PRIMACENES: novel non-cytotoxic primaquine-ferrocene conjugates with *anti-Pneumocystis carinii* activity†

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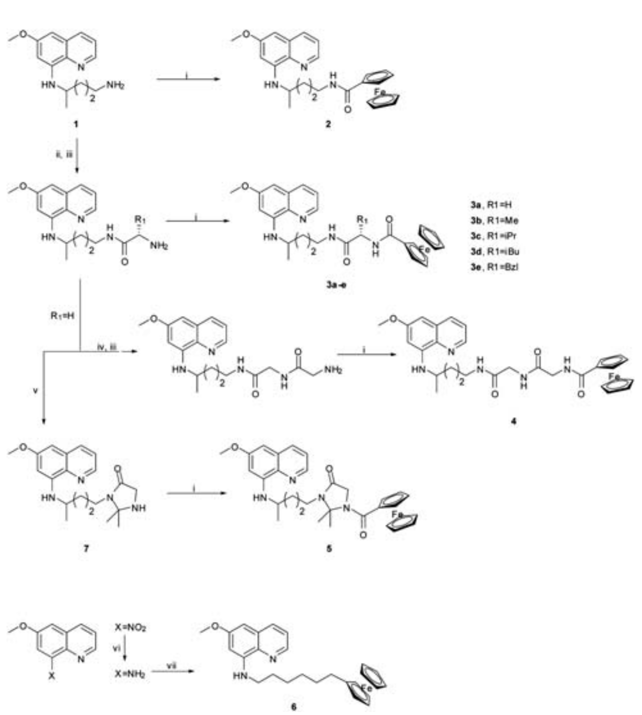
Primacenes, novel ferrocene–primaquine conjugates, were synthesized and screened for their antimalarial and anti-pneumocystis activity. *Primacenes* obtained by coupling primaquine amino acid derivatives to ferrocenoic acid were significantly active against *Pneumocystis carinii* and devoid of cytotoxicity, thus being more selective than the parent drug.

Primaquine (PQ, **1** in Scheme 1) is an antimalarial drug with potent gametocytocidal and modest blood-schizontocidal activity, against which no clinically relevant resistance has been associated to date, as the few PQ-tolerant strains of *Plasmodium vivax* that were so far identified were considered as most likely to present *a priori* differences regarding their sensitivity to PQ than true development of resistance to the drug.[1] PQ is also useful for the treatment of *Pneumocystis* infections[1–4] caused by *Pneumocystis jirovecii* (formerly *P. carinii f. sp.* hominis[2,3]), which is a common cause of pneumonia in immunocompromised individuals and frequently the first serious illness encountered by HIV-infected patients.[4–8] *P. jirovecii* also infects other immunocompromised individuals such as those undergoing cancer therapy and organ and bone marrow transplants.[9] The mechanisms by which PQ exerts its activity against either of these two pathogens are yet unveiled,[1,4,10] but interference with electron transport in the respiratory chain, oxidative stress and impairment of mithocondrial function have been considered.[1,10]

Ferrocene (Fc) has been used in the preparation of bioactive organometallic structures, such as Fc-conjugates of peptides and nucleotides.[11] Fc-conjugates of clinically relevant drugs have also been described as, for instance, arene isosteres in dopamine receptor ligands[12] or organometallic antimalarials derived from 4-aminoquinolines.[13] Indeed, the most emblematic example of the contribution of the Fc moiety to the improvement of a drug is that of Ferroquine (FQ), a chloroquine (CQ) isostere with an Fc-based side chain that is 22 times more potent than CQ itself and now under clinical trials.[14,15] FQ was found to, like CQ, inhibit hemozoin formation[14,15] but, remarkably, it is equally active against CQ-sensitive and CQ-resistant strains of both *P. falciparum*[14,15] and *P. vivax*.[16] This prompted Biot and co-workers to further investigate other mechanisms of action for FQ complementary to a CQ-like interference with hematin



Scheme 1 Synthetic routes to Primacenes **2–6**: (i) 1 eq. ferrocene-carboxylic acid, 1.1 eq. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), 1.1 eq. triethylamine (Et_3N), dry CH_2Cl_2, 90 min in ultra-sound bath (USB) at r.t.; (ii) 1.1 eq. N^{α}-*tert*-butoxycarbonyl-protected amino acid *N*-hydroxy-succinimide ester ($BocAA_1OSu$), 1.1 eq. Et_3N, dry CH_2Cl_2, 24 h at r.t.; (iii) neat CF_3COOH, 30 min at r.t., then drop-wise aq. Na_2CO_3 to pH 11 followed by extraction with $CHCl_3$; (iv) 1.1 eq. BocGlyOSu, dry CH_2Cl_2, 24 h at r.t.; (v) dry propanone (2 eq. per day), 3 days in refluxing CH_3OH with 4 Å molecular sieves; (vi) 5 eq. $SnCl_2$, dropwise conc. HCl, 24 h at r.t., then drop-wise aq. 8 M NaOH to pH 11 followed by extraction with $CHCl_3$; (vii) 1.5 eq. 6-bromo-hexylferrocene, 3.5 h in refluxing Et_3N.

