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Novel Potent Metallocenes against Liver Stage Malaria

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Novel conjugates of the antimalarial drug primaquine (compound 1) with ferrocene, named primacenes, have been synthesized and screened for their activities against blood stage and liver stage malaria *in vitro* and host-vector transmission *in vivo*. Both transmission-blocking and blood-schizontocidal activities of the parent drug were conserved only in primacenes bearing a basic aliphatic amine group. Liver stage activity did not require this structural feature, and all metallocenes tested were comparable to or better than primaquine in this regard. Remarkably, the replacement of primaquine's aliphatic chain by hexylferrocene, as in compound 7, led to a ~45-fold-higher level activity against liver stage parasitemia than that of primaquine.

Resistance to antimalarials, especially in *Plasmodium falciparum*, is a serious problem that threatens to undermine the efficacy of even the artemisinin components of new first-line artemisinin-based combination therapies (ACTs) (5, 7, 24). In addition to *P. falciparum*, *Plasmodium vivax* malaria is an increasing source of concern (1, 3, 11, 12, 14, 21, 23, 28, 32, 34). The benign nature once ascribed to *P. vivax* is being questioned, as we have gained an appreciation of the potential for severe and even fatal *P. vivax* malaria (1, 11). Furthermore, *P. vivax* is the plasmodial species most adaptable to temperate climate conditions (3, 14, 21). The biological robustness of *P. vivax* parasites is also reflected in their ability to form hypnozoites, dormant liver forms that can cause relapses after the initial eradication of blood stage parasites (18, 33), a feature shared with *Plasmodium ovale* (3). Importantly, hypnozoites elude the action of almost any available antimalarial drugs, which makes hypnozoite reservoirs a serious obstacle to malaria eradication (33).

Recently, there has been new enthusiasm for malaria eradication. Eradication efforts will be greatly facilitated by the availability of drugs that act against multiple stages of the parasite, including the liver forms of all malaria parasites and the dormant liver hypnozoites of *P. vivax* and *P. ovale* (3, 14, 33). Unfortunately, primaquine (PQ) (compound 1) (Fig. 1) is currently the only clinically available 8-aminoquinoline active against all liver stages of plasmodia (3, 30). PQ also offers activity against gametocytes, thereby blocking transmission to mosquitoes. However, PQ has low oral bioavailability and is hemotoxic, causing hemolytic anemia in glucose-6-phosphate dehydrogenase (G-6PD)-deficient individuals after the primary induction of methemoglobinemia (30). Many PQ derivatives have been developed over the past 2 decades, including tafenoquine and bulaquine, but none, except for bulaquine in India, has yet been approved for clinical use (30). With the aim of minimizing the drawbacks of PQ while conserving its valuable antimalarial properties, we recently developed imidazoquines (Fig. 1, compound 2, where X is H or an amino acid residue), which were shown to be promising leads for novel transmission-blocking antimalarials, given their significant activity in blocking the transmission of *Plasmodium berghei* malaria from mice to mosquitoes and their remarkable stability in physiological media (2, 31). However, imidazoquines are less active than PQ against liver stage parasites (31), which led us to seek alternatives. Previous reports of enhanced antimalarial activity upon the introduction of ferrocene-based moieties (4, 7, 9, 10) led us to synthesize a new set of PQ metallocene derivatives, named primacenes (compounds 3 to 8) (Fig. 1). These were screened for their activities *in vivo* as transmission-blocking agents and *in vitro* against liver stage *P. berghei* malaria parasites. Encouraging results included the discovery of a metallocene with a level of liver stage activity about 45-fold higher than that of the parent drug.

MATERIALS AND METHODS

Chemistry. N^{α}-protected amino acids were purchased from Bachem (Switzerland). Solvents of high quality and thin-layer chromatography (TLC) aluminum foil plates covered with silica 60 F_{254} (0.25 mm) and silica gel 60 (70 to 230 mesh; ASTM) for preparative column chromatography were all obtained from Merck (VWR International, Portugal). When required, solvents were previously dried with preactivated molecular sieves (4 Å), also from Merck. Racemic primaquine bisphosphate (product no. 160393) and all remaining chemicals were obtained from Sigma-Aldrich (Germany).

General synthetic procedures and chromatographic/spectroscopic data were reported elsewhere previously for all compounds (19), except for compounds 4f, 4g, and 8, for which relevant procedures and data are available upon request (www.fc.up.pt/pessoas/pgomes/Matos-et-al_AAC_SuppInfo.pdf).

