Graphical Abstract

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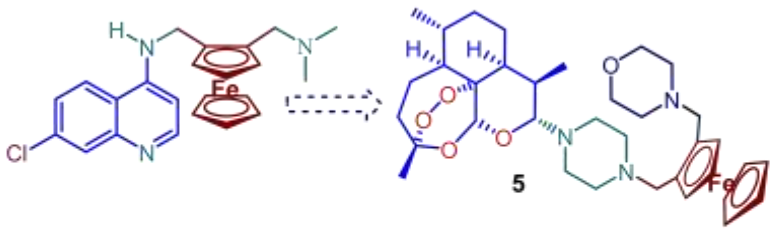

***P. falciparum* blood stage:**
asexual IC_{50} nM NF54 3.3
K1 0.8; W2 1.4
sexual %inhib. at 100 nM:
NF54 87-99%
Cytotoxicity: IC_{50} µM
Hek293 1.0; A375 1.0

Synthesis, antimalarial activities and cytotoxicities of amino-artemisinin-1,2-disubstituted ferrocene hybrids

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ABSTRACT

Artemisinin-ferrocene conjugates incorporating a 1,2-disubstituted ferrocene analogous to that embedded in ferroquine but attached *via* a piperazine linker to C10 of the artemisinin were prepared from the piperazine artemisinin derivative, and activities were evaluated against asexual blood stages of chloroquine (CQ) sensitive NF54 and CQ resistant K1 and W2 strains of *Plasmodium falciparum* (*Pf*). The most active was the morpholino derivative **5** with IC_{50} of 0.86 nM against *Pf* K1 and 1.4 nM against *Pf* W2. The resistance indices were superior to those of current clinical artemisinins. Notably, the compounds were active against *Pf* NF54 early and late blood stage gametocytes – these exerted >86% inhibition at 1 µM against both stages; they are thus appreciably more active than methylene blue (~57% inhibition at 1 µM) against late stage gametocytes. The data portends transmission blocking activity. Cytotoxicity was determined against human embryonic kidney cells (Hek293), while human malignant melanoma cells (A375) were used to assess their antitumor activity

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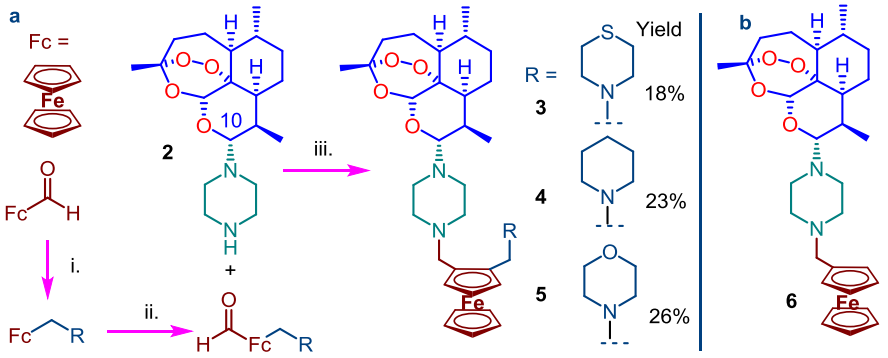
The use of artemisinin based combination therapies (ACTs), currently the most effective for treatment of malaria, is under threat. ACTs are becoming less effective with resistance being reported towards both the artemisinin and non-artemisinin components of ACTs.[1,2] This emphasizes the need for new artemisinin derivatives that cannot be metabolized to the common artemisinin metabolite dihydro-artemisinin implicated in artemisinin resistance.[3-5] This metabolite can be avoided by replacing the oxygen atom attached to C10 of the current clinical artemisinins with an amino group.[6,7] In addition, by incorporating the ferrocene pharmacophore, a mode of action complementary to that of the artemisinin comes into play. Ferrocene (in the Fe^{2+} state) undergoes facile oxidation to ferrocenium (Fe^{3+}), for example by hydrogen peroxide that is thereby reduced to hydroxyl radical in the Fenton reaction.[8] The hydroxyl radical is potently bioactive.[9] In turn, ferrocenium is reduced by NADH and glutathione (GSH) to ferrocene.[10-15] The ensuing redox cycling involving ferrocene and ferrocenium will greatly enhance hydroxyl radical flux. The most successful ferrocene-containing antimalarial drug is ferroquine, based on the chloroquine (CQ) template. The 1,2-disubstituted ferrocene is embedded within the side chain of chloroquine in close proximity to the two amino groups that allows the ferrocene to adopt a uniquely exposed configuration.[16] Ferroquine is able to generate micromolar amounts of hydroxyl radicals from H_2O_2.[17] The ability of ferrocene to generate hydroxyl radical in principle can be exploited further through conjugation to an artemisinin derivative, wherein the latter is able to induce oxidative stress by oxidizing reduced flavin cofactors that normally modulate levels of endogenous thiols required for expunging reactive oxygen species (ROS).[7,18] If redox cycling of the embedded ferrocene in the artemisinin-ferrocene hybrid can indeed maintain the reactive oxygen source, the additional oxidative stress would greatly amplify intracellular damage. Artemisinin-ferrocene hybrids were first prepared some time ago,[19,20] although the original rationalization of their antimalarial activities in terms of binding to ferroprotoporphyrin IX is open to question.[21,22] More recently, artemisinin-acyl ferrocene hybrids prepared from DHA were reported to display antimalarial activities against CQ-