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polymerization.[15,17] These studies allowed important differences to be established between CQ and FQ in terms of basicity and lipophilicity, with FQ being 100 times more lipophilic than CQ at cytosolic pH and to be expectedly 50-fold more concentrated than CQ at the putative acidic pH of the parasite's food vacuole (FV).[15] This led to the assumption that FQ properties could be due to a highly efficient: (i) partition into, and/or hydrophobic collapse with, parasitic membrane lipids; (ii) concentration at the lipid site of hemozoin formation; (iii) maintenance of toxic hematin in the aqueous environment, by creating a barrier at the water-lipid interface.[15] Additionally, the same research group found that under conditions mimicking the environment of the parasite's FV, FQ undergoes a Fenton-like redox reaction that generates highly reactive hydroxyl radicals known both to be rather pernicious towards membrane unsaturated fatty acids, and to promote chain reactions through peroxidation products.[17] Such redox behaviour, which cannot be reproduced by CQ under similar conditions, possibly underlies the distinctive properties of FQ, not only concerning its blood-schizontocidal potency but also, and especially, its apparent lack of propensity to rapidly induce parasite resistance.[15–17]

The above findings have sprang a number of research studies where Fc was used to enhance the antimalarial activity of fluoroquinolones like ciprofloxacin,[18] or to create novel dual-action hybrid drugs with highly promising results.[19–22] In this connection, we hypothesized that insertion of the ferrocene core into either PQ or its derivatives might enhance their anti-plasmodial and anti-pneumocystis activities, mainly due to the redox properties of the Fc moiety.

Our previous research has been focused on ***Imidazoquines***, a new class of imidazolidin-4-one peptidomimetic derivatives of PQ that display antimalarial and anti-pneumocystis activity while being resistant to premature metabolic inactivation by oxidative deamination.[23–31] Thus, we have prepared a first generation of ferrocene–PQ conjugates (**2–5**) that we have called the ***Primacenes***, by coupling ferrocenecarboxylic acid to amino groups present in PQ-based scaffolds (Scheme 1). We have also synthesized structure **6**, a flexible ferrocene derivative of 8-amino-6-methoxyquinoline (MAQ), the heteroaromatic core of PQ (Scheme 1). To the best of our knowledge, there is no precedent in the literature concerning the preparation and study of any kind of ferrocene–primaquine conjugates. Detailed procedures on compound synthesis, chromatographic and spectroscopic data as well as *in vitro* biological evaluation against *P. falciparum* and *P. carinii* are given in the ESI.†

Table 1 summarizes *in vitro* data concerning the antimalarial and anti-pneumocystis activity of the first set of nine primacenes prepared. The table also includes data on the cytotoxicity of the four most active compounds against *P. carinii*, on A549 human lung fibroblast carcinoma and L2 rat lung epithelial cell lines. All assays were run in duplicate.

An immediately eye-catching observation is that, while ferrocenoylation of PQ and of its derivatives, including of *Imidazoquine* **7** (precursor of *Primacene* **5**), is generally detrimental for blood-schizontocidal activity against the CQ-resistant *P. falciparum* strain W2, the same does not apply to the compounds' anti-*Pneumocystis* activity. Thus, all tested compounds, except for the parent drug PQ (**1**) and for the PQ–Leucine–Ferrocene conjugate (**3d**), had IC_{50} over 10 μM against *P. falciparum*. Artemisinin, a potent blood-schizontocidal, was also included in the screening as a comparator, and displayed an IC_{50} of 6.06 nM.

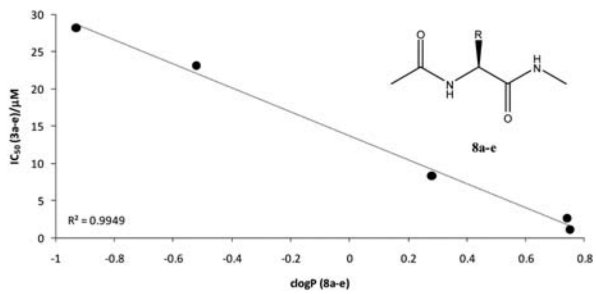

Fig. 1 Linear correlation found between IC_{50} values of Primacenes **3a–e** and clogP values estimated (see Table 1) for model amides **8a–e**.

