates probably react with vital biomolecules, inhibit their activity and cause the death of the parasite. It was proved that one of the side products is a high-valent iron-oxo species Fe(IV)=O, which could also be toxic to the parasite.[101,102] In the absence of a suitable target that would be alkylated, the products **152** and **153** were formed.[100b] The adducts **154**–**158** (Fig. 27) were found indicative for primary and secondary radicals and the ability of ART and its derivatives to act as alkylating agents.[96c,99,100] The results obtained with artesunic acid and trioxaquine **126** additionally confirmed the capability of peroxides or chimeras to alkylate haem.[103]

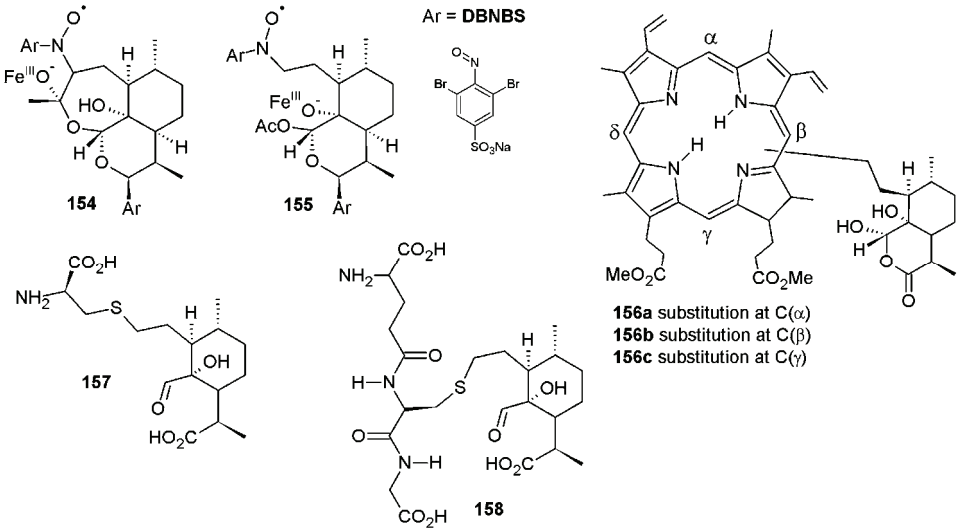


Fig. 27. Structures of covalent adducts **154**–**158** derived from C-centred radicals.

After incubation of erythrocytes infected with D6 or FCR3 *P.f.* strains and with isotope-labelled [10-^{3}H]-**2** or [15-^{3}H]-**3b,** radioactivity was detected in protein fractions originating from the parasite.[104] After treatment with EtSH or 8 M urea, it was concluded that the proteins and isotopic-labelled fragments of **2** and **3b** were covalently bonded. No radioactivity was detected after incubation with non-infected erythrocytes or with radiolabelled deoxoART. 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).[105,106] TCTP possesses bonded haem and to some extent is present in FV membrane. Compound **2** reacted *in vitro* with recombined TCTP in the presence of *in situ* generated haem and formed a covalent adduct in a 1:1 ratio. The amount of covalent adduct decreased to 60 % when one of the cysteines was chemically blocked.

Results of very important research[107] revealed that ART is a very effective inhibitor of sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) orthologue (*P. falciparum* ATP6) and that a catalytic amount of Fe(II) enhanced the inhibiting activity of **1**. It was evidenced that a 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 was investigated.[108] It was found that the resistance was positively correlated only with a mutation of the 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.

Artemisinin **1** and arteether **3b** effectively inhibit FV proteolytic activity of enzymes that degrade haemoglobin, specifically cysteine-protease.[109] Compound E-64 (for structure see ref. 110) a specific inhibitor of cysteine-protease is both a **1** and **3b** antagonist. These observations were further confirmed by *ex vivo* experiments showing accumulation of haemoglobin in the 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, when they inhibit the catabolism of haemoglobin and the liberation of haem.

Recently,[111] 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 °C). The authors claim that the reaction selectively occurs at the haem sites and consists of the progressive, slow decay of the Soret band, as a consequence of haem alkylation and subsequent loss of π-electron delocalization. This finding further complicates the elucidation of the mechanism of action of artemisinins.

