Antiplasmodial Activity and Cytotoxicity of 10β-Aminoquinolinylethylethers of Artemisinin

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Key words
- malaria
- *P. falciparum*
- selectivity

Abstract

Background: Each year roughly 800 000 people die of malaria, with 95 % being African children. The shortcomings of the current drugs and the emergence of *P. falciparum* resistance to the artemisinin class of compounds warrant the search for new classes or derivatives. In search for such compounds, a series of 10β-aminoquinolinylethylethers of artemisinin, previously synthesized from this laboratory were screened for antimalarial activity against both the chloroquine-susceptible 3D7 and -resistant K1 strains of *P. falciparum*. Their cytotoxicity was also assessed against HEK 293 and HepG2 cell lines.
Methods: The parasitic and mammalian cells were incubated with compounds at various concentrations for 72 h. The antimalarial activity was determined using SYBR Green I-based fluorescence. For cytotoxicity determination, cells were grown to confluence and CellTiter-Glo luminescent cell viability assay was used.
Results: All derivatives proved to be active against both strains with good selectivity towards the parasitic cells. The derivative **11** featuring 2 artemisinin moieties and an aminoethylpiperazine linker was the most active of all. It possessed 17- and 166-fold more potency than artemether against 3D7 (EC_{50}: 9.5 vs. 166 nM) and K1 (10.9 vs. 1723.3 nM), respectively, while was found to be as potent as artesunate against both strains.
Conclusion: Derivative **11** stands as a good candidate to be further investigated primarily in vitro in comparison with an equimolar combination of chloroquine (CQ) and artemisinin to ascertain its advantages, if any, over the combination.

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Bibliography
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Introduction

It is estimated that malaria kills about 655 000 people each year, 91 % of whom are living in Africa and, most of them children under the age of 5 years [1]. The development and spread of multidrug resistant (MDR) *P. falciparum* (*P. falciparum*) has led to the adoption of artemisinin-based combination therapies (ACTs) as the first-line treatment for *falciparum* malaria in most malaria-endemic countries of the world [2]. However, the recently confirmed emergence of artemisinin resistance in western Cambodia is a major threat for current initiatives to control and eliminate malaria [3–5]. While artemisinin resistance has not yet spread to other areas [6], the World Health Organization (WHO) is coordinating a large-scale elimination campaign in this region aiming to contain the spread of resistance [7, 8].
The ACTs combine fast-acting artemisinin (ART) derived drugs with other antimalarials possessing longer half-lives such as mefloquine. Because the utility of artemisinin is limited by its solubility in both oil and water, this sesquiterpene has been structurally modified by derivatization into short-chain oil soluble ether derivatives of dihydroartemisinin (DHA, **2**), such as artemether (AM, **2a**) and arteether (AE, **2b**), and the water soluble sodium artesunate (AS, **2c**) (**Fig. 1**).
Thus, all derivatives currently in use are either alkyl acetals or an ester acetal derivative of dihydroartemisinin (DHA, **2**). The problem with these semi-synthetic compounds, however, is their short pharmacological half-lives, a reflection of their acid lability, and facile metabolism to DHA [9–12]. In particular, artesunate is hydrolytically unstable, even at neutral pH, and has a half-life of only a few minutes [13].
A large amount of work has been carried out with the aim of generating new derivatives [14–16]. Artemisone (**Fig. 2**) is thus far the only second-generation semi-synthetic artemisinin derivative

that has been found suitable for further clinical development, progressing through Phase II but showing no major benefits over existing derivatives [17]. A trioxaquine (PA1103/SAR116242) based on hybrid strategy with a dual mode of action proved to be highly active *in vitro* on several sensitive and resistant strains of *P. falciparum* at nanomolar concentrations and also on multi-drug-resistant strains obtained from isolates. It has been selected as an antimalarial drug candidate for development [18]. Arterolane [19], a synthetic ozonide (**Fig. 2**, OZ277) has a longer half-life, is fast acting, well tolerated, and effective. It was in Phase III clinical trials in combination with piperaquine [20,21], and its maleate salt is now registered in India as Synriam, a drug combination product with piperaquine phosphate [22]. OZ439 (**Fig. 2**), a purely synthetic ozonide, is a drug candidate designed to provide a single-dose oral cure in humans. It has successfully completed Phase I clinical trials, where it was shown to be safe at doses up to 1.6 g, and is currently undergoing Phase IIa trials in malaria patients [23]. On the basis of their structural differences, it is believed that these synthetic ozonides may replace the standard artemisinins if they become obsolete [17]. Nevertheless, the shortcomings of the current drugs, the emergence of *P. falciparum* resistance to the artemisinin class of compounds in South East Asia, and the fact that the mechanism by which the parasite acquires resistance is still largely unknown, warrant the search for new classes or derivatives.

