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Parallel inhibition of amino acid efflux and growth of erythrocytic *Plasmodium falciparum* by mefloquine and non-piperidine analogs: Implication for the mechanism of antimalarial action

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ABSTRACT

Despite the troubling psychiatric side-effects it causes in some patients, mefloquine (MQ) has been used for malaria prophylaxis and therapy, due to its activity against all *Plasmodium* species, its ease of dosing, and its relative safety in children and pregnant women. Yet at present there is no consensus on the mechanism of antimalarial action of MQ. Two leading hypotheses for the mechanism of MQ are inhibition of heme crystallization and inhibition of host cell hemoglobin endocytosis. In this report we show that MQ is a potent and rapid inhibitor of amino acid efflux from intact parasitized erythrocytes, which is a measure of the in vivo rate of host hemoglobin endocytosis and catabolism. To further explore the mechanism of action of MQ, we have compared the effects of MQ and 18 non-piperidine analogs on amino acid efflux and parasite growth. Among these closely related compounds, an excellent correlation over nearly 4 log units is seen for 50% inhibition concentration (IC_{50}) values for parasite growth and leucine efflux. These data and other observations are consistent with the hypothesis that the antimalarial action of these compounds derives from inhibition of hemoglobin endocytosis.

According to the World Health Organization, in 2014 there were an estimated 438,000 deaths from malaria.[1] To meet the growing challenge of drug-resistant parasites, new drugs are needed, and a better understanding of the mechanism of action of known antimalarials will help in this search. Of particular interest is mefloquine (MQ), which is safe for malaria prophylaxis in childhood and pregnancy,[2] and (in combination with artemisinin) provides a first-line therapy in Asia.[1] Concerns over the idiosyncratic neuropsychiatric sequelae associated with MQ use have prompted researchers to explore MQ analogs in which the piperidine ring has been excised;[3] a few such compounds (e.g. (*S*)-**1a**,**b**) have shown efficacy against *Plasmodium berghei* malaria.[3d,f]

A common mechanism of action has been put forward for most quinoline-containing antimalarials such as chloroquine (CQ), amodiaquine, MQ, and non-piperidine analogs of MQ (Fig. 1): inhibition of heme crystallization to hemozoin within the acidic food vacuole.[4] Like CQ, MQ and its non-piperidine analogs inhibit heme crystallization in vitro, albeit less potently.[3e,5] MQ has been reported to associate with parasite-derived hemozoin,[4b] and reduces hemozoin levels in vivo.[4d,6] However other mechanisms of antimalarial action for MQ have been proposed,[3e,7] in large part due to the different mechanisms of CQ and MQ resistance.

Two food vacuole membrane-associated transporters (*Pf*CRT, *Pf*MDR1) play key roles in modulating sensitivity to quinoline-containing antimalarials. CQ resistance principally derives from mutations in *Pf*CRT,[8] and a wide range of studies have demonstrated that mutant *Pf*CRT transports CQ out of the food vacuole,[8d,e,9] thereby reducing its exposure to the heme target.

Clinical MQ resistance in contrast is most closely linked to amplification[13] and specific alleles[14] of *Pf*MDR1, which pumps solutes into the food vacuole,[8e,15] where heme resides. In vitro MQ resistance selection experiments confirm upregulation of *Pf*MDR1,[16] whereas genetic downregulation increases MQ sensitivity.[17] Lastly, in vitro experiments have confirmed that *Pf*MDR1 polymorphisms can increase sensitivity to MQ.[18]

Together these observations suggest that the principal antimalarial target of MQ is outside of the food vacuole, and is therefore unrelated to inhibition of heme crystallization. While it is possible that the target of MQ is *Pf*MDR1 itself,[15a,19] it has been observed that MQ significantly inhibits cytostomal endocytosis of host erythrocyte hemoglobin,[7c,20] a critical process that occurs during