Antimalarial activity does not necessarily correlate with chemical reactivity. Amino artemisinins **14a** and **14b** reacted readily with haem giving the expected products but derivatives **159** and **160** did not (Fig. 28).[28,112] On the contrary, derivative **159** reacted with aqueous Fe(II) but **160**, **14a** and **14b** were inert.[28,113] Very interesting information came from the discovery that compounds, such as **161**–**164,** which can generate neither primary nor secondary C-radicals, exhibited pronounced antimalarial activity.[98] Similarly to the 10-deoxy derivative **159**, artemisone **14e** readily reacted with aqueous Fe(II) affording the corresponding products.[113] Both compounds reacted with $Fe(OAc)_2$ and in the presence of the radical scavenger 4-oxo-TEMPO gave the corresponding covalent adducts (3 % for **159**, 10 % for **14e** and 73 % for **165**). DFO, an iron chelator, antagonized the antimalarial activity of aqueous Fe(II)-susceptible artesunate and trioxane **159**

but had no observable effect on either the aqueous Fe(II)-resistant derivative **160** or on artemisone **14e.**[113] It was found that **14e** efficiently alkylates haem.[114]

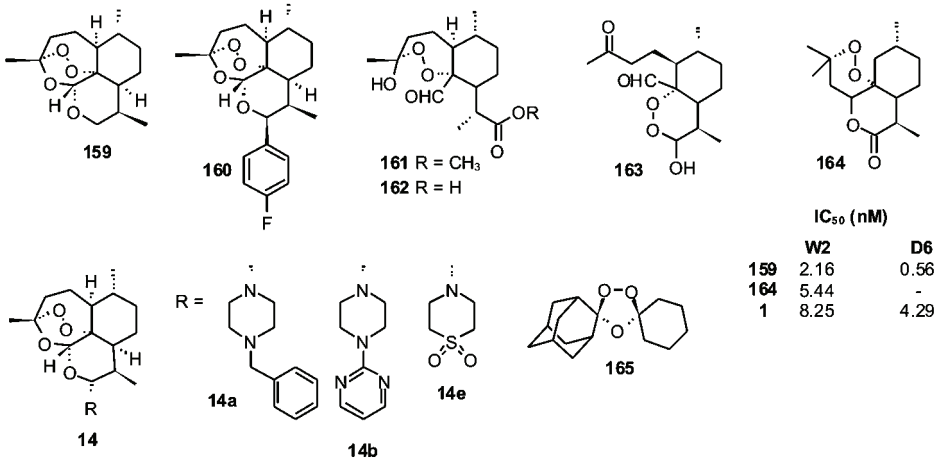


Fig. 28. Structures and antimalarial activities of derivatives **159** – **165** and selected derivatives **14**.

Due to the great specificity of ARTs to malaria parasite SERCA ATPase and inhibition of SERCA orthologues of *P. falciparum* (*Pf*ATP6) and *P. vivax* (*Pv*SERCA), it was found that artemisone **14e** is an even better inhibitor having K_i 1.7±0.6 nM for *Pf*ATP6 and K_i 0.072±0.012 nM for *Pv*SERCA, in comparison to ART, K_i 169±31 nM for *Pf*ATP6 and K_i 7.7±4.9 nM for *Pv*SERCA.[29] In contrast to these derivatives, compound **160** has a low inhibitory activity for *Pf*ATP6 (K_i 277±39 nM), which is in contrast to its high *in vitro* potency (IC_{50} 3D7 = = 1.44).[113]

Studies with conjugates **166**–**168** of ART derivatives, ozonides and 1,2,4,5--tetraoxanes with acridine and nitrobenzyldiazole (NBD) fluorochromes (Fig. 29) showed that they accumulate only in infected erythrocytes, both within the cytoplasm and FV of the parasite.[69,115] The formation of stabile adducts of acridine and NBD-tagged peroxides with biomolecules within the 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 the iron chelators DFO and DFP.[115] All the investigated conjugates showed high *in vitro* antimalarial activity in the 5–13 nM range against the 3D7 *P. falciparum* strain. These results suggest that both the cytoplasm and FV of the parasite could be equally the possible site of action of antimalarial peroxides, and that both haem from the FV or chelatable "free" iron from the cytoplasm could trigger the scission of the peroxide bond.