As part of our contribution to these efforts, we embarked on an on-going program focusing on the design, synthesis and antimalarial activity evaluation of new antimalarials. The structural modification of the artemisinin molecule to generate new derivatives is at the core of this program. We have already reported the synthesis and antiplasmodial activity of several artemisinin derivatives including oligomeric ethers with ethylene glycol [24], hybrids and hybrid dimers with aminoquinoline [25,26] and more recently 10-aminoethylethers [27].

We herein report the antimalarial activity and cytotoxicity of previously synthesized 10β-aminoquinolinylethylethers of artemisinin to highlight their inhibitory potential against a variety of *P. falciparum* strains.

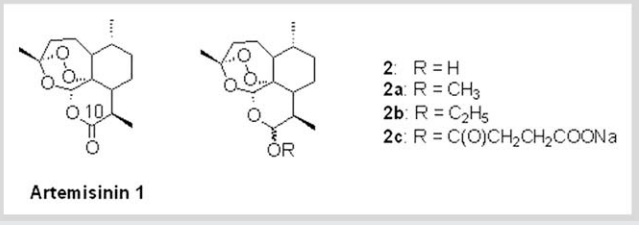

Fig. 1 Structures of artemisinin **1** and its clinically used derivatives; dihydroartemisinin **2**, artemether **2a**, arteether **2b** and sodium artesunate **2c**.

Methods

10β-(Aminoquinolinyl)ethylethers of artemisinin 3–11

The aminoquinolinylethers of the present study were previously synthesized by our group in a 3-step process involving dihydroartemisinin, 4,7-dichloroquinoline and various amines, and briefly described as follows: The condensation of DHA **2** with 2-bromoethanol in the presence of boron trifluoride etherate (BF_3. Et_2O) yielded the 10β-bromoethylether artemisinin intermediate (step 1). In parallel reactions, the quinoline ring was amino-functionalized at its position 4 by bimolecular nucleophilic substitutions involving 4,7-dichloroquinoline and various diamines to afford 4-amino-functionalized quinoline intermediates (step 2). Another bimolecular nucleophilic substitution reaction between both 10β-bromoethylether artemisinin and amino-functionalized quinoline intermediates led to the targeted 10β-(aminoquinolinyl)ethylethers of artemisinin in yields varying from 15 to 59% (Step 3). They were all obtained as the 10-β-isomers and had the *cis* configuration at carbon C-10 of the artemisinin moiety. For stability and solubility reasons, the free base target compounds were converted into oxalate salts and were tested as such for in vitro antiplasmodial activity as well as cytotoxicity. The detailed synthesis and characterization of these compounds are reported elsewhere [25,26]. Their structures are delineated in the **Fig. 3**.

Methodology for *in vitro* biological evaluation

Determination of antimalarial effective concentration (EC_{50})

The artemisinin derivatives **3–11** were screened against the chloroquine-sensitive (CQS, 3D7) and chloroquine-resistant (CQR, K1) strains of *P. falciparum*. Continuous in vitro cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen [28]. Asynchronous parasites were grown in the presence of fresh group O-positive erythrocytes (Lifeblood, Memphis, TN) in Petri dishes at a hematocrit of 4–6% in RPMI-based medium consisting of RPMI 1640 supplemented with 0.5% AlbuMAX II, 25 mM HEPES, 25 mM $NaHCO_3$ (pH 7.3), 100 µg/mL hypoxanthine, and 5 µg/mL gentamicin. Cultures were incubated at 37 °C in a gas mixture of 90% N_2, 5% O_2, and 5% CO_2. For EC_{50} determinations, 20 µL of RPMI 1640 with 5 µg/mL gentamicin were dispensed per well in an assay plate (384-well microtiter plate, clear-bottom, tissue-treated). Next, 40 nL of each compound, previously serial diluted in a separate 384-well white polypropylene plate, were dispensed in the assay plate, and then 20 µL of a synchronized culture suspension (1% rings, 10% hematocrit) were added per well to make a final hematocrit and parasitemia of 5% and 1%, respectively. Assay plates were incubated for 72 h, and the parasitemia was determined by a method previously described [29]. Briefly, 10 µL of 10X Sybr Green I, 0.5% v/v Triton, and 0.5 mg/mL saponin

