Antimalarial Drugs

Highly Antimalaria-Active Artemisinin Derivatives: Biological Activity Does Not Correlate with Chemical Reactivity**

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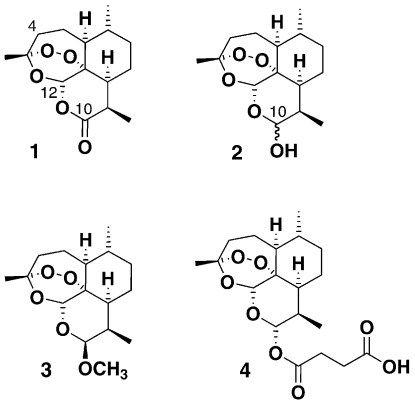
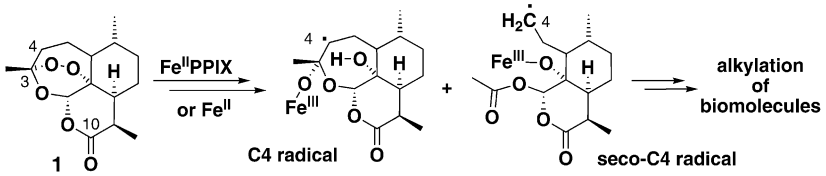
Artemisinin (**1**, qinghaosu), and the derivatives dihydroartemisinin (DHA, **2**), artemether (**3**), and artesunate (**4**) are the most potent and rapidly acting antimalarial drugs.[1–3] Nevertheless, artemether (**3**) and artesunate (**4**) have short terminal half-lives, a consequence of facile metabolism to DHA,[4] a highly neurotoxic compound.[5–8] Therefore, there is an urgent need to develop practical routes to new derivatives that are more stable and do not undergo metabolism to DHA.[2,3,9]



The compounds act on all stages of the intraerythrocytic parasite, induce ultrastructural changes in parasite membranes,[10,11] and affect membrane and other proteins.[12] The bioactive agents are held to be C-centered radicals, generated by scission of the peroxide to give an alkoxyl radical, followed by abstraction of a hydrogen atom at C4 and cleavage of the C3–C4 bond (Scheme 1). Peroxide scission is induced either by heme within the parasite food vacuole or by non-heme, "exogenous" iron. In both cases, iron must be in the +II state.[13,14] The C-centered radicals are held to alkylate undefined sensitive biomolecules in the parasite.[15–21] As artemisinin (**1**) reacts with heme to give covalent adducts,[22,23] a correlation has been drawn between this type of reaction and antimalarial activity for trioxanes in general.[24] For exogenous iron, trapping experiments with cysteine are also interpreted in terms of C-centered radicals.[25] On the basis of the hypothesis,[26] "rational design" in the synthesis of new analogues[15] and modular antimalarial drugs[27] has been claimed.



Scheme 1. Proposed formation of C-centered radicals from artemisinin (**1**) by ferrous heme [Fe^{II}PPIX] or exogenous Fe^{II}. Fe^{III} ≡ ferric heme or Fe^{III}. Detailed discussions are given in refs.[25,27].

We are preparing new artemisinin derivatives in a program in which we address the problems of metabolism to DHA and of neurotoxicity, and seek to improve efficacy through the application of pharmacokinetic parameters

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[**] This work was supported by grants from Bayer AG (Leverkusen, Germany) and by the Government of the HKSAR University Grants Council (grant no. HKUST 6091/02P).
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

 DOI: 10.1002/ange.200352343

designed to enhance solubility and adsorption of the neutral drug.[2] We now report that some of our compounds display very high activity in vivo against the malaria parasite, and they thereby provide an opportunity to test the current hypothesis for the mode of action.