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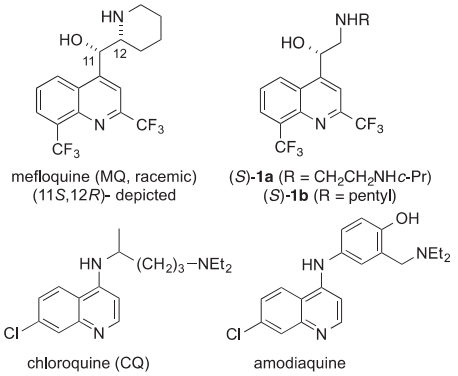
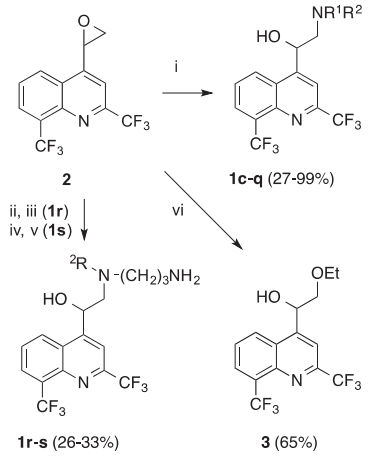


Figure 1. Selected quinoline antimalarial drugs and drug candidates.

the asexual replication cycle. In contrast CQ was found to only weakly inhibit endocytosis, but inhibited vesicle trafficking.[20b] Thus it has been proposed that the antimalarial action of MQ derives from inhibition of hemoglobin endocytosis.[7c,20a]

As a test of this hypothesis, in this work we compare the antimalarial potency (growth inhibition, SYBR Green) of MQ and 18 non-piperidine analogs to their potency to inhibit amino acid efflux from *Plasmodium falciparum*-infected erythrocytes. We have recently shown that amino acid efflux provides a reliable surrogate measure for hemoglobin endocytosis and subsequent catabolism.[12] The required non-piperidine MQ analogs **1c**–**q** (12 known, 6 new, all racemic) were prepared from the commercial epoxide **2** and the required amines by heating in ethanol in a sealed tube at 130 °C for 2–24 h (Scheme 1). In general our yields with conventional heating were in the 70–99% range, similar to the yields reported by other authors using microwave heating.[3b] Only in the cases of **1k**, **1l**, **1n**, **1o** & **1s** were yields below 60% observed. Reaction with cyanamide under these conditions however gave compound **3**, due to reaction with the solvent. Diamine derivatives **1r**–**s** were prepared by ring-opening **2** with the indicated phthalimide-protected 1° amines, and removal of the phthalimide group. Interestingly the standard hydrazine deprotection protocol was not successful, perhaps due to unwanted reaction with the electron-deficient quinoline ring.



Scheme 1. Synthesis of racemic MQ analogs **1c**-**s** and ethanol opening product **3**. See Table 1 for the definition of R^1 and R^2. Reagents and conditions: (i) R^1R^2NH (2–7 equiv), EtOH, sealed tube, 130 °C, 1–24 h. (ii) $BuNH(CH_2)_3NPhth$ (2 equiv), EtOH, sealed tube, 130 °C, 2 h. (iii) $NaBH_4$ (10 equiv), *i*-PrOH/H_2O, rt, 22 h; HOAc (1 equiv) 80 °C, 5 h. (iv) $HC{\equiv}C(CH_2)_2NH$-$(CH_2)_3NPhth$ (2 equiv), EtOH, sealed tube, 130 °C, 2 h. (v) $NaBH_4$ (10 equiv), cyclohexene (10 equiv), *i*-PrOH/H_2O, rt, 22 h; HOAc (1 equiv) 80 °C, 5 h. (vi) NH_2CN (2 equiv), EtOH, sealed tube, 130 °C, 2 h.

Instead a reductive protocol[21] was applied to give **1r** in 27% yield over two steps. When this protocol was applied to the synthesis of acetylene-containing **1s**, reduction of the C≡C triple bond was observed. Suspecting that borane formed in the reaction was responsible for this outcome, cyclohexene was added as a trap. This modification proved successful and **1s** was isolated in 33% yield over 2 steps.

