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Synthesis and in vitro activities of ferrocenic aminohydroxynaphthoquinones against *Toxoplasma gondii* and *Plasmodium falciparum*

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Abstract—Fourteen ferrocenyl aminohydroxynaphthoquinones, analogues of atovaquone, were synthesized from the hydroxynaphthoquinone core. These novel atovaquone derivatives were tested for their in vitro activity against two apicomplexan parasites of medical importance, *Toxoplasma gondii* and *Plasmodium falciparum*, including resistant strains to atovaquone (*T. gondii*) and chloroquine (*P. falciparum*). Three of these ferrocenic atovaquone derivatives composed of the hydroxynaphthoquinone core plus an amino-ferrocenic group and an aliphatic chain with 6–8 carbon atoms were found to be significantly active against *T. gondii.* Moreover, these novel compounds were also effective against the atovaquone-resistant strain of *T. gondii* (Ato^R).

1. Introduction

Malaria and toxoplasmosis infections are caused by two related apicomplexan parasites named *Plasmodium falciparum* and *Toxoplasma gondii*. Recently, more attention has been given to *T. gondii* because toxoplasmosis is the most common cause of focal encephalitis or focal central nervous system abnormalities in immunocompromised patients with AIDS or in transplant recipients.[1,2] Although pyrimethamine plus sulfadiazine or clindamycin are the drugs of choice for therapy, they display several toxic effects including bone marrow suppression and allergies in AIDS patients.[3–5] There is currently a need for alternative and effective anti-microbial agents for the treatment of toxoplasmosis. In the case of malaria, it is estimated that 300 million to 500 million of the world's population are infected with Plasmodia which led to about 2 million deaths each year in developing countries.[6]

Even if some effective drugs against malaria are currently used, the present chemotherapies are proving to be inadequate, toxic or are becoming ineffective because of an increase and widespread resistance of *P. falciparum* to chloroquine.[7] After five decades, chloroquine is still a mainstream drug in the fight against malaria but its efficacy is being steadily eroded by the development of resistant Plasmodia.[8,9] Here again, the development of new chemotherapeutic agents is urgently required. It is interesting to note that a number of anti-microbial agents effective in the treatment of malaria have also been proven to cure toxoplasmosis.[10]

Atovaquone is the unique hydroxynaphthoquinone derivative with broad-spectrum activity against numerous protozoan parasites (Fig. 1).[11] For example, it has been shown to be effective in combination with proguanil for the treatment and prevention of malaria[12,13] and it also exerts high level of activity against tachyzoites of *T. gondii* in vitro.[14] However, high concentrations of atovaquone were required to kill bradyzoites within

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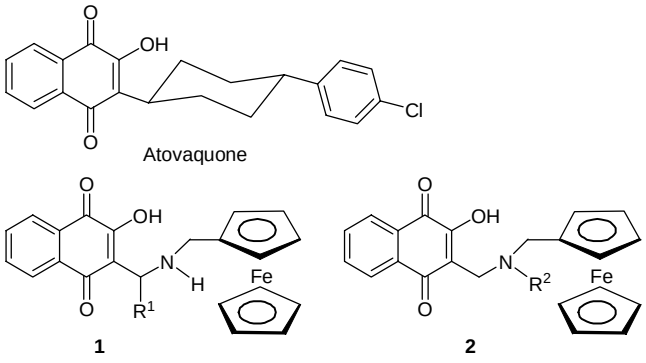


Figure 1. Atovaquone and ferrocenyl derivatives **1** and **2**.

cysts.[14] It appears that its combination with proguanil represents the best treatment at the moment even if the emergence of few atovaquone–proguanil-resistant strains has recently been reported.[15,16] Atovaquone is structurally similar to the coenzyme Q, which is an important component of electron flow in aerobic respiration. The mechanism of action of atovaquone has been most completely elucidated for *Plasmodium*.[11,17,18] It has also been demonstrated that a mechanism operating through the inhibition of ubiquinone binding to cytochrome *b* could explain its mode of action in *T. gondii*.[19]

The use of metal complexes capable of enhancing the activity of biological compounds has become a relevant strategy of research in the communities of both organometallic chemists and biologists. In fact, the introduction of metals can lead to profound changes in drug's biological activities.[20–22] Recently, many different metals have been incorporated into anti-malarial inhibitors. The combination of the chloroquine (CQ) structure for which the 4-aminoquinoline moiety is known to target the parasite with the hydrophobic and cytotoxic ferrocene has led to the design of ferroquine (FQ).[23,24] The high lipophilicity of ferrocene and its electrochemical behaviour render it very attractive for drug design. This strategy, which is based on the incorporation of a ferrocenyl moiety into the 'standard' drug, offers new possibilities in therapeutic applications and reversal of drug resistances.[25,26]

In this work, we report the synthesis of fourteen novel ferrocenic atovaquone derivatives which possess the hydroxynaphthoquinone backbone with different lateral chains (Fig. 1). We have shown that a subset of these compounds inhibits the growth of both sensitive and resistant atovaquone strain of *Toxoplasma gondii* in vitro. In addition, these compounds were also shown to inhibit both chloroquine-sensitive and -resistant strains of *P. falciparum*.

2. Chemistry

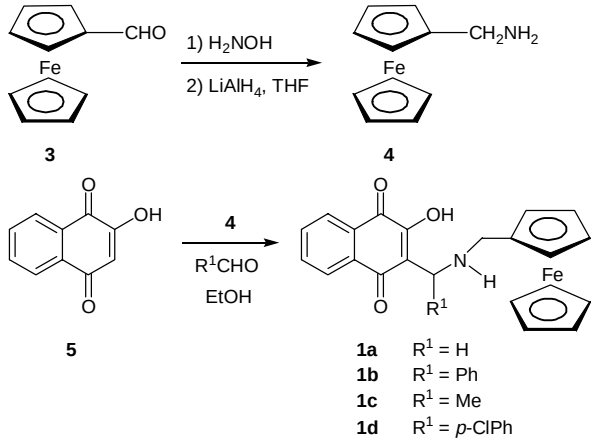
The synthesis of ferrocenyl derivatives **1** and **2** was achieved by a Mannich reaction between 2-hydroxyquinone, ferrocenyl amines and aldehydes.

Ferrocenylmethylamine **4** was first synthesized from ferrocene carboxaldehyde **3** by condensation of hydroxylamine on the aldehyde function followed by a reduction with $LiAlH_4$. The amine **4** was obtained in 93% global yield. The ferrocenyl derivatives **1a–d** were then prepared by condensation of 2-hydroxynaphthoquinone **5** with the corresponding aldehyde in the presence of ferrocenylmethylamine **4** in EtOH (Scheme 1). Compounds **1a–d** were obtained in a range of 60–67% yields.