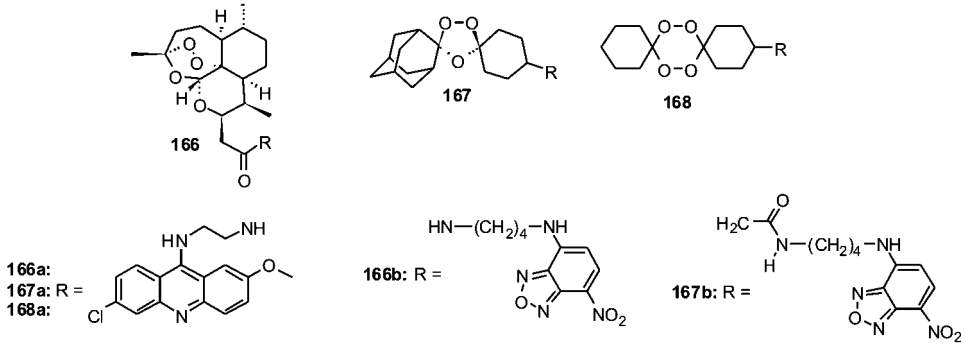


Fig. 29. Structures of conjugates **166**–**168**.

Adamantyl tetraoxane **169**, ozonide **170** and 1,2,4-trioxane **171** (Fig. 30) were subjected to reaction with different ferrous salts. It was shown that the tetraoxane **169** showed significant stability under the applied reaction conditions,[69] while the ozonide **170** and the trioxane **171** readily reacted and formed secondary carbon radicals that were scavenged by TEMPO.[116]

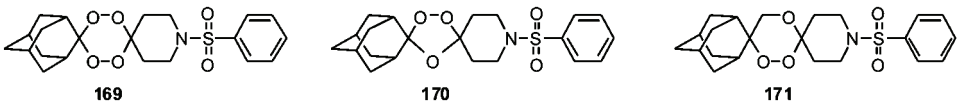


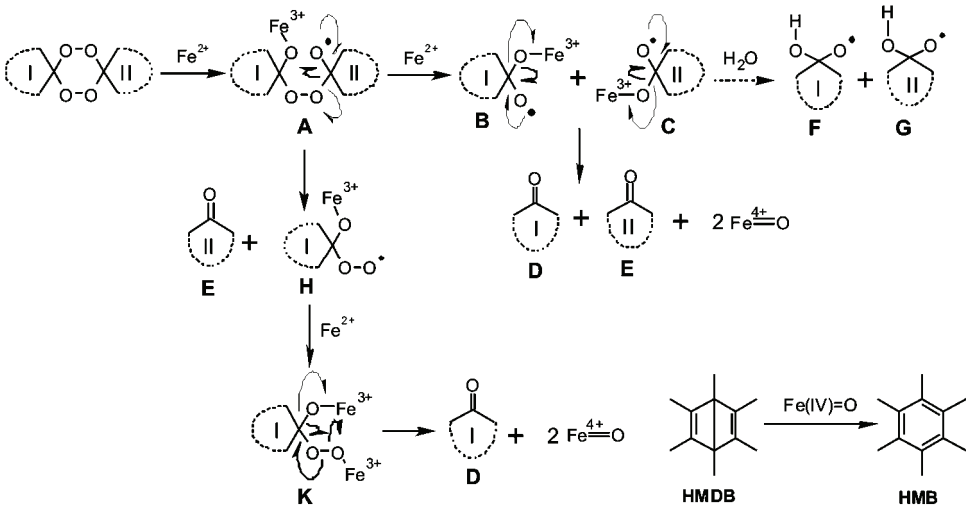
Fig. 30. Structures of peroxides **169**–**171**.