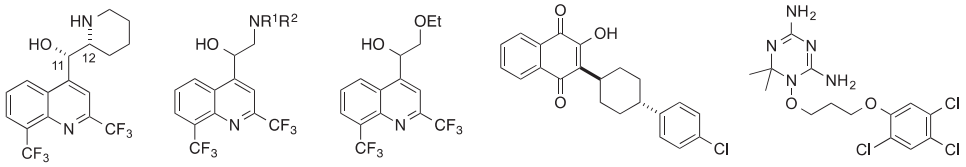
To assess antimalarial activity of these compounds, the well-established SYBR Green method[22] was used to measure growth inhibition of erythrocytic *Plasmodium falciparum* 3D7, a CQ- and MQ-sensitive parasite line, over one replication cycle (Table 1).[23] Atovaquone and WR99210 were selected as positive controls, and as expected, they very potently inhibited growth (IC_{50} values of 0.6 and 0.12 nM, respectively). MQ inhibited growth with an IC_{50} value of 9.1 nM, and the non-piperidine MQ analogs featured IC_{50} values ranging from 4 to 22,000 nM. To benchmark the growth inhibition data obtained for 3D7 strain parasites, in Table 1 we also list published[10] data on the W2 strain, which like 3D7 is MQ-sensitive. Although these data are derived from a different assay, i.e. measurement of [^{3}H]hypoxanthine incorporation,[24] and from a different calculated parameter (IC_{90} vs IC_{50}), others have shown that SYBR Green- and [^{3}H]hypoxanthine-derived IC_{50} values on a single strain can be very closely correlated.[23] Where comparisons can be made, compounds that were potent (IC_{50} < 10 nM) against 3D7 strain in the SYBR Green assay (MQ, **1c**, **1f**, **1i**, **1j**, **1m**), also potently inhibited [^{3}H]hypoxanthine incorporation in W2 strain.

Likewise compounds that are weakly potent against 3D7 strain in the SYBR Green assay (**1d**, **1e**, **1k**, **1o**) are weak growth inhibitors of W2 strain in the [^{3}H]hypoxanthine incorporation assay. A log–log plot of our measured 3D7 strain IC_{50} values versus published W2 strain IC_{90} values is shown in Figure 2. In view of the fact that these data were obtained using two different parasite lines, two different growth inhibition assays, and reflect two different parameters the correlation seen in Figure 2 is surprisingly good and recapitulates the previously reported structure–activity relationships,[3b] namely: i) non-piperidine MQ analogs lacking an pendant amine (**3**) are much less potent than those that do (**1c**–**1s**); ii) phenethyl-substitution (**1m**) confers good potency, but anilino- (**1k**) does not; iii) a pendant 1° alcohol reduces potency (cf. **1e** & **1c**); and iv) within the mono- and dialkylamine-substituted compounds an intricate relationship exists between structure and potency (cf. **1d** & **1c**, **1g** & **1d**, **1n** & **1m**).

With *P. falciparum* growth inhibition activities of MQ, **1c**–**s** and **3** established, their effects on hemoglobin uptake and catabolism was assayed using a published procedure for quantitation of leucine (Leu) efflux from intact *P. falciparum* 3D7-infected erythrocytes into the culture medium.[12] In this assay, parasites are cultured in medium lacking the amino acids Leu and Val, and containing the internal standard D-norvaline. These modifications do not affect parasite growth.[12] After defined time periods, conditioned culture medium is recovered and the Leu concentration is determined by UPLC (AccQ-Tag). The effect of MQ (160 nM) on Leu efflux is shown in Figure 3.

As can be seen, DMSO-treated *P. falciparum* 3D7-infected erythrocytes efflux Leu steadily from 0 to 240 min. However, for infected erythrocytes treated with 160 nM MQ, Leu efflux substantially slows after 60 min, resulting in a 4 h Leu concentration roughly 1/8 of the DMSO-treated erythrocytes. Several lines of evidence indicate that Leu efflux serves as a reliable surrogate measure of rates of hemoglobin uptake and catabolism: (i) amino acid efflux from uninfected erythrocytes is negligible; (ii) relative efflux rates of amino acids correspond well to their abundance in hemoglobin; and (iii) administration of a protease inhibitor greatly suppresses amino acid efflux.[12] Thus MQ (160 nM) dramatically

Table 1
Inhibition of *Plasmodium falciparum* growth and Leu efflux by MQ, analogs **1c-s** & **3**, atovaquone, and WR99210.[a]