The purities of all compounds were confirmed to be at least 98%, as determined by high-performance liquid chromatography (HPLC) using a Merck Hitachi Elite LaChrom instrument equipped with an L-2130 pump, an L-2200 autosampler, and an L-2455 diode array detector. Samples were injected onto a Merck Purospher Star RP-18e 125-cm by 4.6-mm (5-μm) column equipped with a Merck Lichrocart precolumn (Merck, Germany). Elution programs (eluent A consisted of H_2O with 0.05% trifluoroacetic acid [TFA]; eluent B consisted of acetonitrile with

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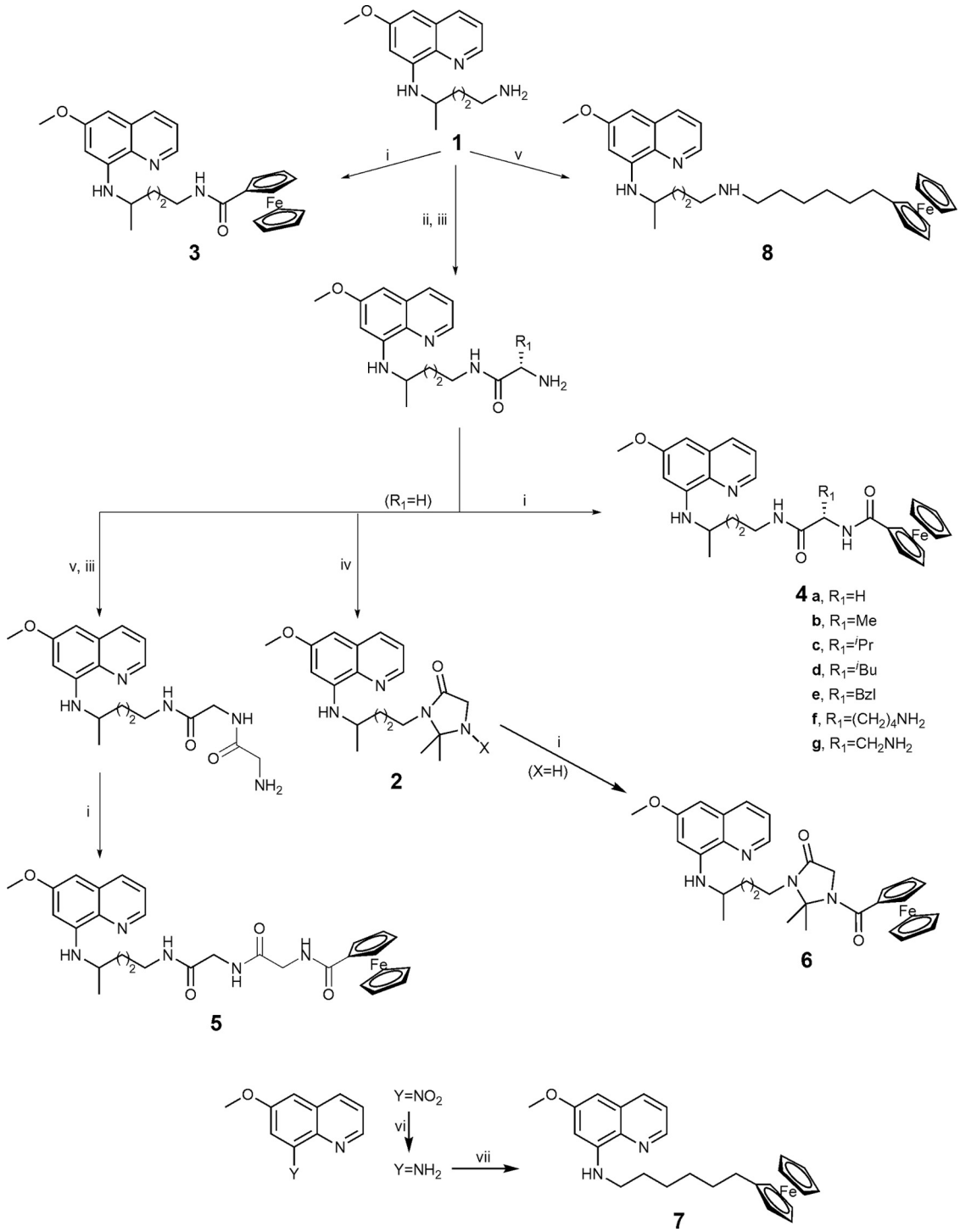