Ozonide **49** inhibits *Pf*ATP6 with a lower potency in comparison to ART, *i.e.*, K_i (**49**) = 7.7 mM *vs.* K_i (ART) = 79 nM, which thus suggests that these two peroxides may have different mechanisms of action.[117] However, the two compounds have certain similarities, such as abrogation of the activity of **49** in the presence of DFO, antagonism in combination with DFO[117] and the formation of a C-centred radical during reaction with Fe(II) and the formation of the corresponding adduct with TEMPO.[48] A fluorescent derivate of **49** 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 **49** showed antagonism in combination with artesunate.

A three-dimensional QSAR pharmacophore model for the antimalarial activity of bis-steroidal and mixed steroidal 1,2,4,5-tetraoxanes was developed.[118] 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 a necessity for good activity of this class of compounds. Docking calculations with haem suggest 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 the mechanism of action.

Differences between 1,2,4,5-tetraoxanes and the other peroxides were emphasised in studies of the mechanism of action of bis- and mixed steroidal tetraoxanes.[76] Performing experiments under the same conditions as for other antimalarial active peroxides, unexpectedly the steroidal tetraoxanes generated only oxygen centred radical that did not further rearrange to a carbon centred radical. Using DMPO and DEPMPO, both O- and C-radical traps, EPR experiments revealed only DMPO–•OR and DEPMPO–•OR spin-trapped adducts. As the only organic products, the corresponding starting ketones were isolated from the reaction mixture and no traces of rearranged products that would result from C-radical intermediates were detected. Indirect evidence of the existence of high valent Fe(IV)=O species was obtained from the rearrangement HMDB → HMB. Based on these evidences, two pathways (Scheme 5) were proposed, in which it was suggested that tetraoxane peroxides serve as an 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.



Scheme 5. Proposed mechanism of action of tetraoxanes.

10. CONCLUSIONS

Synthetic and semi-synthetic peroxides are effective drugs and are employed with success in the treatment of severe malaria. They are especially efficient against CQR strains of *P. falciparum*, the cause of cerebral malaria. Accessi-

bility, relatively inexpensive preparation and the stability of 1,2,4-trioxane and 1,2,4,5-tetraoxane function to a broad spectrum of reaction conditions enables the syntheses of derivatives with 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 the appearance of resistance of Plasmodium species, except in the cases when drugs were used without proper control. These facts, together with the possibility for combination with other non-peroxide drugs, chiefly aminoquinolines, open unrestricted possibilities in combating malaria.

Reported results concerning the mechanism of antimalarial peroxide action are contradictory at a first glance. However, bearing in mind that the compounds differ significantly in their structures that are responsible for different log *P* values, bioavailability, passage through cellular membranes and stereospecific interactions with assumed receptors or trigger species, the observed differences are logical. In our opinion, antimalarial peroxides may themselves have different action pathway and they may have different targets. This said, certain common behaviour, such as the capability of acting as alkylating agents, is not excluded. In addition, this does not exclude the possibility that one compound is simultaneously activated against several different targets. Perhaps it should not be expected that all peroxides could be fitted into a unique mechanism of action. It is more likely that they have a complex, multi-targeted mechanism, including oxidative stress.[113]

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И З В О Д

ПЕРОКСИДНИ АНТИМАЛАРИЦИ

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Ширење маларије је стално присутан проблем на глобалном нивоу. Од маларије годишње оболи 40 % светске популације и око 1,5–2,7 милиона људи умре. Према подацима Светске здравствене организације, 90 % смртних случајева је у земљама подсахарске Африке, међу којима доминирају деца старости до 5 година. Услед немогућности развоја вакцине, хемотерапија остаје као једини поуздан облик лечења од ове болести. Последњих година проблем борбе против маларије постаје ургентан из бројних разлога, међу којима је најзначајнији развој хлорокин-резистентних сојева паразита. Откриће да артемизинин (АРТ, **1**) и његови деривати показују изузетну ефикасност према хлорокин-резистентним сојевима отворило је велике могућности у борби против маларије. Од тада, посебно током 80-тих година, синтетисан је велики број једињења и резултати њихове активности описани су у многим научним публикацијама. Осим тога, у клиничкој пракси нису забележени примери по-

јаве резистенције паразита према овој класи антималарика. У овом ревијалном раду описани су најновији резултати у развоју пероксидних антималарика.

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