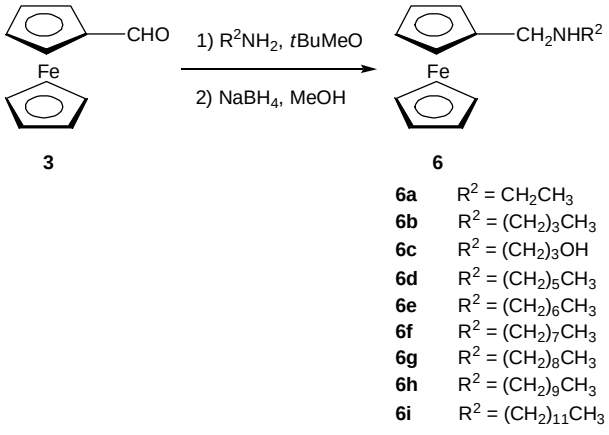
Condensation of commercial amines on ferrocene carboxaldehyde **3** followed by reduction with $NaBH_4$ led to secondary ferrocenyl amines **6a–i** in high global yield (82–93%) (Scheme 2).

The ferrocenyl derivatives **2a–i** were then prepared by condensation of 2-hydroxynaphthoquinone **5** with the formaldehyde in the presence of the corresponding ferrocenylmethylamines **6a–i** in EtOH. Compounds **2a–i** were obtained in 64–77% yield (Scheme 3).

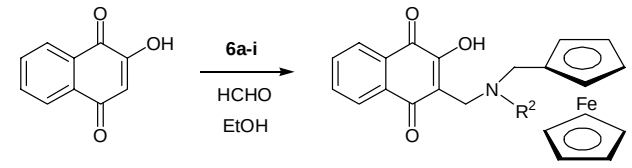
Following the procedure described in the literature,[23] the diamine **7** was prepared from commercially available *N*,*N*-dimethylaminomethylferrocene. The treatment of compound **7** with 2-hydroxynaphthoquinone in the



Scheme 1. Synthesis of ferrocenyl compounds **1a–d**.



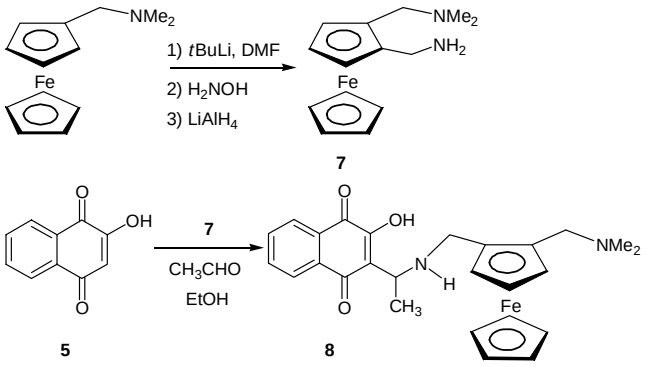
Scheme 2. Synthesis of ferrocenyl amines **6a–i**.



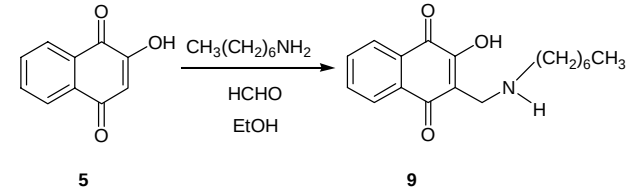
5 2

2a	$R^2 = CH_2CH_3$
2b	$R^2 = (CH_2)_3CH_3$
2c	$R^2 = (CH_2)_3OH$
2d	$R^2 = (CH_2)_5CH_3$
2e	$R^2 = (CH_2)_6CH_3$
2f	$R^2 = (CH_2)_7CH_3$
2g	$R^2 = (CH_2)_8CH_3$
2h	$R^2 = (CH_2)_9CH_3$
2i	$R^2 = (CH_2)_{11}CH_3$

Scheme 3. Synthesis of ferrocenyl compounds **2a–i**.



Scheme 4. Synthesis of ferrocenyl compounds **7** and **8**.



Scheme 5. Synthesis of aminonaphthoquinone **9**.

presence of acetaldehyde afforded the compound **8** as a mixture of diastereomers in 55/45 ratio (92% yield) which can not be separated by silica gel column chromatography (Scheme 4).

Compound **9**, which has not ferrocenyl group on the amino function, was obtained in 70% yield using a direct condensation of *n*-heptylamine on 2-hydroxynaphthoquinone in the presence of formaldehyde (Scheme 5).

3. Biological results and discussion

3.1. In vitro activity of ferrocenic derivatives of atovaquone on *T. gondii*

The 14 novel ferrocenic atovaquone derivatives generated by replacement of the lateral chain of hydroxynaphthoquinone core by either hydrophilic or hydrophobic groups (see above) were tested for their abilities to inhibit *T. gondii* growth. For this purpose, intracellular parasites (tachyzoites) of PLK strain were incubated with different concentrations of drugs ranging from 0.05 to 50 μM. Inhibition of parasite growth was monitored by measuring the specific incorporation of [^{3}H]uracil in the parasite's nucleic acids.[27] Among the 14 compounds tested, three drugs, named **2d**, **2e** and **2f**, showed significant and reproducible anti-parasitic effects (Table 1). Identical results were obtained using a second strain of *T. gondii* named 76K (data not shown). The three compounds comprised the hydroxynaphthoquinone core with a modified ferrocenic lateral chain composed of 6, 7 and 8 carbons, respectively. Cytotoxicity testing of these three most active compounds showed that they exhibit selective activity against intracellular *T. gondii*. In other words, they displayed no detectable inhibitory effects on the host cells (human foreskin fibroblasts) using [^{3}H]hypoxanthine labelling of these host cells in the presence of these drugs (data not shown). The remaining eleven compounds showed no significant activity or they only inhibited the parasite growth at higher drug concentrations that also killed the host cells. However, the IC_{50} of the three active compounds was three- to sixfold higher than that of the atovaquone, suggesting that the replacement of the parental lateral chain of atovaquone by the ferrocenic derivatives gave rise to a lower parasitic activity. Nevertheless, we discovered that these compounds were equally active on the *T. gondii* atovaquone-resistant strain (ATO^R), which has been chemically generated in vitro.[19] It has been demonstrated that mutations present within the cytochrome *b* gene of the ATO^R strain represent alterations in the ubiquinol-binding pocket (Qo domain). That this atovaquone-resistant ATO^R strain is sensitive to these three ferrocenic atovaquone analogues indicates that the mode of action of these novel drugs is different from that of the atovaquone alone. Thus, it is suggested that these atovaquone derivatives may not interfere with the electron transport at the cytochrome *bc*1 complex as previously shown for *T. gondii*[19] and *P. falciparum*.[18]