Compound	R^1	R^2	3D7 Growth IC_{50} (nM)[b]	W2 Growth IC_{90} (nM)[c]	3D7 Leu efflux IC_{50} (nM)[d]
MQ	na	na	9.1 ± 2.8	16	22 ± 6
1c[3a]	Pr	H	4.2 ± 0.5	1	10 ± 5
1d[10]	$CH_2C{\equiv}CH$	H	110 ± 20	1400	140 ± 30
1e[3b]	CH_2CH_2OH	H	340 ± 70	710	500 ± 70
1f[3b]	Bu	H	4.9 ± 1.3	5	9 ± 3
1g	$CH_2CH_2C{\equiv}CH$	H	34 ± 6	nd	51 ± 14
1h	$(CH_2)_3NH_2$	H	1600 ± 200	nd	5000 ± 600
1i[3a]	*i*-Pr	H	7.4 ± 1.7	13	14 ± 3
1j[3a]	*c*-C_6H_{11}	H	3.8 ± 1.5	6	9.7 ± 5.4
1k[3b]	Ph	H	2000 ± 200	1200	2600 ± 400
1l	1-Adamantyl	H	5.9 ± 1.2	nd	15 ± 5
1m[3a]	CH_2CH_2Ph	H	8.5 ± 2.9	11	13 ± 4
1n	$(CH_2)_2c$-C_5H_9	H	120 ± 20	nd	210 ± 70
1o[3d]	$(CH_2)_2NMe_2$	H	240 ± 20	1300	320 ± 110
1p[11]	Pr	Pr	4.7 ± 1.6	nd	9.3 ± 5.1
1q[3b]	*i*-Pr	*i*-Pr	350 ± 90	12	590 ± 80
1r	$(CH_2)_3NH_2$	Bu	49 ± 4	nd	180 ± 40
1s	$(CH_2)_3NH_2$	$(CH_2)_2C{\equiv}CH$	140 ± 20	nd	360 ± 110
3[3b]	na	na	22,000 ± 13,000	nd	22,000 ± 10,000
Atovaquone	na	na	0.6 ± 0.4	nd	>1000[e]
WR99210	na	na	0.12 ± 0.03	nd	>1000[e]

[a] Compounds previously reported are appropriately cited; na and nd designate "not applicable" and "not determined".
[b] 3D7 strain is CQ-sensitive and MQ-sensitive; growth inhibition was determined in a SYBR Green I-based replication assay. Values are the mean and standard deviation from three independent experiments.
[c] W2 strain is CQ-resistant and MQ-sensitive; growth inhibition IC_{90} values are calculated from ng/mL values reported by the Walter Reed Group,[3a,b,10] as determined by [^{3}H]hypoxanthine incorporation.
[d] Assay performed using strain *P. falciparum* 3D7 (2% hematocrit, 10% parasitemia), according to the published procedure.[12] Values are the mean and standard deviation from three independent experiments.
[e] No inhibition of efflux seen at 1000 nM.

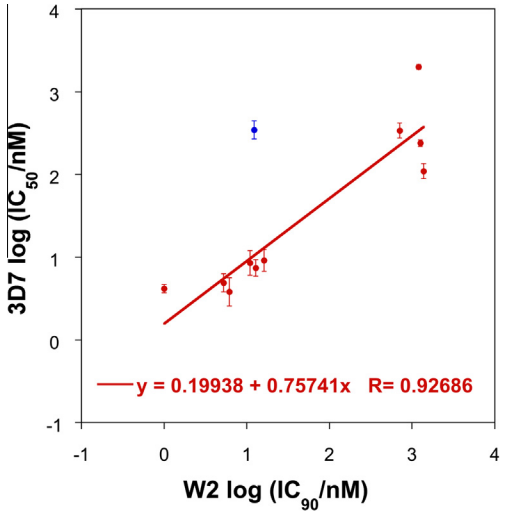


Figure 2. Comparison of 3D7 and W2 growth inhibition activities for MQ and 9 non-piperidine analogs (red points). Log IC_{90} ([^{3}H]hypoxanthine incorporation) is plotted vs log IC_{50} (SYBR Green) (Table 1). Compound **1q** (blue) was considered an outlier and was not included in the linear regression. Error in 3D7 (log IC_{50}/nM) is indicated; error for the literature W2 strain IC_{90} values was not available.

reduces hemoglobin endocytosis and subsequent catabolism. It has recently been reported that 146 nM MQ (an IC_{90} dose) causes a cell cycle delay in 3D7 strain *P. falciparum*.[25] However at this dose, differences in stage morphology are not evident before 12 h, whereas at 160 nM MQ the Leu efflux rate has slowed considerably within 2 h (Fig. 3B). Thus the reduced efflux of Leu does not appear to be the consequence of cell cycle delay.

Full concentration–response curves were then obtained for MQ and 18 non-piperidine analogs of MQ, using a 4 h endpoint assay (see Fig. 4 for selected curves).