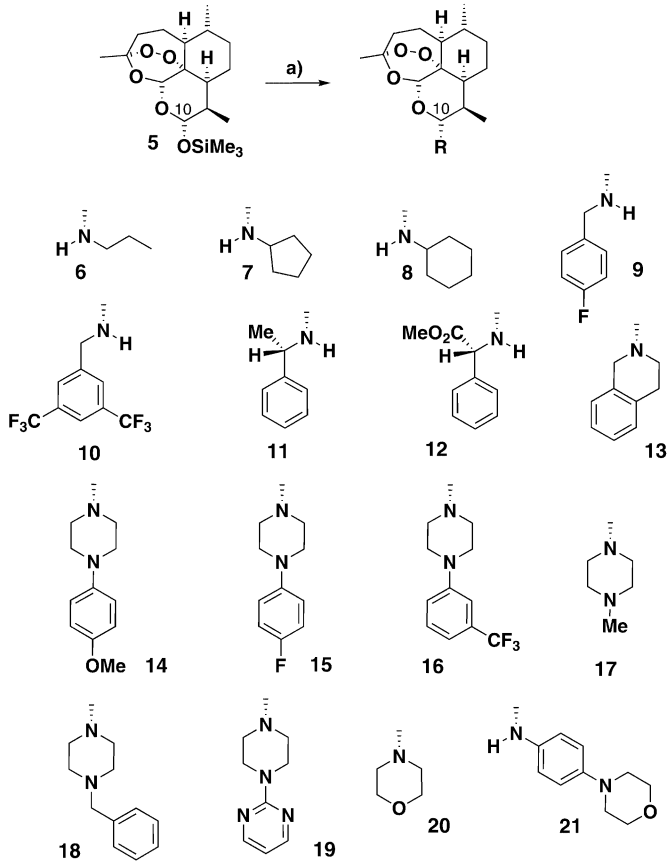
A solution of the DHA trimethylsilyl ether **5** in dichloromethane was treated with bromotrimethylsilane[28] and then with an excess of a primary or secondary alkylamine to give exclusively the 10α-alkylamino derivatives **6**–**20** (Scheme 2). Arylamino derivatives, for example, **21**, may also be prepared.[29] The reaction proceeds by means of nucleophilic displacement by the amine on the unstable 10β (axial) bromide intermediate, which is detectable in reaction mixtures by ^{1}H NMR spectroscopy.[28] Whilst yields are moderate (up to 60%), the method is straightforward and applicable to primary and secondary alkylamines, and primary aromatic amines.

Results of in vivo screens for compounds **17**–**20** conducted against chloroquine(CQ)-sensitive *P. berghei* and CQ-resistant *P. yoelii* in mice according to the Peters' four-day test are given in Table 1.[30] For comparative purposes, data acquired in the same screens for artesunate (**4**), the most active of the artemisinin derivatives currently in clinical use, and the aryl derivative **22** are also included.[28] For *P. berghei*, the amino derivatives are 3–26 times more active than artesunate when administered subcutaneously (*sc*), and 3–7 times more active when administered orally (*or*). For *P. yoelii*, the compounds are 2–81 times more active by the *sc* route (Table 1). The activities of compounds **18**–**20** in the murine malaria models appear to be superior to those of any other peroxide-containing antimalarial compound, either derived from artemisinin or of the synthetic trioxane-type, ever recorded. Thus, we have identified a new class of artemisinin derivatives with the potential for further development.

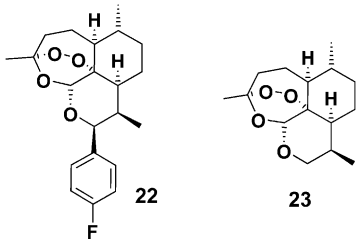
Table 1: In vivo screens against the chloroquine (CQ)-sensitive *P. berghei* N strain and the CQ-resistant *P. yoelii* NS strain according to the Peters four-day test; mice treated daily subcutaneously (*sc*) or orally (*po*) from day of infection (D0) through D+3; results (ED_{90} [mg kg^{-1}]) evaluated from parasite counts in peripheral blood on D+4.