Table 1. Biological activities of the ferrocenyl aminohydroxynaphthoquinones **2d**, **2e**, and **2f** on *Toxoplasma gondii* and *Plasmodium falciparum*[a]

Compound	Anti-toxoplasma activity (IC_{50}, μM)		Anti-malarial activity (IC_{50}, μM)	
	PLK strain	ATO strain	3D7 strain	Dd2 strain
Atovaquone	0.5 ± 0.1	15 ± 2	0.6 ± 0.2	0.7 ± 0.35
2d	1.2 ± 0.37	1.4 ± 0.27	5 ± 0.4	2.5 ± 0.3
2e	2.1 ± 0.5	1.1 ± 0.35	2.5 ± 0.3	5.0 ± 0.4
2f	3.0 ± 0.4	1.2 ± 0.15	6.25 ± 1.5	6.0 ± 1.25

[a] The effect of 17 novel ferrocenic atovaquone derivatives was compared to that of atovaquone on the PLK wild-type *T. gondii* strain, an atovaquone (ATO^R) derived from the parental PLK strain, the chloroquine-sensitive (3D7) and -resistant (Dd2) strains of *P. falciparum*. Each drug has been tested at doses from 0.5 to 50 μM. IC_{50} values ($P < 0.05$) correspond to means ± SD from three independent experiments and each drug concentration has been tested in triplicate.

3.2. Effect of ferrocenic atovaquone analogues on *Plasmodium falciparum*

The effect of the three novel atovaquone analogues, **2d**, **2e** and **2f** that showed significant anti-parasitic activities against *T. gondii*, were evaluated on another related apicomplexan parasite, *P. falciparum*. The chloroquine-sensitive strain (3D7) and chloroquine-resistant strain (Dd2) of *P. falciparum* were tested for drug susceptibility by measuring the incorporation of [^{3}H]hypoxanthine of intra-erythrocytic stages grown under asynchronous culture conditions.[27] The treatment of *P. falciparum* with drug concentrations in the range from 0.5 to 50 μM led to a sharp decline of the red blood cells infected by the parasites irrespective of its chloroquine resistance or sensitivity state. For the chloroquine-sensitive 3D7 strain, the IC_{50} values of the compounds **2d**, **2e** and **2f** were estimated at 5.0, 2.5 and 6.25 μM, while their IC_{50} in the chloroquine-resistant strain (Dd2) were shown to be 2.5, 5.0 and 6.0 μM, respectively (Table 1). There is a good correlation between the decreased incorporation of [^{3}H]hypoxanthine in the drug-treated *P. falciparum* and the sharpness decline of the parasitemia as described above using the classical Giemsa-staining of thin red blood smears and counting by light microscopy. We found that the inhibitory effect of these novel ferrocenic atovaquone derivatives on both *T. gondii* and *P. falciparum* was dose dependently significant for the three drugs **2d**, **2e** and **2f**. Importantly, their similar inhibitory activities on *T. gondii* atovaquone and *P. falciparum* chloroquine-resistant strains suggest that these novel compounds could be considered as potential alternative chemotherapeutic agents against malaria and toxoplasmosis.

3.3. Discussion

In this study, we have evaluated the effect of ferrocenic hydroxynaphthoquinone derivatives on two apicomplexan parasites *T. gondii* and *P. falciparum*. Atovaquone has been described as an active inhibitor against the tachyzoites of *T. gondii* in vitro.[14,19] This drug is currently used to treat acute toxoplasmosis in humans. In addition, atovaquone is currently used with proguanil as synergistic partner for malarial treatment. The implications and the mechanisms of developing resistance to atovaquone are not yet fully understood. To anticipate the emergence of such resistant strains in the field, it may be important to develop new atovaquone derivatives based on the strategy previously described by our laboratory, which has successfully synthesized novel ferrocenic chloroquine analogues.[23,24] We have now synthesized a series of compounds derived from atovaquone and tested them for their potent activity against atovaquone-sensitive or -resistant (ATO^R) strains of *T. gondii*. The mutations present within the cytochrome *b* gene of the ATO^R strain are responsible for alterations in the ubiquinol-binding pocket (Qo domain) which lead to atovaquone resistance in *T. gondii*.[19] Therefore, it can be hypothesized that the three active ferrocenic atovaquone analogues identified here have modes of action which are different from that of atovaquone alone. We do not have in our hands a *P. falciparum* strain that is resistant to atovaquone. However, a previous work, which has generated a laboratory strain of *P. falciparum* resistant to atovaquone, has demonstrated that cytochrome *bc*1 complex is also involved in the mechanism by which this parasite can also become resistant to this drug. Thus, it can be expected that the atovaquone derivatives tested here can function in both *T. gondii* and *P. falciparum* by similar mechanisms. Most importantly, we have been able to show that these atovaquone derivatives can also inhibit both chloroquine-sensitive and -resistant strains of *P. falciparum*.

It is interesting to note that the atovaquone derivatives that display significant anti-parasitic activity are composed of a hydroxynaphthoquinone core with lateral chain of an amino-ferrocenic group and aliphatic side chain comprised between 6, 7 and 8 carbons. The compounds with shorter or longer lateral carbon chains than 6 and 8 carbons show no significant biological activity under our experimental conditions. As control, we have tested the *N*,*N*-dimethylaminomethylferrocene and the 2-hydroxy-1,4-naphthoquinone alone. In addition, compound **9** (Scheme 5, Table 1), which has an identical structure with that of the best anti-parasitic compound **2e**, except that it lacks the ferrocenic moiety, has also been evaluated. These latter three compounds were tested individually or as a mixed solution. The data demonstrated that none of these compounds has anti-parasitic activities when tested alone or as a mixed solution, suggesting that a covalent linkage of the three partners is required for proper biological activity.

4. Conclusion

Because malaria and toxoplasmosis are caused by two related apicomplexan parasites and a number of antimicrobial agents are effective in the treatment of both infections, we have obtained three novel atovaquone derivatives showing some significant activities against both *T. gondii* and *P. falciparum* in vitro. The data reported herein provide a foundation for the generation of new atovaquone derivatives with better IC_{50} values using other lateral chemical moieties. Since chloroquine resistance is becoming more widespread in the developing countries that suffer from malaria, the lead compounds identified during this study can be considered as alternative and potential chemotherapy for both malaria and toxoplasmosis.

5. Experimental

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC300 spectrometer using tetramethylsilane (TMS) as the internal standard and $CDCl_3$, MeOH-d_4 or DMSO-d_6 as the solvents. MS-MALDI TOF spectra were obtained using a Vision 2000 time-of-flight instrument (Finnigan MAT, Bremen, Germany) equipped with a nitrogen laser operating at a wavelength of 337 nm. The matrix used was trihydroxyacetophenone (thap). HRMS were performed on a JEOL JMS-700m Station mass spectrometer. Thin layer chromatography

(TLC) was carried out on aluminium-baked Merck silica gel 60 F254. Column chromatography was performed on silica gel (35–70 mesh). Melting points were determined with a Kofler apparatus and are uncorrected.