FIG 1 Synthetic routes for primacenes 3 to 8. (i) Ferrocene-carboxylic acid (FcCOOH) (1 molar equivalent, eq.), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl) (1.1 eq.), triethylamine (TEA) (1.1 eq.), and dry dichloromethane (DCM) for 90 min in an ultrasound bath (USB) at room temperature (RT), to give compounds 3, 5, and 6 (15) (a) or 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU) (1 eq.), *N*-ethyl-*N*,*N*-diisopropylamine (DIEA) (2 eq.), and dry DCM at 0°C at RT for 24 h, to give compounds 4a to 4g. (ii) N^{α}-*tert*-Butoxycarbonyl-protected amino acid succinimidyl ester (Boc-AA-OSu) (1.1 eq.), TEA (1.1 eq.), and dry DCM for 24 h at RT. (iii) Neat trifluoroacetic acid (TFA) for 30 min at RT and then 30% aqueous (aq.) Na_2CO_3 dropwise to pH 11, followed by extraction with DCM. (iv) Dry acetone (2 eq. per day), 3-Å molecular sieves, and refluxing methanol for 3 days. (v) Same as described above for step ii but using Boc-Gly-OSu. (vi) $SnCl_2$ (5 eq.) and dropwise concentrated HCl for 24 h at RT and then 8 M aq. NaOH dropwise to pH 11, followed by extraction with DCM (15). (vii) 6-Bromohexylferrocene (0.6 eq.) and TEA at 120°C for 24 h.

0.05% TFA) were as follows: 45 to 60% solvent B in solvent A (0 to 10 min), 60% solvent B in solvent A (10 to 18 min), 60 to 100% solvent B in solvent A (18 to 19 min), and 100% solvent B in solvent A (19 to 23 min) for compound 4 and 70 to 100% solvent B in solvent A (0 to 5 min) and 100% solvent B in solvent A (5 to 15 min) for compound 8. Nuclear magnetic resonance (NMR) spectra of compounds dissolved in either deuterated chloroform ($CDCl_3$) or hexadeuterated dimethyl sulfoxide (DMSO-d6), containing tetramethylsilane (TMS) as an internal reference, were acquired on a Bruker AMX-300 spectrometer. Mass spectrometry (MS) was performed by use of an electrospray ionization-ion trap (ESI-IT) technique with a Finnigan Surveyor LCQ Deca XP Max quadrupole mass spectrometer.

Biology. (i) *In vivo* transmission-blocking activity assays. BALB/c mice were infected by intraperitoneal inoculations of 10^7 erythrocytes parasitized with a green fluorescent protein (GFP)-expressing strain of *P. berghei* ANKA. After 4 days, the presence of gametocytes was observed by the microscopic observation of Giemsa-stained blood films and microgamete exflagellation. For each compound and PQ, mice were randomly separated into groups of three animals, and each group was treated by the intraperitoneal administration of one single dose of each compound and PQ (10 and 50 μmol/kg of body weight in inoculation volumes of 0.1 to 0.2 ml; controls consisted of infected mice given a phosphate-buffered saline [PBS] solution). Two hours after administration, mice were anesthetized and placed on top of individual cages containing ca. 50 glucose-starved *Anopheles stephensi* female mosquitoes, which were allowed to feed for 1 h. After the blood meal, unfed female mosquitoes were removed from each cage. Ten days after the blood meal, mosquitoes were dissected for the microscopic detection of oocysts in midguts.

(ii) *In vitro* blood-schizontocidal activity assays. *In vitro* blood-schizontocidal activity assays were conducted as previously reported (31). Briefly, synchronized ring stage W2 strain *P. falciparum* parasites were cultured with multiple concentrations of test compounds (added from 1,000× stocks in DMSO) in RPMI 1640 medium with 10% human serum or 0.5% Albumax serum substitute. After 48 h of incubation, when control cultures contained new rings, parasites were fixed with 1% formaldehyde in PBS (pH 7.4) for 48 h at room temperature (RT) and then labeled with YOYO-1 (1 nM; Molecular Probes) in 0.1% Triton X-100 in PBS. Parasitemias were determined from dot plots (forward scatter versus fluorescence) acquired on a FACSort flow cytometer using CELLQUEST software (Becton Dickinson). Fifty-percent inhibitory concentrations (IC_{50}s) for growth inhibition were determined from plots of percentages of the control parasitemia relative to the inhibitor concentration by use of GraphPad Prism software. In each case, the goodness of the curve fit was documented by R^2 values of >0.95.