Cmpd.	*P. berghei* ED_{90} [mg kg^{-1}: *sc*]	*P. berghei* ED_{90} [mg kg^{-1}: *po*]	*P. berghei* artesunate index[a] *sc*	*po*	*P. yoelii* ED_{90} [mg kg^{-1}: *sc*]	*P. yoelii* ED_{90} [mg kg^{-1}: *po*]	*P. yoelii* artesunate index[a] *sc*
4	4.6	9.3	1.0	1.0	42.0	–	1.0
17	1.45	3.5	3.2	2.66	22.0	–	1.9
18	0.78	2.4	5.9	3.88	0.85	3.0	49.4
19	0.46	1.5	10.0	6.2	0.52	2.0	81.0
20	0.18	1.3	25.6	7.15	1.25	1.84	33.6
22	1.16	5.0	4.0	1.86	1.08	–	39.6

[a] ED_{90}(**4**)/ED_{90}(derivative).



Scheme 2. Preparation of C10 aminoalkyl and aminoaryl derivatives **6**–**21** of DHA (**2**). a) 1. $SiMe_3Br$, CH_2Cl_2, 0°C, 45 min; 2. alkylamine (2.0 equiv), 0°C to room temperature, 12 h, then NH_4Cl/H_2O.



In order to evaluate the correlation between the antimalarial efficacy of these compounds with their reactivity towards ferrous heme or Fe^{III}, we also include the hydrolytically stable compounds **22** and **23**. The former possesses very good in vivo activities, especially against CQ-resistant *P. yoelii* (Table 1), and the latter, unlike artemisinin (**1**) and DHA (**2**), does not interact at all, for example, by complex formation, with ferric heme, and thereby does not interfere in formation of β-hematin or hemozoin, the heme dimer.[31] In comparison with artemisinin (**1**), compound **23** possesses superior in vitro activity against *P. falciparum* and is approximately equipotent in vivo against *P. berghei*.[32]

The heme experiments were carried out as previously described.[13] The artemisinin derivative, heme (ferriprotoporphyrin IX chloride), and L-cysteine (1 equiv of each) were stirred in buffered solution of deionized water/acetonitrile (1:1, pH 7.4) under nitrogen in the dark for 24 h.[33] After extraction with ether and dichloromethane, and then filtra-

tion through Celite, the crude product mixture was examined by ^{1}H NMR spectroscopy with an internal standard (1,3,5-trimethoxybenzene) to determine the amounts of the unreacted derivative. The signal for H12 (for numbering, see **1**), a singlet which appears away from other signals in the spectra of these compounds, was used as the reference signal. The major products are presumed to be heme adducts of the artemisinin derivatives, which are insoluble in organic solvents;[13] no attempt was made to isolate them. The heme adduct from artemisinin has been thoroughly characterized.[23] For the amino derivatives **18** and **19**, the most significant by-product was DHA (**2**); very little of any other product was obtained for the other compounds (Table 2). The most striking observation is that compounds **22** and **23** do not undergo significant decomposition.

Table 2: Reaction of artemisinin derivatives with ferriprotoporphyrin IX chloride/L-cysteine (each 1 equiv) in buffered MeCN/H_2O (pH 7.4) during 24 h at 23 °C.

Cmpd.	Unreacted cmpd. [%]	Other products (%)
18	18	**2** (6), **24** (6), **25** (4)
19	62	**2** (18), **25** (2.5)
20	67	not determined
22	>99	not determined
23	>99	**27** + **30** (<1)

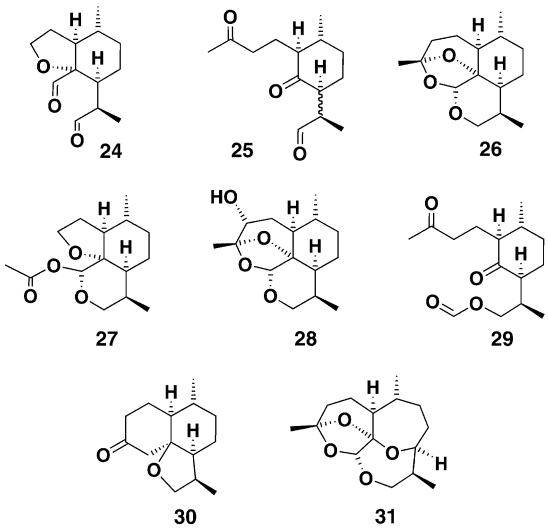
Next, the reactivity of the derivatives towards Fe^{II} was assessed (Table 3). Iron(II) sulfate in aqueous MeCN was used, in line with established practice in this type of