sensitive *Pf* 3D7 ranging from 7.2 - 30.2 nM that were inferior to those of the parent artemisinin; however, the compounds were notably cytotoxic towards multidrug-resistant leukemia cell lines.[23] Similarly an artemisinin acyl ferrocene hybrid intriguingly incorporating the redox-active thymoquinone unit was active against leukemia cell lines, but less active than artemisinin control compounds against malaria.[24] As aminoartemisinins appear to display optimal antimalarial activities,[7] we used the C10 piperazino artemisinin derivative **2**[25] to prepare hybrids bearing a terminal acyl ferrocene or alkyl ferrocene that elicited IC_{50} activities against CQ-sensitive and –resistant *Pf* of 2.9-24.1 nM.[22] We now describe the use of the artemisinin **2** for preparation of new hybrids incorporating the 1,2-disubstituted ferrocene moiety according to the precept for ferroquine outlined above. The methods are presented in Scheme 1a. The terminal alkyl ferrocene hybrid **6** (Scheme 1b) prepared as previously described[22] is included here for comparative purposes.

The synthesis of the ferrocene derivatives was carried out in two steps. Ferrocene carboxaldehyde was submitted to reductive amination with sodium triacetoxyborohydride[26] in the presence of the secondary cyclic amine (thiomorpholine, piperidine and morpholine) to give the corresponding aminoferrocenes in yields above 80%. The aminoferrocene derivatives were then treated with *n*-butyllithium-potassium *tert*-butoxide to give the lithiated intermediate.[27,28] Treatment of the lithiated intermediate with *N*,*N*-dimethyl formamide (DMF) provided the corresponding amino-ferrocenealdehydes in yields after purification of 30%. The foregoing products were then coupled through reductive amination with the 10α-piperazino artemisinin **2** by using sodium triacetoxyborohydride to deliver the amino-artemisinin-1,2-disubstituted ferrocene derivatives (Scheme 1a).



Scheme 1. **a**. Preparation of the amino-artemisinin-1,2-disubstituted ferrocene derivatives i. Ferrocene carboxaldehyde (1.1 eq.), secondary amine (1.2 eq.), sodium triacetoxyborohydride (2 eq.), dichloromethane, N_2, room temperature, 4 h. ii. Aminoferrocene (1 eq.), potassium *tert*-butoxide (0.1 eq.), *n*-BuLi (1.1 eq.), Et_2O, Ar, room temperature, 16 h, then addition of DMF (3 eq.), 4 h. iii. **2** (3 eq.), aminoferrocenealdehyde (1.0 eq.), sodium triacetoxy-borohydride (3 eq.), THF, N_2, room temperature, overnight; **b**. Compound **6** prepared from **2** according to the previously published procedure (ref. 22).

Biological activities for the artemisinin-ferrocene conjugates are given in Tables 1 and 2. *In vitro* antimalarial activities were determined against the asexual blood stages of three *Pf* strains – the drug sensitive NF54, and drug-resistant K1 and W2 strains.[29] The resistance index RI is the ratio of the IC_{50} values of the resistant to sensitive strains IC_{50} K1/IC_{50} NF54 and IC_{50} W2/IC_{50} NF54, and was used as an indication of potential for cross resistance formation for each drug resistant strain (Table 1). The gametocytocidal activities were determined with *Pf* NF54 early and late stage gametocytes at two concentrations, 1 µM and 100 nM (Table 2).[30] The cytotoxicities of the derivatives were evaluated *in vitro* with human embryonic kidney cells Hek293 while anti-tumor screening was carried out with the human malignant melanoma cell line (A375) (Table 1).[31] The selectivity indexes (SI) indicate the selectivity of the compounds towards parasitized cells or cancer cells with respect to the non-proliferating mammalian cell line. Details are given in the Supplementary Material.