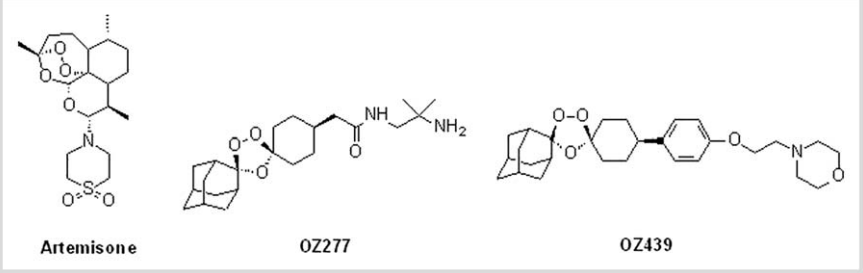

Fig. 2 Structures of the semi-synthetic artemisinin derivative artemisone and the synthetic ozonides OZ277 and OZ439.

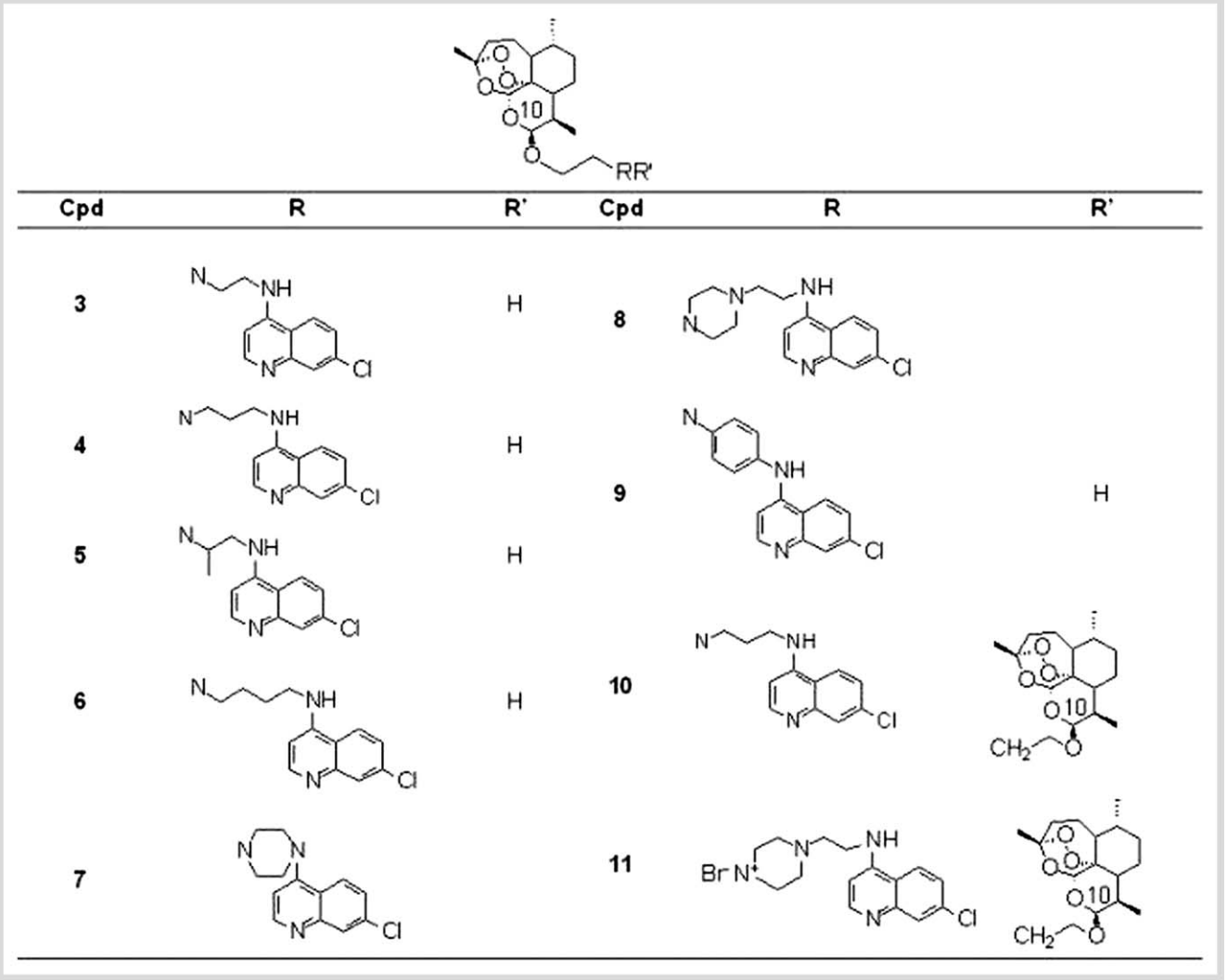

Fig. 3 Structures of 10β-(aminoquinolinyl)ethylethers of artemisinin **3–11** obtained from DHA **2**, 4,7-dichloroquinoline and various diamines in a 3-step process [25, 26].

solution in RPMI were added per well. Assay plates were shaken for 30 s, incubated in the dark for 4 h, and then read with the Envision spectrofluorometer at Ex/Em 485 nm/535 nm. EC_{50}s were calculated using proprietary software developed in house [30] in the Pipeline Pilot environment that pools data from all replicates of the experiment and fits a consensus model. The values reported in the Table are the results of a statistical analysis performed by pooling the data of 6 replicates (2 runs and 3 replicates per run) and not the arithmetic means of the 2 independent runs.

In vitro cytotoxicity against HEK293 and HEP G2 cells

HEK293 and HepG2 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured according to recommendations. Cell culture media were purchased from ATCC. HEK 293 (human embryonic kidney) and HepG2 (human liver hepatocellular carcinoma) are both mammalian cell lines. Exponentially growing cells were plated in Corning 384 well white custom assay plates, and incubated overnight at 37 °C in a humidified, 5 % CO_2 incubator. DMSO inhibitor stock solutions, the same as in the antiplasmodial assay, were added the following day to a top final concentration of 25 µM, 0.25 % DMSO and then diluted 1/3 for a total of 10 testing concentrations. Cytotoxicity was determined following a 72-h incubation using Promega Cell Titer Glo Reagent according to the manufacturer's recommendation. Luminescence was measured on an Envision plate reader (Perkin Elmer). EC_{50}s were calculated using proprietary software as in the antiplasmodial assay [30].