(iii) *In vitro Plasmodium* liver stage infection assays. The inhibition of liver stage infection was determined by measuring the luminescence intensity in Huh-7 cells infected with a firefly luciferase-expressing *P. berghei* line, *Pb*GFP-Luc_{con}, as previously described (27). Huh-7 cells, a human hepatoma cell line, were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, 1% (vol/vol) nonessential amino acids, 1% (vol/vol) penicillin-streptomycin, 1% (vol/vol) glutamine, and 10 mM HEPES (pH 7) and maintained at 37°C with 5% CO_2.

For infection assays, Huh-7 cells (1.2×10^4 cells per well) were seeded into 96-well plates the day before drug treatment and infection. The medium in the cells was replaced with medium containing the appropriate concentration of each compound approximately 1 h prior to infection with sporozoites freshly obtained through the disruption of the salivary glands of infected female *Anopheles stephensi* mosquitoes. The addition of sporozoites was followed by centrifugation at $1,700 \times g$ for 5 min. At 24 h postinfection, the medium was replaced with fresh medium containing the appropriate concentration of each compound. The inhibition of parasite development was measured 48 h after infection. The effect of the compounds on the viability of Huh-7 cells was assessed by the AlamarBlue assay (Invitrogen, United Kingdom) according to the manufacturer's protocol.

RESULTS

Chemistry. Primacenes were prepared, as depicted in Fig. 1, according to previously described methods (19). Detailed synthetic and spectroscopic data on these compounds were previously reported (19), except for primacenes 4f, 4g, and 8, for which relevant data are available upon request. In all cases, target compounds were successfully isolated with a high level of purity, and their structures were conveniently confirmed.

All compounds whose synthesis involved the coupling of racemic primaquine to enantiomerically pure L-amino acids (compounds 4b to 4g) were isolated as mixtures of coeluting diastereomers. All attempts to separate the two diastereomers of compounds 4b to 4g using column chromatography, flash chromatography, and preparative TLC were unsuccessful.

Also, reverse-phase HPLC using Merck RP-8 and RP-18 columns (both 125 and 250 mm) consistently gave single peaks for derivatives 4b to 4g (not shown). Similar observations were reported previously for the N^{α}-aminoacyl-primaquine precursors (2, 31).

Biology. (i) *In vivo* transmission-blocking activity. The abilities of primacenes 3, 4a, 4f, 4g, 6, and 8 to inhibit the sporogonic cycle of plasmodia within the mosquito gut were studied by using a model consisting of *P. berghei* ANKA-GFP-infected BALB/c mice and *Anopheles stephensi* mosquitoes and were compared to that of PQ (2, 31). Activities were measured at two different compound concentrations by determining the percentage of mosquitoes with oocysts (infection rate) and the mean number of oocysts per infected mosquito (oocyst burden) (Table 1). Although this model cannot distinguish gametocytocidal (inhibiting the stage infective for mosquitoes) from sporontocidal (inhibiting mosquito stages) activity, it can clearly elucidate whether a compound is effective at interrupting the transmission of infection from mammalian hosts to mosquitoes. Of all the compounds tested, only compounds 4a, 4f, and 8 showed an ability to decrease the level of parasitemia in mosquitoes. Compound 8 was the only compound, at a concentration of 50 μmol/kg, able to completely inhibit the sporogonic cycle of *Plasmodium*.

(ii) *In vitro* activity against blood stage *P. falciparum*. Compounds 4f and 8 were evaluated for *in vitro* antiplasmodial activity against chloroquine-resistant *P. falciparum* strain W2 (Table 1), as previously described for compounds 3, 4a to 4e, and 7 (19). Unlike what was found previously for compounds 3, 4a to 4e, and 7, which were completely devoid of blood-schizontocidal activity (19), compounds 4f (IC_{50} = 3.48 μM) and 8 (IC_{50} = 1.25 μM) were moderately active, matching or even slightly outdoing the activity of the parent drug (IC_{50} = 3.3 μM).

(iii) Anti-*Plasmodium* liver stage activity. The ability of primacenes to inhibit the development of *P. berghei* schizonts in Huh-7 human hepatoma cells was assessed according to previously reported methods (27), as described in Materials and Methods.