6. Synthesis

6.1. Ferrocenylmethylamine (4)

A mixture of ferrocene carboxaldehyde (1.00 g, 4.67 mmol), sodium hydroxide (1.10 g, 27.5 mmol) and hydroxylamine chlorohydrate (0.65 g, 9.3 mmol) in EtOH (50 mL) was stirred at reflux for 3 h. After cooling, the mixture was hydrolysed and extracted by CH_2Cl_2 (3× 100 mL). The organic layer was dried over Na_2SO_4 and the solvent was evaporated under vacuum pressure to give ferrocenylcarboxaldehyde oxime as an orange solid (1.05 g, 98%). Mp 106 °C. ^{1}H NMR ($CDCl_3$): δ 8.00 (1H, s, CH=N), 4.54 (2H, m, C_p), 4.35 (2H, m, C_p), 4.22 (5H, s, $C_{p'}$). ^{13}C NMR ($CDCl_3$): δ 50.0, 70.0, 69.0, 67.0.

Ferrocenecarbaldehyde oxime (0.400 g, 1.75 mmol) in anhydrous THF (10 mL) was added dropwise to $LiAlH_4$ (0.351 g, 9.24 mmol) in anhydrous THF (10 mL). The mixture was stirred under reflux for 6 h. After cooling, the mixture was hydrolysed and extracted by diethyl ether (3× 100 mL). The organic layer was dried over Na_2SO_4 and evaporated under vacuum pressure to give **4** as an orange oil (0.357 g, 95%). ^{1}H NMR ($CDCl_3$): δ 4.16 (2H, m, C_p), 4.14 (5H, s, $C_{p'}$), 4.11 (2H, m, C_p), 3.52 (2H, s, CH_2). ^{13}C NMR ($CDCl_3$): δ 90.8, 68.3, 67.7, 67.1, 41.1.

6.2. General procedure for synthesis of 2-hydroxynaphthoquinone derivatives 1a–d

2-Hydroxynaphthoquinone (0.5 mmol) and *N*-ferrocenylmethylamine **4** (0.5 mmol) were dissolved in ethanol (10 mL). The mixture was heated at 45 °C for 5 min. The aldehyde (0.6 mmol) was then added with stirring vigorously. The product occurred as a red purple precipitate in 1 h. After 3 h, the mixture was filtered, washed with ethanol and then with diethyl ether. After evaporation of solvent, **1a**–**d** were obtained as a red purple solid.

6.3. 3-(*N*-(Ferrocenylmethyl)aminomethyl)-2-hydroxynaphthoquinone (1a)

Yield = 60%. Mp 196 °C. ^{1}H NMR (DMSO): δ 7.92 (1H, d, J = 7.4 Hz, ArH), 7.81 (1H, d, J = 6.9 Hz, ArH), 7.67 (1H, t, J = 7.2 Hz, ArH), 7.59 (1H, t, J = 7.2 Hz, ArH), 4.44 (2H, s, NaphtCH_2N), 4.23–4.11 (4H, m, C_p), 4.19 (5H, s, $C_{p'}$), 3.87 (2H, s, FcCH_2). HRMS, calcd for $C_{22}H_{19}FeNO_3$: 401.0714 [M]. Found: 401,0711.

6.4. 3-(*N*-(Ferrocenylmethyl)aminobenzyl)-2-hydroxynaphthoquinone (1b)

Yield = 60%. Mp 226 °C. ^{1}H NMR (DMSO, 300 MHz): δ = 7.89 (1H, d, J = 7.2 Hz, ArH), 7.81 (1H, d, J = 7.8 Hz, ArH), 7.69 (1H, t, J = 7.9 Hz, ArH), 7.57 (1H, t, J = 6.8 Hz, ArH), 7.48 (2H, d, J = 6.9 Hz, ArH), 7.29 (3H, m, ArH), 5.40 (1H, s, NaphtCHN), 4.25–4.20 (4H, m, C_p), 4.15 (5H, s, $C_{p'}$), 3.82 (2H, s, FcCH_2). HRMS, calcd for $C_{28}H_{24}FeNO_3$: 478.1105 [M+H$^+$]. Found: 478.1100.

6.5. 3-(*N*-(Ferrocenylmethyl)-1-aminoethyl)-2-hydroxynaphthoquinone (1c)

Yield = 67%. Mp 178 °C. ^{1}H NMR (DMSO): δ 7.92 (1H, d, J = 7.4 Hz, ArH), 7.83 (1H, d, J = 7.3 Hz, ArH), 7.71 (1H, t, J = 7.1 Hz, ArH), 7.58 (1H, t, J = 7.2 Hz, ArH), 4.43 (1H, q, J = 6.6 Hz, NaphtCHN), 4.35 (2H, d, J = 5.1 Hz, C_p), 4.26 (2H, d, J = 4.8 Hz, C_p), 4.14 (5H, s, $C_{p'}$), 3.76 (2H, s, FcCH_2), 1.35 (3H, d, J = 6.6 Hz, CH_3). HRMS, calcd for $C_{23}H_{21}FeNO_3$: 416.0949 [M+H$^+$]. Found: 416.0950.

6.6. 3-(*N*-(Ferrocenylmethyl)amino-*p*-chlorobenzyl)-2-hydroxynaphthoquinone (1d)

Yield = 61%. Mp 190 °C. ^{1}H NMR (DMSO): δ 7.92 (1H, d, J = 7.4 Hz, ArH), 7.80 (1H, d, J = 6.9 Hz, ArH), 7.70 (1H, t, J = 7.2 Hz, ArH), 7.59 (1H, t, J = 7.2 Hz, ArH), 7.50 (2H, d, J = 8.4 Hz, ArHCl), 7.38 (2H, d, J = 8.4 Hz, ArHCl), 5.42 (1H, s, NaphtCHN), 4.25–4.20 (4H, m, C_p), 4.14 (5H, s, $C_{p'}$), 3.87 (2H, s, FcCH_2). HRMS, calcd for $C_{28}H_{22}{}^{35}ClFeNO_3$: 512.0716 [M+H$^+$], Found: 512.0721, calcd for $C_{28}H_{22}{}^{37}ClFeNO_3$: 514.0686 [M+H$^+$]. Found: 514.0701.