Dose-response curve fitting

Dose-response curves were calculated from percent activity values and log10-transformed concentrations the proprietary robust interpretation of screening experiments (RISE) application written in Pipeline Pilot (Accelrys, v. 8.5) and the R program [30]. A non-linear regression was performed using the R *drc* package with the 4-parameter log-logistic function (LL2.4) [31]

Results

The in vitro biological evaluation of the compounds was performed at the Department of Chemical Biology and Therapeutics, St Jude Children's Research Hospital, Memphis, Tennessee, US. 10β-(Aminoquinolinyl)ethylethers of artemisinin **3–11**, also artemisinin-quinoline hybrids **3–9** and artemisinin-quinoline hybrids dimers **10–11**, were screened for antimalarial activity

Table 1 *In vitro* antimalarial activity of 10β-aminoquinolinylethylethers of artemisinin 3–11 against 3D7 and K1 strains of *P. falciparum*, and their cytotoxicity against HEK293 and HepG2 cell lines.

	Activity EC_{50} (nM)[a]		Activity IC_{50} (nM)[b]				Cytotoxicity EC_{50} (μM)[e]		Cytotoxicity IC_{50} (μM)[f]			
Comp.	3D7	K1	D10	Dd2	RI[c]	RI[d]	HEK293	HepG2	CHO	SI_1[g]	SI_2[h]	SI[i]
3	41.6	44.2	21.5	25.7	1.1	1	>24.4	>24.4	1.7	>587	>587	77
4	52.7	54.9	14.3	19.8	1.0	1	9.8	4.6	0.2	186	87	12
5	35.5	34.1	14.9	20.8	1.0	1	14.0	16.1	3.4	394	454	184
6	85.6	99.7	nd	nd	1.2		>24.6	>24.6		>287	>287	
7	63.4	68.2	nd	nd	1.1		>23.3	>23.3		>368	>368	
8	199.4	479.3	29.0	29.2	2.4	1	>26.1	>26.1	2.3	>130	>130	80
9	23.5	22.2	17.3	30.2	1.0	2	>26.3	>26.3	35.2	>1119	>1119	2039
10	36.6	33.8	5.3	28.4	0.9	5	>22.0	>22.0	0.7	>601	>601	128
11	9.5	10.9	19.6	55.7	1.2	3	>23.0	>23.0	74.8	>2421	>2421	3813
AM **2a**	166	1723.3			10.4		>26.0	>26.0		>0.2	>0.2	
AS **2c**	6.6	6.6			1		>26.0	>26.0		>3.9	>3.9	