Table 1 Cytotoxicity, anti-*Pneumocystis* activity and anti-*Plasmodium* activity of compounds **1–7**

Compound	Activity against blood-stage *P.falciparum* W2[a] IC_{50} in μM	Activity against *P. carinii* (72 h) IC_{50} in μM/μg mL^{-1}[d]	Cytotoxicity IC_{50} in μM/μg mL^{-1}: A549 cells	Cytotoxicity IC_{50} in μM/μg mL^{-1}: L2 cells	clogP for model structures **8a–e**[e]
1	3.3[b]	**1.77 (0.46)**	**84.6 (22.0)**	**83.9 (21.8)**	—
2	>10	(>100)	ND		—
3a	>10	28.2 (14.9)	ND		−0.93
3b	>10	23.1 (13.2)	>175 (>100)		−0.52
3c	>10	8.33 (4.52)	ND		0.28
3d	8.33	2.69 (1.57)	>171 (>100)		0.74
3e	>10	**1.15 (0.71)**	**>162 (>100)**		0.75
4	>10	46.8 (27.4)	ND		—
5	>10	35.5 (20.2)	ND		—
6	>10	5.76 (2.55)	>226 (>100)		—
7	9.1[c]	23 (8.3)[c]	ND		—

[a] CQ-resistant strain, against which highly active drugs have IC_{50}s at the low nanomolar range [*e.g.*, IC_{50} for artemisinin = 6.06 nM]. [b] Taken from ref. 23 [c] Taken from ref. 19 [d] Drug *anti*-Pneumocystis activity scale: highly active (compounds with an IC_{50} of <0.010 μg ml^{-1}), very marked (IC_{50}s of 0.011 to 0.099 μg ml^{-1}), marked (IC_{50}s from 0.10 to 0.99 μg ml^{-1}), moderate (IC_{50}s from 1.0 to 9.99 μg ml^{-1}), slight (IC_{50}s from 10.0 to 49.9 μg ml^{-1}), and none (*i.e.*, inactive; IC_{50}s of ≥50 μg ml^{-1});[32] [e] Calculated using the OSIRIS Property Explorer (http://www.organic-chemistry.org/prog/peo/); ND, not determined.

In turn, ferrocenoylation was almost never deleterious for anti-*Pneumocystis* activity, whose levels depended on the specific compound: while direct ferrocenoylation of PQ, as in **2**, deleted the marked activity of the parent drug, the presence of an amino acid residue between the PQ moiety and the ferrocene core (**3a–e**) preserved activity, which was higher for more lipophilic structures (*i.e.*, derived from amino acids leucine, **3d**, and phenylalanine, **3e**). Considering clogP values for the series of model amides **8a–e**, which reflect the same amino acid variation as on primacenes **3a–e**, we find a very good linear correlation between IC_{50} (**3a–e**) values and clogP (**8a–e**) values (Fig. 1).

Overall, we found that, on a molar basis, primacene **3e** was the best anti-*Pneumocystis* agent of the set, being even more active than PQ. In addition, neither **3e** nor the other three most active primacenes (**3c**, **3d** and **6**) were cytotoxic against any of the two cell lines used (IC_{50}>100 μg mL^{-1}), in clear contrast with the parent drug (IC_{50} *ca.* 22 μg mL^{-1} in both cases).

Finally, it should be mentioned that the distinct performance of primacenes as blood-schizontocidals *vs.* anti-pneumocystis agents was quite surprising, since our previous findings with PQ derivatives drove us to the assumption that there was a certain degree of correlation between these two types of bioactivity.[27,31]

At the present stage, we believe that the deletion of blood-schizontocidal activity caused by ferrocen(o)ylation of PQ derivatives or of MAQ is due to the fact that the final primacene structures lack a basic amine. The relevance of such a group has been proposed earlier[33] and confirmed by our previous studies with PQ peptide and peptidomimetic (imidazoquine) derivatives.[23–31] To check this hypothesis, we are now working on the synthesis and *in vitro* evaluation of second-generation primacenes bearing basic amino groups.

Concluding remarks

Novel primaquine-ferrocene conjugates, the *Primacenes*, have been prepared and screened for their anti-*Plasmodium* (blood-stage parasites) and anti-*Pneumocystis* activities. Primacenes were seen to be devoid of significant antimalarial action, but one subset was found to be moderately to markedly active against *Pneumocystis carinii*. This subset included one compound, **3e**, that was more active and markedly less cytotoxic than the parent drug, which is an unparalleled finding for PQ-based anti-*Pneumocystis* agents. Also, to the best of our knowledge, there is no precedent in the literature concerning the study of primaquine-ferrocene conjugates.

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