6.7. *N*-1-Ethyl-ferrocenylmethylamine (6a)

Ferrocene carbaldehyde (200 mg, 0.934 mmol) was dissolved in dried diethyl ether (15 mL) at room temperature under nitrogen. Ethylamine (2 mL, 2 mmol) in THF (2 M) was added dropwise. The solution was stirred at room temperature for 1 h. The solvent was then evaporated and the collected oil was dissolved in MeOH (20 mL). The reaction mixture was cooled at 0 °C and $NaBH_4$ (250 mg, 0.71 mmol) was slowly added. After 1 h, the mixture was hydrolysed with water (20 mL) and extracted with diethy lether (2× 30 mL). The organic layer was dried over Na_2SO_4 and evaporated to give **6a** as a yellow oil (168 mg, 74%). ^{1}H NMR ($CDCl_3$): δ 4.16 (2H, m, C_p), 4.08 (7H, s, $C_{p'} + C_p$), 3.50 (2H, s, FcCH_2), 2.65 (2H, q, J = 7.1 Hz, NCH_2), 1.09 (3H, t, J = 7.1 Hz, CH_3). ^{13}C NMR ($CDCl_3$): δ 86.8, 68.4, 68.3, 67.7, 48.8, 43.7, 15.2.

6.8. General procedure for synthesis of *N*-alkyl-ferrocenylmethylamine 6b–i

A mixture of ferrocene carbaldehyde (428 mg, 2.0 mmol), *n*-alkylamine (4.0 mmol) and molecular sieves (**6g**) in *t*-butylmethylether (20 mL) was stirred at room temperature. After 4 h, MeOH (10 mL) and $NaBH_4$ (110 mg, 2.9 mmol) were added to the reaction mixture. After 15 min, the mixture was filtered and the solvent was evaporated. A mixture of water (10 mL) and diethyl ether (10 mL) was added to the crude product. After extraction, the organic layer was dried over Na_2SO_4, evaporated to give ferrocenylmethylamine **6b**–**i**.

6.9. *N*-1-Butyl-ferrocenylmethylamine (6b)

Yellow oil. Yield = 95%. ^{1}H NMR ($CDCl_3$): δ 4.15 (2H, m, C_p), 4.09 (7H, m, $Cp' + C_p$), 3.48 (2H, s, $FcCH_2$), 2.61 (2H, t, J = 6.9 Hz, NCH_2), 1.46 (2H, q, J = 6.9 Hz, CH_2), 1.33 (2H, sept, J = 7.3 Hz, CH_2), 0.90 (3H, t, J = 7.3 Hz, CH_3). ^{13}C NMR ($CDCl_3$): δ 87.0, 68.4, 68.3, 67.7, 49.4, 49.0, 32.2, 20.5, 14.0.

6.10. 3-(*N*-Ferrocenylmethylamino)-1-propanol (6c)

Yellow oil. Yield = 82%. ^{1}H NMR ($CDCl_3$): δ 4.14 (2H, m, C_p), 4.11 (7H, m, $C_{p'} + C_p$), 3.79 (2H, m, Cp), 3.79 (2H, t, J = 5.2 Hz, CH_2OH), 3.50 (2H, s, $FcCH_2$), 2.89 (2H, t, J = 5.6 Hz, NCH_2), 1.70 (2H, q, J = 5.4 Hz, CH_2). ^{13}C NMR ($CDCl_3$): δ 86.1, 68.4, 68.2, 67.8, 65.8, 49.5, 48.7, 30.5.

6.11. *N*-1-Hexyl-ferrocenylmethylamine (6d)

Yellow oil. Yield = 82%. ^{1}H NMR ($CDCl_3$): δ 4.21 (2H, m, C_p), 4.12 (5H, s, $C_{p'}$), 4.11 (2H, m, C_p), 3.55 (2H, s, $FcCH_2$), 2.62 (2H, t, J = 7.4 Hz, NCH_2), 1.50 (2H, m, CH_2), 1.28 (6H, m, CH_2), 0.87 (3H, t, J = 6.8 Hz, CH_3). ^{13}C NMR ($CDCl_3$): δ 85.3, 68.7, 68.4, 67.9, 48.9, 48.5, 31.7, 29.3, 26.9, 22.6, 14.0.

6.12. *N*-1-Heptyl-ferrocenylmethylamine (6e)

Yellow oil. Yield = 85%. ^{1}H NMR ($CDCl_3$): δ 4.18 (2H, m, C_p), 4.11 (5H, s, $C_{p'}$), 4.10 (2H, m, C_p), 3.49 (2H, s, $FcCH_2$), 2.60 (2H, t, J = 7.4 Hz, NCH_2), 1.48 (2H, m, CH_2), 1.27 (8H, m, CH_2), 0.87 (3H, t, J = 6.8 Hz, CH_3). ^{13}C NMR ($CDCl_3$): δ 86.9, 68.4, 68.3, 67.7, 49.5, 48.9, 31.8, 30.0, 29.2, 27.3, 22.6, 14.1.

6.13. *N*-1-Octyl-ferrocenylmethylamine (6f)

Yellow oil. Yield = 83%. ^{1}H NMR ($CDCl_3$): δ 4.19 (2H, m, C_p), 4.12 (5H, s, $C_{p'}$), 4.10 (2H, m, C_p), 3.49 (2H, s, $FcCH_2$), 2.60 (2H, t, J = 7.3 Hz, NCH_2), 1.48 (2H, m, CH_2), 1.27 (10H, m, CH_2), 0.87 (3H, t, J = 6.8 Hz, CH_3). ^{13}C NMR ($CDCl_3$): δ 86.9, 68.4, 68.3, 67.7, 49.6, 48.9, 31.8, 30.0, 29.5, 29.2, 27.4, 22.6, 14.1.

6.14. *N*-1-Nonyl-ferrocenylmethylamine (6g)

Yellow oil. Yield = 83%. ^{1}H NMR ($CDCl_3$) δ 4.17 (5H, s, $C_{p'}$), 4.14 (4H, m, C_p), 3.49 (2H, s, $FcCH_2$), 2.60 (2H, t, J = 7.2 Hz, NCH_2), 1.49 (2H, m, CH_2), 1.27 (12H, m, CH_2), 0.87 (3H, t, J = 6.9 Hz, CH_3).

6.15. *N*-1-Decyl-ferrocenylmethylamine (6h)

Yellow oil. Yield = 93%. ^{1}H NMR ($CDCl_3$): δ 4.13 (4H, m, C_p), 4.11 (5H, s, $C_{p'}$), 3.49 (2H, s, $FcCH_2$), 2.59 (2H, t, J = 6.9 Hz, NCH_2), 1.47 (2H, m, CH_2), 1.26 (14H, m, CH_2), 0.87 (3H, t, J = 6.8 Hz, CH_3).