[a] Effective concentration of compound inducing 50% reduction in parasitic cells count; [b] reported antimalarial activity [25,26]; [c] resistance index (RI) = EC_{50} K1/EC_{50} 3D7; [d] resistance index (RI) = IC_{50} Dd2/IC_{50} D10 [25,26]; [e] effective concentration of compound selectively inducing 50% reduction in parasitic cells count in the presence of mammalian cells; [f] effective concentration of compound selectively inducing 50% reduction in parasitic cells count in the presence CHO (Chinese Hamster Ovarian) cells [25,26]; [g] Selectivity index (SI_1) = EC_{50} HEK293/EC_{50} 3D7; [h] selectivity index (SI_2) = EC_{50} Hep G2/EC_{50} 3D7; [i] Selectivity index (SI) = IC_{50} CHO/IC_{50} D10 [25,26]; nd (not determined)

against 3D7 and K1, 2 strains of *P. falciparum*. 3D7 and K1 are CQ-S and CQ-R strains, respectively. Also tested alongside them, were the clinically used derivatives, artemether **2a** and sodium artesunate **2c**. The results of both antiplasmodial activity and cytotoxicity are reported in the ◉ **Table 1**.

Furthermore, antiplasmodial activity and cytotoxicity, expressed as inhibitory concentration (IC_{50}) against D10 and Dd2 strains of *P. falciparum*, and CHO cells, respectively, previously determined at the Department of Pharmacology, University of Cape Town, South Africa [25,26], are also compiled in the Table for comparison sake.

As can be seen, all tested derivatives were active against both strains with good selectivity towards the parasitic cells, even better than AM and AS. Hybrid **8** was the least whereas dimer **11** was the most active of all. Derivative **8** showed activity comparable to that of artemether against 3D7 while all compounds were found to possess activities 7–17 times higher. Against the K1 strain, hybrid **8** was still the least active, however, being 4 times more potent than AM. All others were significantly (>10-times) more potent, dimer **11** displaying an outstanding 160-times higher activity than AM.

In comparison to ARS, the derivatives **3**–**10** proved to be less potent against both strains while **11** was found as potent as artesunate against both strains.

Furthermore, derivatives **3–7** and **9–11** have RI (resistance index) values ~ 1 indicating that these compounds retained their activity even against the CQR K1 strain. On the contrary, derivative **8** and artemether possess RI values of 2.4 and 10.4, respectively.

Discussion

The *in vitro* antiplasmodial activity of compounds **3–11** have already been evaluated against the D10 (CQS) and Dd2 (CQR) strains of *P. falciparum*. In that early work, all compounds were active against both strains. The oxalate salt of compound **5** stood as the most active of all. This compound was found to be less potent than **2** but displayed a significant 7 times higher potency than CQ against Dd2 [26].

In the current study, compounds **3–11** proved to be active against both strains as well. The EC_{50} values varied in the 10–90 nM range. The dimeric compound **11** displayed the most effective activity and was found to be as potent as artesunate against both 3D7 and K1, though, it had previously been reported as less potent than that reference drug against the Dd2 strain. The comparison of the activity of **11** against the CQS (D10 vs. 3D7) and CQR (Dd2 vs. K1) strains reveals a meaningful difference. It was found to be slightly more active against 3D7 than D10, and also a 5 times more active against K1 than Dd2.

Compound **5**, the most active compound in the previous study carried over its performance in the present one. There was no meaningful difference between activity against D10 and 3D7, and against Dd2 and K1. When the comparison was extended to the remaining compounds viz. **3–4** and **6–10**, it clearly appeared that these were more active against D10 than 3D7, and more active against Dd2 than K1 strain. Thus, the artemisinin derivatives **3–11** were overall more active against D10 than 3D7, and Dd2 than K1. This suggests that although both D10 and 3D7 are considered to be CQ sensitive, these strains of different cell lines have different behaviors in the presence of the compounds. Similarly, Dd2 and K1 are considered to be CQ resistant, and yet these artemisinin derivatives have different behaviors against them.