Figure 2 shows the results obtained upon the initial screening of both cell viability and the inhibition of the *P. berghei* infection of Huh-7 cells at two different concentrations. The parent drug, PQ, was included at 15 μM as a positive control. None of the compounds affected cell confluence, as assessed by AlamarBlue fluorescence, except for compound 8 at 2.5 μM or above. At lower concentrations, this compound was not active against liver stage parasites. All other primacenes (compounds 3 to 7) were active

TABLE 1 Antiplasmodial activities of primacenes 3 to 8[e]

Compound	R_1	Charton's ν constants for R_1 (ref. 6)	clogP for model amides 9a–9f[a]	Transmission-blocking activity (infection rate [%]) (mean oocyst burden [no. of oocysts/infected mosquito] ±SEM)[b]		Activity against blood stage *P. falciparum* W2 (IC_{50} [μM])	Activity against liver stage *P. berghei* (IC_{50} [μM])
				10 μmol/kg	50 μmol/kg		
1				45.7 (8.8 ± 3.40)	26.9 (10.0)[d]	3.3[c]	7.50
3				98.3 (46.5 ± 6.10)	88.1 (57.9 ± 8.10)	>10[c]	1.74
4a[f]	H	0	−0.93	33.8 (40.8 ± 10.3)	41.8 (80.2 ± 7.60)	>10[c]	9.33
4b	Me	0.52	−0.52	ND	ND	>10[c]	6.46
4c	^{i}Pr	0.76	0.28	ND	ND	>10[c]	3.09
4d	^{i}Bu	0.98	0.74	ND	ND	8.33[c]	1.90
4e	Bzl	0.70	0.75	ND	ND	>10[c]	2.40
4f	$(CH_2)_4NH_2$		−0.62	93.3 (106 ± 4.40)	42.4 (26.0 ± 4.40)	3.48	6.46
4g	CH_2NH_2			68.2 (62.5 ± 6.70)	73.2 (57.6 ± 4.00)	ND	ND
5				ND	ND	>10[c]	7.41
6				75.6 (30.2 ± 4.2)	86.7 (47.4 ± 4.40)	>10[c]	2.82
7				80.0 (70.7 ± 10.3)	95.8 (85.1 ± 7.70)	>10[c]	0.17
8				65.2 (61.7 ± 6.30)	0.00 (0.00)	1.25	ND

[a] Calculated logarithm of the partition coefficient; calculated by using the OSIRIS Property Explorer (http://www.organic-chemistry.org/prog/peo/).
[b] See the text.
[c] Data taken from reference 19.
[d] Only two data points were available.
[e] Primaquine (compound 1) is also included for comparison. ND, not determined.
[f] In 4a to 4g, R_1 stands for amino acid side chain; H, hydrogen; Me, methyl; ^{i}Pr, isopropyl; ^{i}Bu, isobutyl; Bzl, benzyl.

against liver stage *P. berghei*, in contrast to their behavior as transmission-blocking or blood-schizontocidal agents. Primacenes 4a, 4b, 4f, and 5 presented activities similar to that of PQ, while primacene 4c had a 2.5-fold-higher level of activity, and primacene 7 had a 45-fold-higher level of activity (Table 1).

DISCUSSION

Concerning the ability of primacenes to block host-to-vector malaria transmission, Table 1 shows that only compounds 4a, 4f, and 8 were able to decrease the level of parasitemia in mosquitoes. However, the activity of compound 4a must be seen skeptically, as there was no dose-effect dependency. In turn, both compounds 4f and 8 showed dose-dependent activity, with compound 8 being able to fully impair the parasite's sporogonic cycle at 50 μmol/kg, performing better than the parent drug PQ at the same concentration. The common feature of compounds 4f and 8 is the presence of a basic aliphatic amine group connected to a polymethylene chain: in compound 4f, this corresponds to the R_1 lysine side chain [$—(CH_2)_4NH_2$], whereas in compound 8, it corresponds to the PQ aliphatic amine bound to ferrocene through a $—(CH_2)_6—$ spacer. Transmission-blocking activity was lost if a basic aliphatic amine was absent, as in compounds 3, 6, and 7, or when this amine was not connected to a polymethylene chain, as in compound 4g. Thus, it seems that the presence of a $—(CH_2)_n—$NHR (where n is >1 and R is H or an alkyl group) motif is required for the preservation of transmission-blocking activity in PQ-ferrocene conjugates.