6.16. *N*-1-Dodecyl-ferrocenylmethylamine (6i)

Yellow oil. Yield = 83%. ^{1}H NMR ($CDCl_3$): δ 4.18 (2H, m, C_p), 4.11 (5H, s, $C_{p'}$), 4.10 (2H, m, C_p), 3.49 (2H, s, $FcCH_2$), 2.60 (2H, t, J = 6.9 Hz, NCH_2), 1.47 (2H, m, CH_2), 1.25 (18H, m, CH_2), 0.88 (3H, t, J = 7.1 Hz, CH_3). ^{13}C NMR ($CDCl_3$): δ 86.0, 68.4, 68.3, 67.7, 49.6, 48.9, 31.9, 30.0, 29.6, 29.3, 27.4, 22.7, 14.1.

6.17. Procedure for synthesis 3-(*N*-alkyl-*N*-ferrocenylmethyl)aminomethyl-2-hydroxynaphthoquinone 2a–i

2-Hydroxynaphthoquinone (87 mg, 0.5 mmol) and ferrocenylamine **6a**–**i** (0.5 mmol) were dissolved in ethanol (10 mL). The mixture was heated at 45 °C for 5 min. The formaldehyde (0.6 mmol) was then added with stirring vigorously. The product appeared as a red purple precipitate in 1 h. After 3 h, the mixture was filtered, washed with ethanol and then with diethyl ether. The solvent was then evaporated to give **2a**–**i**.

6.18. 3-(*N*-Ethyl-*N*-(ferrocenylmethyl)aminomethyl)-2-hydroxynaphthoquinone (2a)

Orange solid. Yield = 64%. Mp 194 °C. ^{1}H NMR ($CDCl_3$): δ 8.08 (1H, d, J = 7.6 Hz, ArH), 7.90 (1H, d, J = 7.5 Hz, ArH), 7.64 (1H, t, J = 7.4 Hz, ArH), 7.48 (1H, t, J = 7.5 Hz, ArH), 4.47–4.07 (6H, m, C_p + NaphtCH_2N), 4.27 (2H, s, $FcCH_2$), 4.09 (5H, s, $C_{p'}$), 3.07 (2H, q, J = 7.0 Hz, NCH_2), 1.39 (3H, t, J = 7.0 Hz, CH_3). MS (EI) m/z 430 $(M+H)^+$. HRMS, calcd for $C_{24}H_{24}FeNO_3$: 430.1105 [$M+H^+$]. Found: 430.1115.

6.19. 3-(*N*-1-Butyl-*N*-(ferrocenylmethyl)aminomethyl)-2-hydroxynaphthoquinone (2b)

Orange solid. Yield = 76%. Mp 176 °C. ^{1}H NMR ($CDCl_3$): δ 8.08 (1H, d, J = 7.5 Hz, ArH), 7.97 (1H, d, J = 7.3 Hz, ArH), 7.65 (1H, t, J = 7.2 Hz, ArH), 7.51 (1H, t, J = 7.2 Hz, ArH), 4.49–4.07 (6H, m, C_p + NaphtCH_2N), 4.28 (2H, s, $FcCH_2$), 4.16 (5H, s, $C_{p'}$), 2.93 (2H, m, NCH_2), 1.75 (2H, m, CH_2), 1.37 (2H, sept, J = 7.2 Hz, CH_2), 0.90 (3H, t, J = 7.2 Hz, CH_3). HRMS, calcd for $C_{26}H_{28}FeNO_3$: 458.1418 [$M+H^+$]. Found: 458.1422.

6.20. 3-(*N*-(Ferrocenylmethyl)-*N*-1-(3-hydroxypropyl)-aminomethyl)-2-hydroxynaphthoquinone (2c)

Orange solid. Yield = 70%. Mp 181 °C. ^{1}H NMR (DMSO): δ 7.94 (1H, d, J = 7.6 Hz, ArH), 7.83 (1H, d, J = 7.4 Hz, ArH), 7.70 (1H, t, J = 7.3 Hz, ArH), 7.58 (1H, t, J = 7.4 Hz, ArH), 4.57 (2H, s, NaphtCH_2N), 4.28 (2H, s, C_p), 4.18 (5H, s, $C_{p'}$), 4.11 (2H, s, C_p), 3.96 (2H, s, $FcCH_2$), 3.40 (2H, t, J = 5.8 Hz, CH_2OH), 2.88 (2H, t, J = 7.1 Hz, NCH_2), 1.86 (2H, quint, J = 5.8 Hz, CH_2). HRMS, calcd for $C_{25}H_{25}FeNO_4$: 460.1211 [$M+H^+$]. Found: 460.1205.

6.21. 3-(*N*-(Ferrocenylmethyl)-*N*-1-hexylaminomethyl)-2-hydroxynaphthoquinone (2d)

Orange solid. Yield = 65%. Mp 178 °C. ^{1}H NMR ($CDCl_3$): δ 8.08 (1H, d, J = 7.5 Hz, ArH), 8.00 (1H, d, J = 7.6 Hz, ArH), 7.66 (1H, t, J = 7.3 Hz, ArH), 7.49 (1H, t, J = 7.5 Hz, ArH), 4.55–4.08 (6H, m, C_p + NaphtCH_2N), 4.29 (2H, s, $FcCH_2$), 4.16 (5H, s, $C_{p'}$), 2.90 (2H, m, NCH_2), 1.75 (2H, m, CH_2), 1.26 (6H, m, CH_2), 0.84

(3H, t, $J = 6.8$ Hz, CH_3). ^{13}C NMR ($CDCl_3$): δ 184.4, 881.3, 172.4, 134.5, 133.6, 131.8, 131.0, 126.1, 125.7, 107.1, 73.9, 70.0, 69.0, 52.2, 51.5, 49.8, 31.1, 26.3, 24.3, 22.3, 13.8. HRMS, calcd for $C_{28}H_{31}FeNO_3$: 486.1731 [M+H$^+$]. Found: 486.1737.

6.22. 3-(*N*-(Ferrocenylmethyl)-*N*-1-heptylaminomethyl)-2-hydroxynaphthoquinone (2e)

Orange solid. Yield = 76%. Mp 170 °C. ^1H NMR ($CDCl_3$): δ 8.08 (1H, d, $J = 7.5$ Hz, ArH), 8.00 (1H, d, $J = 7.2$ Hz, ArH), 7.66 (1H, t, $J = 7.4$ Hz, ArH), 7.53 (1H, t, $J = 7.6$ Hz, ArH), 4.60–4.08 (6H, m, C_p + NaphtCH$_2$N), 4.29 (2H, s, FcCH$_2$), 4.16 (5H, s, $C_{p'}$), 2.90 (2H, m, NCH$_2$), 1.75 (2H, m, CH$_2$), 1.24 (8H, m, CH$_2$), 0.84 (3H, t, $J = 7.2$ Hz, CH$_3$). ^{13}C NMR ($CDCl_3$): δ 134.5, 133.7, 131.1, 126.2, 125.7, 106.7, 73.7, 70.1, 69.0, 52.0, 51.5, 50.2, 31.4, 28.7, 26.5, 24.4, 22.5, 14.0. HRMS, calcd for $C_{29}H_{33}FeNO_3$: 500.1888 [M+H$^+$]. Found: 500.1893.