Furthermore, the fact that hybrid **8** and artemether possess RI values higher than 1 may suggest that perhaps the K1 strain is on the verge of developing resistance against these 2 compounds.

Thus, through this study, the 10β-(aminoquinolinyl)ethylethers of artemisinin **3–11** were confirmed to be active *in vitro* against 4 different strains of *P. falciparum*.

Conclusion

A series of 9 10β-(aminoquinolinyl)ethylether derivatives of artemisinin of proven in vitro antimalarial activity against CQS D10 and CQR Dd2, were screened against another 2 strains of *P. falciparum viz.* CQS 3D7 and CQR K1. They were all also active against both latter strains with good selectivity towards the parasitic cells. However, the dimeric compound **11** featuring 2 artemisinin moieties and an aminoethylpiperazine linker was

distinctively the most active of all. It displayed an outstanding potency in the nanomolar range over artemether and was found equipotent to artesunate against both strains. This compound lends itself as a good candidate to be further investigated to ascertain whether this excellent in vitro activity can be carried over in vivo. It will be interesting to assess through pharmacokinetic study if this compound possess prolonged half-life in comparison to the parent drug artemisinin or its clinical derivatives, and whether it operates as artemisinin or/and chloroquine.

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Conflict of interest

The author(s) declare(s) that they have no conflicts of interest to disclose.