Table 3: Reaction of artemisinin derivatives with Fe^{II} sulfate in 1:1 MeCN/water during 24 h at 23 °C.

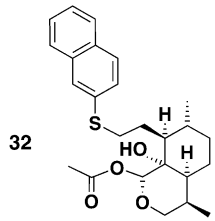
Artemisinin derivative	$FeSO_4 \cdot 7H_2O$ [equiv]	Unreacted derivative [%]	Other products (%)
18	0.3	96	**24** (3), **25** (1)
	1.0	63	**24** (25), **25** (12)
19	0.3	97	**24** (2), **25** (1)
	1.0	78	**24** (16), **25** (6)
23	0.3	0	**26** (1), **27** (41), **28** (51), **29** (5), **31** (1.5)
	1.0	0	**26** (<1), **27** (46), **28** (45), **29** (6), **31** (2.0)
19 + **23** (1 equiv)	0.3	**19:** 96, **23**: 4	**24** (3), **25** (1), **26** (6), **27** (43), **28** (41), **29** (6), **31** (0)
	1.0	**19**: 89, **23**: 0	**24** (7), **25** (4), **26** (13), **27** (41), **28** (41), **29** (5), **31** (0)

study.[20,21,25,34] The artemisinin derivative with iron(II) sulfate in 1:1 deionized water/MeCN was stirred at 23 °C for 24 h. The mixture was extracted with dichloromethane, and the products (**24**–**31**) were isolated by chromatography. Identity was secured by spectroscopic characterization and by comparison with literature data.[35,36]

Notably, the highly antimalarial-active compounds **18** and **19** displayed poor reactivity towards Fe^{II}. In contrast, compound **23** was completely decomposed. It may be argued that



the poor reactivity of the first two compounds towards Fe^{II} arises because the basic piperazinyl groups prevent the iron from entering into the redox chemistry associated with cleavage of the peroxide (cf. Scheme 1) through modulation of the pH of the aqueous reaction medium. However, that the Fe^{II} in fact remains catalytically active was established by treatment of equimolar mixtures of compounds **19** and **23** with iron(II) sulfate under the foregoing conditions. Each compound in the mixture behaved essentially as it did alone; that is, Fe^{II} remains catalytically active in inducing decomposition of **23**, whereas **19** was still unreactive (Table 3). Reactions with compound **23** were also run in the presence of 2-naphthalenethiol and iron(II) sulfate at different concentrations (0.005, 0.3, and 1.0 equiv). This did not greatly affect yields or ratios of products, although the unstable adduct **32** was obtained in variable, but low, yields.[37] Its formation belies the elaborate explanation[25] put forward for formation of cysteine adducts in related experiments.



In summary, we have developed a straightforward route to highly antimalaria-active artemisinin derivatives, which will be amenable for development of selected lead compounds. We also show that the hydrolytically stable compounds **22** and **23** are inert to ferrous heme, whereas under essentially the same conditions, compound **23** is completely decomposed by free Fe^{II}. In further contrast with the reactivity of **23**, the highly antimalaria-active alkylamino derivatives **18** and **19** are relatively unreactive towards free Fe^{II}. Their reactions in the presence of ferrous heme appears to depend partially on the nature of the amino group at C10. In any event, the formation of the hydrolysis product, DHA (**2**), in the heme reactions indicates activation of the piperazinyl substituent by complexation with heme-Fe^{II} or -Fe^{III} to displacement by water in the reaction medium takes place. In the reactions involving free Fe^{II}, it is possible that the piperazine forms a complex with Fe^{II}, and the amine–iron complex blocks access of further iron to the peroxide bridge.