6.23. 3-(*N*-(Ferrocenylmethyl)-*N*-1-octylaminomethyl)-2-hydroxynaphthoquinone (2f)

Orange solid. Yield = 77%. Mp 172 °C. ^1H NMR ($CDCl_3$): δ 8.11 (1H, d, $J = 7.8$ Hz, ArH), 8.05 (1H, d, $J = 7.5$ Hz, ArH), 7.66 (1H, t, $J = 7.0$ Hz, ArH), 7.55 (1H, t, $J = 7.4$ Hz, ArH), 4.44–4.06 (6H, m, C_p + NaphtCH$_2$N), 4.30 (2H, s, FcCH$_2$), 4.20 (5H, s, $C_{p'}$), 2.90 (2H, m, NCH$_2$), 1.72 (2H, m, CH$_2$), 1.24 (10H, m, CH$_2$), 0.85 (3H, t, $J = 7.3$ Hz, CH$_3$). ^{13}C NMR ($CDCl_3$): δ 134.5, 133.7, 131.2, 126.3, 125.6, 106.6, 73.7, 70.1, 69.0, 51.9, 51.5, 50.4, 39.2, 31.6, 28.9, 26.5, 24.4, 22.5, 14.0. HRMS, calcd for $C_{30}H_{35}FeNO_3$: 514.2045 [M+H$^+$]. Found: 514.2051.

6.24. 3-(*N*-(Ferrocenylmethyl)-*N*-1-nonylaminomethyl)-2-hydroxynaphthoquinone (2g)

Orange solid. Yield = 74%. Mp 160 °C. ^1H NMR ($CDCl_3$): δ 8.09 (1H, d, $J = 7.0$ Hz, ArH), 7.97 (1H, d, $J = 6.9$ Hz, ArH), 7.64 (1H, t, $J = 7.2$ Hz, ArH), 7.50 (1H, t, $J = 7.2$ Hz, ArH), 4.52–4.07 (6H, m, C_p + NaphtCH$_2$N), 4.28 (2H, s, FcCH$_2$), 4.10 (5H, s, $C_{p'}$), 2.91 (2H, m, NCH$_2$), 1.76 (2H, m, CH$_2$), 1.22 (12H, m, CH$_2$), 0.85 (3H, t, $J = 7.2$ Hz, CH$_3$). ^{13}C NMR ($CDCl_3$): δ 184.3, 181.3, 172.3, 134.5, 133.6, 131.9, 131.0, 126.1, 125.7, 107.2, 74.0, 69.9, 69.0, 52.2, 51.7, 49.9, 31.7, 29.2, 29.1, 29.0, 26.6, 24.4, 22.6, 14.0. HRMS, calcd for $C_{31}H_{37}FeNO_3$: 528.2201 [M+H$^+$]. Found: 528.2193.

6.25. 3-(*N*-1-Decyl-*N*-(ferrocenylmethyl)aminomethyl)-2-hydroxynaphthoquinone (2h)

Orange solid. Yield = 73%. Mp 152 °C. ^1H NMR ($CDCl_3$): δ 8.09 (1H, d, $J = 7.8$ Hz, ArH), 7.94 (1H, d, $J = 7.5$ Hz, ArH), 7.64 (1H, t, $J = 7.5$ Hz, ArH), 7.49 (1H, t, $J = 7.5$ Hz, ArH), 4.56–4.07 (6H, m, C_p + NaphtCH$_2$N), 4.28 (2H, s, FcCH$_2$), 4.17 (5H, s, $C_{p'}$), 2.94 (2H, m, NCH$_2$), 1.78 (2H, m, CH$_2$), 1.22 (14H, m, CH$_2$), 0.86 (3H, t, $J = 6.9$ Hz, CH$_3$). ^{13}C NMR ($CDCl_3$): δ 184.4, 181.3, 172.3, 134.6, 133.6, 131.9, 130.9, 126.0, 125.7, 107.5, 74.1, 69.9, 69.0, 52.3, 51.7, 49.5, 31.8, 29.4, 29.3, 29.2, 26.7, 24.3, 22.6, 14.1. HRMS, calcd for $C_{32}H_{39}FeNO_3$: 542.2357 [M+H$^+$]. Found: 542.2357.

6.26. 3-(*N*-1-Dodecyl-*N*-(ferrocenylmethyl)aminomethyl)-2-hydroxynaphthoquinone (2i)

Red solid. Yield = 68%. Mp 151 °C. ^1H NMR ($CDCl_3$): δ 8.09 (1H, d, $J = 7.4$ Hz, ArH), 8.03 (1H, d, $J = 6.9$ Hz, ArH), 7.66 (1H, t, $J = 7.4$ Hz, ArH), 7.54 (1H, t, $J = 7.0$ Hz, ArH), 4.45–4.10 (6H, m, C_p + NaphtCH$_2$N), 4.30 (2H, s, FcCH$_2$), 4.16 (5H, s, $C_{p'}$), 2.91 (2H, m, NCH$_2$), 1.75 (2H, m, CH$_2$), 1.22 (18H, m, CH$_2$), 0.88 (3H, t, $J = 7.0$ Hz, CH$_3$). ^{13}C NMR ($CDCl_3$): δ 172.3, 134.5, 133.6, 131.9, 131.1, 126.2, 125.7, 73.9, 70.0, 69.0, 52.1, 51.6, 50.2, 31.9, 29.5, 29.4, 29.3, 29.0, 26.6, 24.4, 22.6, 19.3, 14.1. HRMS, calcd for $C_{34}H_{43}FeNO_3$: 570.2670 [M+H$^+$]. Found: 570.2673.