References

1 *WHO, World Malaria Report 2011*. ([Web:] http://www.who.int/malaria/ world_malaria_report_2011/9789241564403_eng.pdf [Date used: 7 February 2012]) 2011;
2 *White NJ*. Qinghaosu (artemisinin): the price of success. Science 2008; 320: 330–334
3 *Dondorp AM*, *Nosten F*, *Yi P et al.* Artemisinin resistance in P. falciparum malaria. N Engl J Med 2009; 361: 455–467
4 *Noedl H*, *Se Y*, *Schaecher K et al.* Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med 2008; 359: 2619–2620
5 *Noedl H*, *Se Y*, *Sriwichai S et al.* Artemisinin resistance in Cambodia: a clinical trial designed to address an emerging problem in Southeast Asia. Clin Infect Dis 2010; 51: e82–e89
6 *Noedl H*, *Socheat D*, *Satimai W*. Artemisinin-resistant malaria in Asia. N Engl J Med 2009; 361: 540–541
7 *WHO World Health Organization*. Development of a strategy towards elimination of Plasmodium falciparum parasites with altered responses to artemisinins. WHO 2009; 1–52
8 *WHO World Health Organization*. Strategic framework for artemisinin resistance containment in Myanmar (MARC) 2011–2015. WHO 2011; 1–97 http://www.who.int/malaria/publications/atoz/artemisinin_resistance_containment_2011.pdf
9 *Lin AJ*, *Klayman DL*, *Milhous WK*. Antimalarial activity of new water-soluble dihydroartemisinin derivatives. J Med Chem 1987; 30: 2147–2150
10 *Leskovac V*, *Theoharides AD*. Hepatic metabolism of artemisinin drugs – I. Drug metabolism in rat liver microsomes. Comp Biochem Physiol 1991; 99: 383–390
11 *Leskovac V*, *Theoharides AD*. Hepatic metabolism of artemisinin drugs – II. Metabolism of arteether in rat liver cytosol. Comp Biochem Physiol 1991; 99: 391–396
12 *Baker JK*, *McChesney JD*, *Chi H-T*. Decomposition of arteether in simulated stomach acid yielding compounds retaining antimalarial activity. Pharm Res 1993; 10: 662–666
13 *Batty KT*, *Ilett KF*, *Davis TME*. Chemical stability of artesunate injection and proposal for its administration by intravenous infusion. J Pharm Pharmacol 1996; 48: 22–26
14 *China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials*. J Tradit Chin Med 1982; 2: 9–16
15 *Li Y*, *Zhu Y-M*, *Jiang H-J et al.* Synthesis and antimalarial activity of artemisinin derivatives containing an amino group. J Med Chem 2000; 43: 1635–1640 *Haynes RK, Chan H-W, Cheung M-K, Lam W-L, Soo M-K, Tsang H-W, et al.* C-10 Ester and ether derivatives of dihydroartemisinin -10-α artesunate, preparation of authentic 10-β artesunate, and of other ester and ether derivatives bearing potential aromatic intercalating groups at C-10. Eur J Org Chem 2002; 113-132; *Hindley S, Ward SA, Storr RC, Searle NL, Bray PG, Park BK, et al.* Mechanism-based design of parasite-targeted artemisinin derivatives: Synthesis and antimalarial activity of new diamine containing analogues. J Med Chem 2002; 45: 1052-1063; *Haynes RK, Chan H-W, Cheung M-K, Lam W-L, Soo M-K, Tsang H-W, et al.* Stereoselective preparation of 10α- and 10β-aryl derivatives of dihydroartemisinin. Eur J Org Chem 2003; 2098-2114
16 *Few reviews on clinical use, chemistry, pharmacology, and mode of action, see: Meshnick SR*. Artemisinin: mechanisms of action, resistance and toxicity. Inter J Parasitol 2002; 32: 1655–1660 *Gordi T, Lepist E-I*. Artemisinin derivatives: toxic for laboratory animals, safe for humans? *Toxicol Lett* 2004; 147: 99-107; *PrayGod G, de Frey A, Eisenhut M*. Artemisinin derivatives versus quinine in treating severe malaria in children: a systematic review. Malaria J 2008;7: 210; *Chaturvedi D, Goswani A, Sakia PP, Barua NC, Rao PG*. Artemisinin and its derivatives: a novel class of anti-malarial and anti-cancer agents. Chem Soc Rev 2010; 39: 435-454
17 *Enserink M*. If artemisinin drugs fail, what's plan B? Science 2010; 328: 846
18 *Coslédan F*, *Fraisse L*, *Pellet A et al.* Selection of a trioxaquine as an antimalarial drug candidate. PNAS 2008; 105: 17579–17584
19 *Vennerstrom JL*, *Arbe-Barnes S*, *Brun R et al.* Identification of an antimalarial synthetic trioxolane drug development candidate. Nature 2004; 430: 900–904
20 *Olliaro P*, *Wells TNC*. The global portfolio of new antimalarial medicines under development. Clin Pharmacol Ther 2009; 85: 584–595
21 *Valecha N*, *Krudsood S*, *Tangpukdee N et al.* Arterolane maleate plus piperaquine phosphate for treatment of uncomplicated *P. falciparum* Malaria: A comparative, multicenter, randomized clinical trial. Clin Infect Dis 2012; 55: 663–671
22 http://www.rsc.org/chemistryworld/2012/05/ranbaxy-launches-new-anti-malarial-synriam
23 *Charman SA*, *Arbe-Barnes S*, *Bathurst IC et al.* Synthetic ozonide drug candidate OZ439 offers new hope for a single-dose cure of uncomplicated malaria. PNAS Early Edition 2011; 1–6
24 *Steyn M*, *N'Da DD*, *Breytenbach JC et al.* Synthesis and antimalarial activity of ethylene glycol oligomeric ethers of artemisinin. J Pharm Pharmacol 2011; 63: 278–286
25 *Lombard MC*, *N'Da DD*, *Breytenbach JC et al.* Artemisinin-quinoline hybrid-dimers: Synthesis and in vitro antiplasmodial activity. Bioorg Med Chem Lett 2010; 20: 6975–6977
26 *Lombard MC*, *N'Da DD*, *Breytenbach JC et al.* Synthesis, in vitro antimalarial and cytotoxicity of artemisinin-aminoquinoline hybrids. Bioorg Med Chem Lett 2011; 21: 1683–1686
27 *Cloete TT*, *Breytenbach JW*, *de Kock C et al.* Synthesis, antimalarial activity and cytotoxicity of 10-aminoethylether derivatives of artemisinin. Bioorg Med Chem 2012; 20: 4701–4709
28 *Trager W*, *Jensen JB*. Human malaria parasites in continuous culture. Science 1976; 193: 673–675
29 *Smilkstein M*, *Sriwilaijaroen N*, *Kelly JX et al.* Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob Agents Chemother 2004; 48: 1803–1806
30 R Development Core Team, R. A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, URL http://www.R-project.org 2011
31 *Ritz C*, *Streibig JC*. Bioassay analysis using RJ. Statist Software 12, 2005