These results, coupled with those of earlier work,[13,31] and other arguments (see below), seem now to exclude the possibility that an activation pathway involving heme-Fe^{II} underpins antimalarial activity of artemisinin derivatives. In the case of free, that is, non-heme or "exogenous" Fe^{II}, others have sought to demonstrate a correlation between antimalarial activity and reactivity under the reaction conditions described herein.[20,21,25,34,38] However, as no such correlation exists for our compounds,[39] the use of such a probe to establish the chemical basis for antimalarial activity in general has to be treated with considerable caution, especially as so little is known of the biological environment in which the artemisinin derivative purportedly reacts with the iron. Given that the hypothesis involving "activation" of artemisinins by Fe^{II}, and the migration and reaction of the resulting C-centered radicals with sensitive biomolecules has its genesis in such studies,[15] the hypothesis itself must also be brought into question.

We have noted previously that several antimalaria-active trioxanes cannot obviously generate C-centered radicals according to the formalism of Scheme 1.[14,18,40] Whilst many peroxides are susceptible to reductive cleavage by ferrous iron,[41] a large number also display at best feeble antimalarial activity,[36,42] and further, the Fe^{III} formed by oxidation of Fe^{II} by the peroxide (cf. Scheme 1) is capable of oxidizing the C-centered radicals to carbocations.[40,43,44] Artemisinin derivatives do not inhibit but rather are potent inducers of Phase I-metabolizing P450 (CYP) enzymes, in which the heme hydroxylates the periphery of the molecule *syn* to the peroxide without interfering with it.[2] Further, the seco-C4 radical (Scheme 1) held to be produced in the reaction with ferrous heme clearly cannot be trapped by the L-cysteine (present work), or other thiols,[13] used to generate ferrous heme; clearly, if the C-centered seco-C4 is formed at all, it reacts exclusively with the proximate heme. In general, artemisinin and derivatives affect intraerythrocytic stages of the parasite in which catabolism of hemoglobin to produce heme does not take place, namely tiny rings and gametocytes, and further, locate to parasite membranes, not the food vacuole containing the heme.[10,45] Overall, the weight of evidence suggests that binding of the artemisinin to a specific target is involved, as we have pointed out previously.[2,18,31] The peroxide itself may be activated concomitantly with, or subsequent to, binding to generate either hydroperoxide, or derived electrophilic oxygenating agent, or oxygen-centered radical capable of inducing irreversible inhibition.[2,18,31,46]

Received: July 9, 2003 [Z52343]
Published Online: February 16, 2004

Keywords: heme proteins · iron · medicinal chemistry · peroxides · radicals

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[33] The reactivity of artemisinin (**1**) under these conditions has been established; see ref. [13].

[34] W.-M. Wu, Z.-J. Yao, Y.-L. Wu, K. Jiang, Y.-F. Wang, H.-B. Chen, F. Shan, Y. Li, *Chem. Commun.* **1996**, 2213 – 2214; D.-Y. Wang, Y.-L. Wu, Y. Wu, J. Liang, Y. Li, *J. Chem. Soc. Perkin Trans. 1* **2001**, 605 – 609.

[35] For compounds **24**, **27**,and **28**, see ref. [20]; for compounds **25**, **29**, and **30**, see A. J. Lin, A. D. Theoharides, D. L. Klayman, *Tetrahedron* **1986**, *42*, 2181 – 2184; for compound **26**, see ref. [36] and M. Jung, X. Li, D. A. Bustos, H. N. ElSohly, J. D. McChesney, W. K. Milhous, *J. Med. Chem.* **1990**, *33*, 1516 – 1518; for compound **31**, see Y.-M. Pu, H. Yeh, H. Ziffer, *Heterocycles* **1993**, 36, 2099 – 2107. Detailed explanations have been presented previously for the formation of the products; see foregoing references, especially refs. [13,14,20,25,36].