6.27. 3{*N*-(2-(*N*,*N*-Dimethylaminomethyl)ferrocenylmethyl)-1-aminoethyl}-2-hydroxynaphthoquinone (8)

2-Hydroxynaphthoquinone (261 mg, 1.5 mmol) and 2-(*N*,*N*-dimethylaminomethyl)-*N*-ferrocenylmethylamine 7 (408 mg, 1.5 mmol) were dissolved in ethanol (20 mL). The acetaldehyde (79 mg, 1.8 mmol) was then added with stirring vigorously at room temperature. After 5 h, the reaction mixture was evaporated to give an oil, which was purified by column chromatography (eluent Et_2O/CH_2Cl_2/MeOH/TEA 50/30/10/10). Compound **8** was obtained as a mixture of diastereomers 55/45 (647 mg, 92%). ^1H NMR ($CDCl_3$): δ 8.11 (1H, d, $J = 7.6$ Hz, ArH), 8.04 (1H, d, $J = 7.6$ Hz, ArH), 7.66 (1H, t, $J = 7.6$ Hz, ArH), 7.54 (1H, t, $J = 7.6$ Hz, ArH), 4.51 (1H, q, $J = 6.8$ Hz, NaphtCH), 4.53 (1H, d, $J = 13.6$ Hz, FcCH$_2$), 4.09 (1H, d, $J = 12.8$ Hz, FcCH$_2$NMe$_2$), 4.27–4.12 (3H, m, C_p), 4.13 (5H, s, $C_{p'}$), 3.65 (1H, d, $J = 13.6$ Hz, FcCH$_2$), 2.88 (1H, d, $J = 12.8$ Hz, FcCH$_2$NMe$_2$), 2.20 (6H, s, N(CH$_3$)$_2$), 1.41 (3H, d, $J = 6.8$ Hz, CH$_3$). ^1H NMR ($CDCl_3$): δ 8.11 (1H, d, $J = 7.6$ Hz, ArH), 8.04 (1H, d, $J = 7.6$ Hz, ArH), 7.66 (1H, t, $J = 7.6$ Hz, ArH), 7.54 (1H, t, $J = 7.6$ Hz, ArH), 4.69 (1H, q, $J = 6.8$ Hz), 4.43 (1H, d, $J = 13.0$ Hz, FcCH$_2$NMe$_2$), 4.27–4.05 (3H, m, C_p), 4.11 (5H, s, $C_{p'}$), 3.89 (1H, d, $J = 13.1$ Hz, FcCH$_2$), 3.84 (1H, d, $J = 13.1$ Hz, FcCH$_2$), 2.92 (1H, d, $J = 13.0$ Hz, FcCH$_2$NMe$_2$), 2.20 (6H, s, N(CH$_3$)$_2$), 1.41 (3H, d, $J = 6.8$ Hz, CH$_3$).

6.28. 3-(*N*-(1-Heptyl)aminomethyl)-2-hydroxynaphtoquinone (9)

Following the general procedure with 2-hydroxynaphthoquinone (174 mg, 1 mmol), *N*-1-heptylamine (150 μL, 1 mmol) and formaldehyde (100 mg, 1.2 mmol), **9** was obtained as an orange solid (211 mg, 70%). Mp 166 °C. ^1H NMR ($CDCl_3$): δ 7.78 (1H, d, $J = 7.8$ Hz, ArH), 7.40 (1H, d, $J = 7.5$ Hz, ArH), 7.37 (1H, t, $J = 7.8$ Hz, ArH), 7.11 (1H, t, $J = 7.6$ Hz, ArH), 4.04 (2H, s, NaphtCH$_2$N), 3.15 (2H, t, $J = 7.6$ Hz, NCH$_2$), 1.94 (2H, quint, $J = 7.4$ Hz, CH$_2$), 1.52 (2H, sext, $J = 7.4$ Hz, CH$_2$), 1.32 (6H, m, CH$_2$), 0.88 (3H, t, $J = 7.1$ Hz, CH$_3$). HRMS, calcd for $C_{18}H_{24}NO_3$: 301.1678 [M+H$^+$]. Found: 301.1683.

7. Biological studies

7.1. Anti-microbial agents

Atovaquone was kindly provided by Dr. W. E. Gutteridge at the Glaxo-Wellcome (UK). The drugs were dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mg/mL. The different concentrations tested were obtained by dilutions with DMEM or RPMI 1640 (Gibco-BRL) containing 10% calf foetal serum and sterilized using 0.2 μm filters. These media were designated DMEM-FCS or RPMI-FCS, respectively. Neither DMEM nor RPMI 1640 containing DMSO alone was shown to have an effect on *T. gondii* and *P. falciparum* growth.

7.2. In vitro culture of *T. gondii* and drug assay

The experiments were performed using both 76K and PLK strains (atovaquone-sensitive strains) and with an Ato^R strain derived from the parental PLK strain. Tachyzoites of *T. gondii* were grown in human foreskin fibroblasts (HFF) in DMEM-FCS. The assays were conducted in 24-well plates. The monolayer of HFF cells was infected with 2.5×10^5 parasites per well, and 6 h later, drugs (prepared as described above) were added at the range of concentrations of $0–5 \times 10^{-5}$ μM. After a further 48 h, the intracellular tachyzoites were pulse-labelled with 2 μCi [^{3}H]uracil (Amersham; 1 mCi/mL) per well for 6 h. The lack of uracil phosphoribosyltransferase in the host cells allows the specific labelling of *T. gondii*.[23] After labelling, lysis was performed in the wells using 1% sodium dodecyl sulfate containing 100 μg of cold uracil per millilitre. Radiolabelled nucleic acids were precipitated with trichloroacetic acid overnight at 4 °C and then recovered on glass filters. Radioactivity was measured by scintillation counting as previously described.[19] All drugs were tested in triplicate for each experiment, which was repeated three times. The concentrations corresponding to 50% inhibition (IC_{50}) were determined graphically.

7.3. In vitro culture of *P. falciparum* and drug assays

The experiments were performed using two strains of *P. falciparum*, the chloroquine-sensitive strain 3D7 and the mefloquino–chloroquine resistant strain Dd2. The parasites were maintained on human type O^+ erythrocytes in RPMI 1640 culture medium supplemented with 27.5 mM $NaHCO_3$, 20 mM Hepes (pH 7.4), 11 mM glucose and 7.5% (v/v) heat-inactivated human AB^+ serum under 5% CO_2–5% O_2–90% N_2 at 37 °C as described.[27] The assays were conducted in 96-well plates. The different drug concentrations prepared as described above were added to asynchronous parasites cultures (0.5% parasitemia and 1.8% hematocrit) in the presence of 0.5 μCi [^{3}H]hypoxanthine (Amersham; 1 mCi/mL) per well. After incubation for 48 h at 37 °C, the cells were harvested from each well with an automatic cell harvester (1450 Microbeta; Wallac) onto glass filters. The radioactivity was measured by scintillation counting. All drug concentrations were tested in triplicate for each experiment and three independent experiments were performed for each drug tested. Inhibition of parasite growth was determined for each concentration by comparing the radioactivity incorporated in the treated cultures with that in the control cultures (without drug) maintained on the same plate. The concentrations corresponding to 50% inhibition (IC_{50}) were determined graphically. In addition, the morphology, development and replication of asynchronous cultures of *P. falciparum* treated with the different drugs were also evaluated in cultures by light microscopy of Giemsa-stained of thin blood smears. Smears from drug-free cultures were used as controls.

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