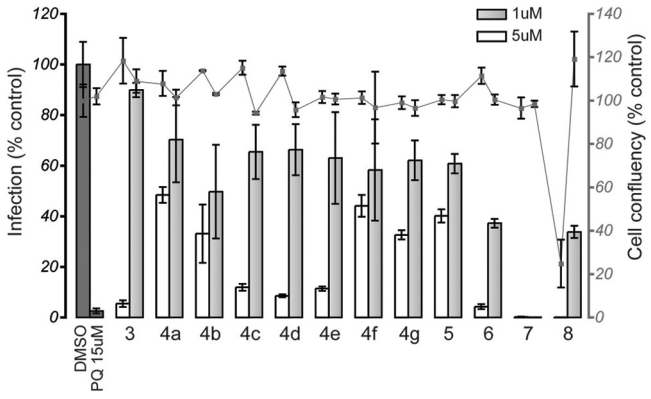

FIG 2 Evaluation of the activities of primacenes 3 to 8 against *P. berghei* liver stages. Shown are data for the screening of anti-infective activity (bars) and toxicity to hepatoma cells (line with square markers).

Interestingly, a previously reported assessment of the activity of primacenes 3, 4a to 4e, and 7 against blood stage *P. falciparum* (chloroquine-resistant strain W2) also revealed that the moderate activity of PQ against blood stage malaria was completely lost in compounds lacking the basic aliphatic amine (19); in turn, the present study (Table 1) showed that such blood-schizontocidal activity was recovered in compounds 4f and 8, again with compound 8 performing slightly better than the parent drug.

Overall, there is strong evidence that the absence of $—(CH_2)_n—$NHR (where n is >1 and R is H or an alkyl group) is detrimental to the activity of primacenes against both host-to-vector transmission and blood stage malaria parasites (Table 1). The relevance for the PQ antimalarial activity of a basic amino group linked to the 8-aminoquinoline core through a carbon chain of 2 to 6 carbons was established long ago (20, 25, 30). In fact, most PQ derivatives that have lately been evaluated in clinical trials as antiparasitics have the parent drug's aliphatic chain either conserved (e.g., tafenoquine) or modified at the amine terminus in a way that reasonably preserves its basicity (e.g., bulaquine or sitamaquine) (30). Many reports from different research groups have demonstrated that the antimalarial activity of PQ is not abolished by the acylation of its primary amine, but in all cases, another basic amine was present elsewhere in the new molecule (2,