[36] Treatment of compound **23** with anhydrous $FeBr_2$ in THF rapidly provides products **26** (8%) and **29** (79%): M. A. Avery, P.-C. Fan, J. M. Karle, J. D. Bonk, R. Miller, D. K. Goins, *J. Med. Chem.* **1996**, *39*, 1885 – 1897.

[37] Compound **32** is easily detected by thin-layer chromatography; however, it is admixed with approximately 15% of another adduct, possibly the C1-naphthyl-linked compound; oil, ^{1}H NMR spectrum (300 MHz, $CDCl_3$): δ = 8.16–7.36 (m, 7H), 5.97 (s, 1H), 3.68–3.60 (m, 1H), 3.48 (t, J = 11.7 Hz, 1H), 3.11–3.06 (m, 2H), 2.55–2.39 (m, 2H), 1.89 (s, 3H), 1.81–1.77 (m, 1H), 1.69–1.52 (m, 2H), 1.53–1.04 (m, 6H), 0.96–0.94 (d, J = 6.3 Hz, 3H), 0.78–0.76 ppm (d, J = 7.2 Hz, 3H).

[38] In ref. [21], it is reported that stereoisomeric naphthyl-10-deoxo-10-dihydroartemisinin derivatives differing in configuration at C9 and C10 react at markedly different rates with $FeSO_4$ in aqueous MeCN; the less reactive isomer in this case displays very low activity against *P. falciparum* in vitro.

[39] A referee contends that differences in reactivity may be due to different solubilities of the artemisinin derivatives in the reaction medium (1:1 MeCN/H_2O). In this respect, compound **20** is observed to be poorly soluble, but it is indeed decomposed by Fe^{II} (0.3 equiv) under the standard conditions (Table 3) to leave unreacted **20** (36%) and provide products **24** (13%) and **25** (6%), in addition to large amounts of as yet uncharacterized products bearing the morpholino substituent. Further, the contrasting reactivities of the benzylpiperazino compound **18** and 10-deoxo compound **23** towards each of heme-Fe^{II} and -Fe^{II} under essentially identical reaction conditions (Tables 2 and 3) are not in key with a solubility factor.

[40] R. K. Haynes, H.-O. Pai, A. Voerste, *Tetrahedron Lett.* **1999**, *40*, 4715 – 4718.

[41] For reviews and examples, see *Organic Peroxides* (Ed.: W. Ando), Wiley, Chichester, **1992**, chap. 3, pp. 174–178; *Organic Peroxides* (Ed.: W. Ando), Wiley, Chichester, **1992**, chap. 6, pp. 295–304.

[42] G. Rucker, E. Breitmaier, D. Manns, W. Maier, A. Marek, B. Heinzmann, K. Heiden, S. Seggeweis, *Arch. Pharm.* **1997**, *330*, 12 – 16.

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[45] F. ter Kuile, N. J. White, P. H. Holloway, G. Pasvol, S. Krishna, *Exp. Parasitol.* **1993**, *76*, 85 – 95.

[46] After submission of the manuscript, it was reported that artemisinin (**1**) exerts its antimalarial activity through irreversible inhibition of the Ca^{2+}-dependent *Plasmodium falciparum* ATPase (PfATP6), as expressed in *Xenopus* oocytes: U. Eckstein-Ludwig, R. J. Webb, I. D. A. Van Goethem, J. M. East, A. G. Lee, M. Kimura, P. M. O'Neill, P. G. Bray, S. A. Ward, S. Krishna, *Nature* **2003**, *424*, 957 – 961. The enzyme is a transmembrane protein associated with the parasite endoplasmic reticulum. This beautiful work convincingly demonstrates the irrelevance of the heme-activation idea. Most importantly, the identification of the target will now allow for a rational examination of the mechanism of action of this remarkable class of antimalarial drug and the precise role, if any, played by iron in artemisinin activation at the binding site.