The activities of derivatives **4** and **5** against asexual blood stage parasites were better than those of dihydroartemisinin (DHA), artesunate (AS) and artemether (AM) towards the resistant K1 and W2 strains but were less active towards the sensitive NF54 (Table 1). In general, however, activities of the 1,2-disubstituted ferrocene hybrids here are superior to those described previously for the acyl and alkyl ferrocene hybrids;[22] activities of the best of the latter, namely compound **6**, are included for comparison in Table 1. Although the SI value of the morpholino ferrocene derivative **5** indicates that it is more selective towards parasites than mammalian cells, this SI value is lower than that of DHA, possibly indicative of generalized toxicity. In this respect, it is intriguing that the amino artemisinin derivative bearing the morpholino group attached directly to the C10 position (*cf*. compound **2**) exhibited acute toxicity.[6] While compound **3** did not

have the same antimalarial potency as the other derivatives, it was relatively quite active towards the A375 melanoma cell line with respect to non-proliferating mammalian cells. The RI values of ferrocene hybrids **3**-**5** indicate a lower potential for resistance formation than compound **6** and the clinically used DHA, AS and AM.

Table 1. *In vitro* anti-malarial activities against *Pf* asexual blood stage parasites determined by SYBR Green I fluorescence proliferation readout, cytotoxicities and selectivity indices of amino-artemisinin ferrocene derivatives[a]

Compd.	Antimalarial activity IC_{50} (± SEM) nM					Cytotoxicity IC_{50} (μM)		Antitumour IC_{50} (μM)	
	NF54	K1	RI[b]	W2	RI[c]	Hek293	SI[d]	A375	SI[e]
CQ	10.0 (3.0)	154.0 (14.0)	15.4	233.0 (49.0)	23.3	nd	nd	nd	nd
DHA	2.5 (0.1)	1.5 (0.3)	0.6	1.7 (0.2)	0.6	4.0[f]	1593	1.0[f]	0.3
AS	3.0 (0.2)	4.0 (1.0)	1.3	2.4 (0.4)	0.8	nd	nd	nd	nd
AM	1.8 (0.1)	9.0 (2.0)	4.8	7.0 (1.0)	3.8	nd	nd	nd	nd
3	7.5 (2.5)	2.9 (0.3)	0.3	3.4 (1.1)	0.4	43.0	5733	11.0	3.9
4	3.8 (1.4)	1.1 (1.1)	0.2	1.7 (0.6)	0.4	60.0	15424	65.0	0.9
5	3.3 (1.3)	0.8 (0.2)	0.2	1.4 (0.7)	0.4	1.0	300	1.0	1.0
6[g]	4.5 (0.6)	2.7 (0.7)	0.6	3.2 (1.0)	0.7	53.0	11597	19.0	2.7

[a]Data are from at least three independent biological replicates, n=3, each performed in technical triplicates; CQ chloroquine; DHA dihydroartemisinin; AS artesunate; AM artemether; nd not determined; Hek293 human embryonic kidney cells, A375 human malignant melanoma cells; [b]Resistance Index = IC_{50} K1/IC_{50} NF54; [c]Resistance Index = IC_{50} W2/IC_{50} NF54; [d]Selectivity Index = IC_{50} Hek293/ IC_{50} NF54; [e]Selectivity Index = IC_{50} Hek293/ IC_{50} A375; [f]historical cytotoxicity and antitumour values for DHA (refs. 32,33); [g]historical values for compound **6** (ref. 22).

The activities of the ferrocene derivatives against early (stages I-III) and late stage gametocytes (IV-V) are noteworthy. When each were applied at a concentration of 1 μM, they were approximately equipotent with methylene blue and DHA against early stage, but were appreciably more active against late stage gametocytes (Table 2). This is the first time gametocytocidal activity is reported for ferrocene-artemisinin hybrids; this is significant, as activity against late-stage gametocytes in particular portends transmission-blocking capability. For any new drug development programme, it is important that drugs have the ability to block transmission to the mosquito, in particular of resistant parasites.

Table 2. % Inhibition *in vitro* of *Pf* NF54 gametocytes by amino-artemisinin ferrocene derivatives at 1 μM and 100 nM against early (I-III) and late stage (IV-V) gametocytes as determined with the luciferase reporter gene assay.[a]

Compound	Early stage (I-III) gametocytes		Late stage (IV-V) gametocytes	
concentration	1 μM	100 nM	1μM	100 nM
MB	95.0±1.7	nd	57.3±3.96	nd
DHA	97.1±0.5	nd	72.0±6.7	nd
3	95.7±0.33	93.8±0.7	86.5±3.56	84.8±0.5
4	95.8±0.21	95.9±0.3	88.7±2.04	87.0±0.4
5	96.1±0.37	99.1±0.2	88.4±0.96	87.6±0.8

[a]see ref. 30; MB Methylene Blue, DHA dihydroartemisinin; data are from a single biological replicate (n=1) performed in technical triplicates, ±SD.

Overall, the data obtained for these derivatives strongly encourages further investigation of these ferrocene-artemisinin linked derivatives, including the accessible derivative **6** described earlier,[22] with attention to be focussed on conducting assays *in vivo* so as to establish the role of the ferrocene group in carrying cytotoxic mode of action, on improving the synthetic routes, and on generating related derivatives wherein polarity of the amino group attached to the ferrocene is modulated so as to enhance drug uptake.

Acknowledgments

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