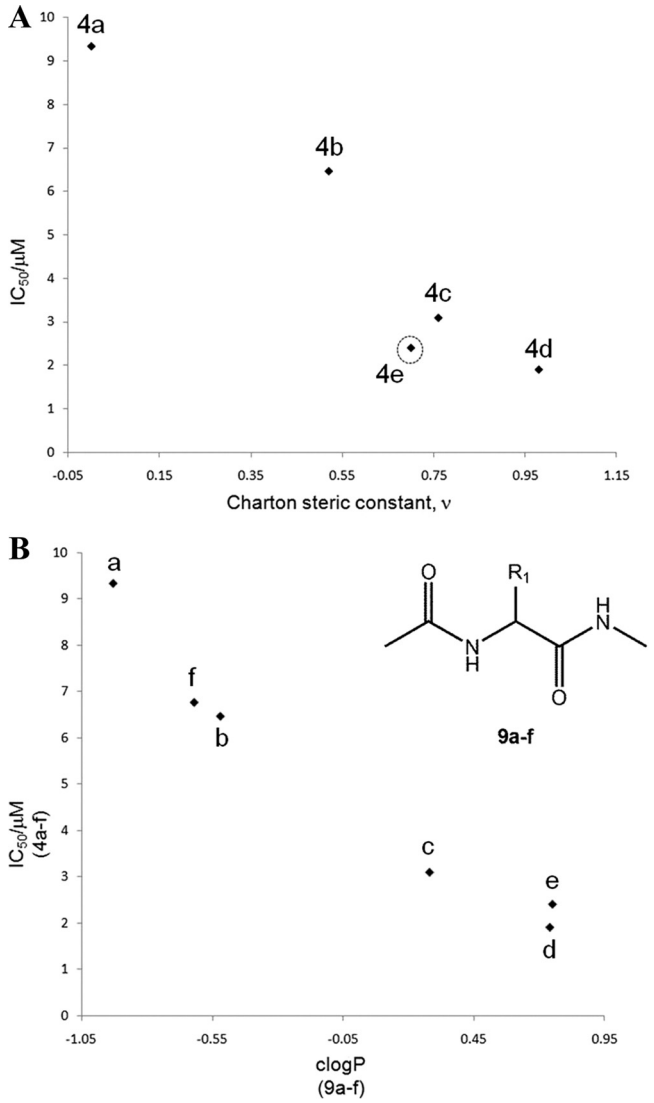

FIG 3 (A) Correlation between IC_{50}s (liver stage activity) (Table 1) and Charton's steric parameters for amino acid side chains (R_1) in compounds 4a to 4e. (B) clogP values estimated for model amides 9a to 9f, whose side chains (R_1) match those of primacenes 4a to 4f.

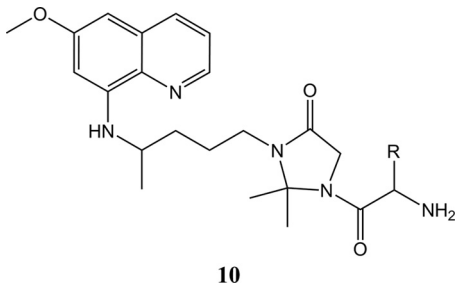

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FIG 4 General structures of imidazoquines.

15–17, 26, 31). Therefore, structural requirements for the activity of primacenes against both host-to-vector transmission and blood stage malaria parasites fully agree with previously established structure-activity relationships (SAR) for PQ. However, any SAR on PQ-based structures must be taken cautiously, as (i) the mode of action of PQ is not yet fully understood and (ii) most SAR reported for PQ-related structures have been taken from *in vitro* blood-schizontocidal activity assays (20, 25, 30) and cannot be used to predict or explain the tissue-schizontocidal activities of novel PQ derivatives. This is dramatically demonstrated by the present work, where all primacenes, except for compound 8, were found to be active against liver stage parasites (Table 1 and Fig. 2). Therefore, the lack of structural requisites found to be crucial for the action of primacenes as blood schizontocidals or transmission blockers was not impeditive to the display of liver stage activity, in some cases at a notably high level. In particular, the treatment of infected Huh-7 cells with compound 7 led to a remarkably potent inhibition of liver stage parasites, as shown in Fig. 2. This exciting finding was confirmed by the subsequent determination of IC_{50}s (Table 1), which substantiated that all primacenes have activity against liver stage parasites equal to or better than that of PQ. Specifically, compared to PQ, primacenes 4a, 4b, 4f, and 5 were comparable in activity; primacene 4c had a 2.5-fold-higher level of activity; and primacene 7 had a 45-fold-higher level of activity (Table 1).

Another interesting observation was that for the subfamily of compounds 4a to 4f, where natural amino acids are used as spacers between PQ and the ferrocenoyl group, the IC_{50}s correlated with the nature of the amino acid side chains (Fig. 3A). These data suggest a near-linear dependency between IC_{50}s and Charton's steric constants, ν (6), for the hydrocarbon side chains present in compounds 4a to 4e (H, Me, ^{i}Pr, ^{t}Bu, and Bzl); however, the phenylalanine derivative compound 4e (Fig. 3A, dashed circle) falls out of this linear correlation, which may indicate that side-chain bulkiness is not the only structural factor affecting activity. In fact, since bulkier hydrocarbon side chains are increasingly lipophilic, compound activity may depend not (or not only) on their size but (also) on their lipophilicity. In support of this hypothesis, clogP values estimated for the model amides 9a to 9f (Fig. 3B), having the same side-chain variation as that of primacenes 4a to 4f, were found to correlate linearly ($r^2 = 0.96$) with IC_{50}s for these compounds (Fig. 3B). It is interesting that we have previously found a similar trend for compounds 4a to 4e in terms of their activities against another PQ-sensitive pathogen, *Pneumocystis jirovecii* (19).

The effect of ferrocene on the liver stage activity of primacenes, compared to the parent drug, is not uniquely demonstrated by the remarkable activity of compound 7: primacene 6 (IC_{50} = 2.82 μM) is not only 2.6-fold more active than PQ (7.5 μM) but also 3.5- to 11-fold more active than similar imidazoquines 10 possessing natural amino acids instead of the ferrocenoyl group (31) (Fig. 4). Thus, it is sensible to assume that iron must be an important player in the tissue-schizontocidal activity displayed by primacenes.

The antimalarial activities of other quinoline-derived metallocenes, as ferroquine, are thought to be multifactorial, including the generation of reactive oxygen species (ROS), like hydroxyl radicals, under the oxidizing conditions of the food vacuole of blood stage parasites; these radicals may be produced by a Fenton-like redox reaction, represented by equation 1 (10):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\cdot} \quad (1)$$

Although the mechanism of action of PQ has not yet been deter-

mined, the drug is known to interfere with electron transport in the respiratory chain, possibly leading to oxidative stress and an impairment of mitochondrial function (30). Therefore, it is reasonable to hypothesize that a ferrocene-based moiety, as in primacenes, might increase the generation of hydroxyl radicals in the liver, reinforcing the ROS-related tissue-schizontocidal activity of PQ. In fact, using a ferrocene-based compound as an iron donor, Moon et al. recently established that iron potentiates acetaminophen-induced oxidative stress and mitochondrial dysfunction in cultured primary mouse hepatocytes, following earlier reports on the increased production of hydroxyl radicals and lipid peroxidation in rats with an iron overload in the liver (22). While this indicates that the presence of iron in primacenes will eventually bring about a higher level of hepatotoxicity than that of the parent drug PQ, it may also explain why primacenes like primacene 7 are so good at killing liver stage malaria parasites. Upcoming toxicological studies will provide the grounds to establish a useful therapeutic window for primacenes; also, bioavailability studies must be undertaken to confirm our belief that these PQ derivatives do not undergo the metabolic oxidative deamination that underlies the low bioavailability of PQ (19, 31). It is possible that a highly active primacene, such as compound 7, will exhibit a significantly improved therapeutic index over that of PQ.

A future definition of therapeutic windows/indices for primacenes is also relevant for what concerns PQ-induced hemotoxicity: methemoglobinemia and hemolytic anemia are dose-limiting side effects of PQ, which hold back its therapeutic use and efficacy. The hemotoxicity of PQ arises from hydroxylated metabolites such as *N*-hydroxy-8-amino-6-methoxy-quinoline (MAQ-NOH) or 5-hydroxylated species, which tautomerize into hemotoxic quinone-imines (30). To avoid the formation of the latter, much effort has been put into the development of PQ derivatives substituted at quinoline's C-5, of which tafenoquine (TFQ) has been the emblematic, but not perfect, example (29, 30). TFQ is a 5-phenoxyl derivative of PQ with a longer half-life and lower toxicity than those of PQ but is not devoid of hemotoxicity *in vivo*, so new 5-aryl analogues are now being explored as TFQ surrogates (29). Also, as for PQ, TFQ is not protected from undergoing oxidative deamination on its aliphatic chain. In primacenes, quinoline's C-5 is not substituted, so hemotoxic 5-hydroxylated metabolites can arise; however, such problem would be minimized if the oral bioavailability of the primacenes is confirmed to be significantly higher than that of PQ or TFQ. These aspects remain to be evaluated for primacenes, but it is interesting that novel bis(8-aminoquinolines) derived from PQ, bearing an unsubstituted quinoline C-5, have a negligible propensity to induce the formation of methemoglobin, together with an improved antimalarial activity over that of PQ (17).

Furthermore, although the molecular masses of some primacenes are above 500 Da, which is a bit high according to known ADMET (adsorption-distribution-metabolism-excretion-toxicity) rules of thumb (13), most promising compounds are close to (compound 8) or even below (compound 7) this value; compared to the above-mentioned promising bis(8-aminoquinolines), whose average molecular masses lie above 600 Da (17), primacenes have "more drug-like" sizes.

In conclusion, we describe novel conjugates of PQ with ferrocene that are superior to PQ against liver stage *Plasmodium* parasites. The presence of an aliphatic basic amine linked to a polymethylene chain was required for transmission-blocking and blood-schizontocidal activity but not for liver stage activity. It remains to be determined whether the liver stage activity of metallocenes as compound 7 will affect *P. vivax* hypnozoites, since a suitable *in vitro* model of these parasite forms for drug screening is still unavailable (8). Nonetheless, the remarkably potent activity of compound 7 makes it a highly promising lead for novel agents against liver stage plasmodia. To the best of our knowledge, these are unprecedented findings that pave the way toward the development of novel primaquine-derived metallocenes as highly potent agents against liver stage malaria.

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