The residual Leu efflux (10–20% of control at 4 h) seen at high [drug] likely derives from endocytosis and proteolysis of hemoglobin prior to target engagement (cf. MQ curve in Fig. 3). As can be seen in Table 1, Leu efflux IC_{50} values for these compounds ranged from 9 nM (**1f**) to 22,000 nM (**3**). MQ has an IC_{50} value of 22 ± 6 nM and thus potently inhibits Leu efflux. Atovaquone (a selective inhibitor of the parasite electron transport chain) and WR99210 (a selective inhibitor of parasite dihydrofolate reductase) were also evaluated. As expected from their mechanisms of action, neither showed any effect on Leu efflux at 1000 nM, a concentration that is over 1000-fold higher than their respective growth inhibition IC_{50} values.

Interestingly, a strong correlation is found between Leu efflux inhibition and growth inhibition for MQ and the 18 non-piperidine analogs (Table 1). A log–log plot of Leu efflux IC_{50} vs. growth inhibition IC_{50} affords an R^2 value of 0.98 (Fig. 5). The data span nearly 4 log units, giving a slope of 0.94. Thus structural modifications of MQ affect growth inhibition and Leu efflux in nearly identical ways. In contrast the data for atovaquone and WR99210 (blue points, Fig. 5) fall well off this line, since they potently inhibit growth (IC_{50} = 0.6, 0.12 nM, respectively), but they do not affect

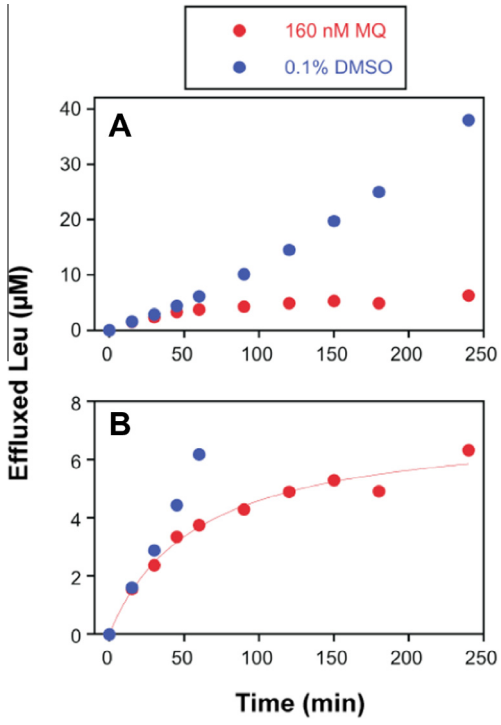


Figure 3. Plots of the concentration of Leu effluxed to the culture medium vs time for DMSO- and MQ-treated *P. falciparum* 3D7-infected erythrocytes. (A) Full data set. (B) Expansion of the ordinate to better visualize the difference in efflux profiles at early time points, including a hyperbolic fit of the MQ-treated data set. In the presence of 160 nM MQ, Leu efflux substantially slows after 60 min of exposure. Data points are the average of two technical replicates.

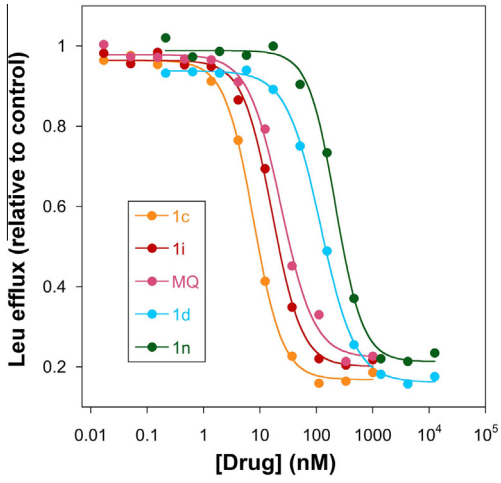


Figure 4. Effects of MQ and select non-piperidine analogs on Leu efflux from trophozoite stage *P. falciparum*-infected erythrocytes over 4 h.

Leu efflux at the highest concentration tested (1000 nM). *These data therefore suggest that inhibition of hemoglobin endocytosis and subsequent catabolism provides a major component of the mechanism of antimalarial action of both MQ and its non-piperidine analogs.*

Can the effects of MQ on endocytosis and catabolism be distinguished? Two literature observations are helpful in this regard. First, it has been reported that MQ (100 nM) did not deplete ATP in cultured *P. falciparum* over 4 h.[7g] Thus the significant (~70%, Fig. 3) inhibition of Leu efflux we observe at this concentration of MQ over the same time period cannot be attributed to global metabolic collapse. Secondly, incubation of parasites with

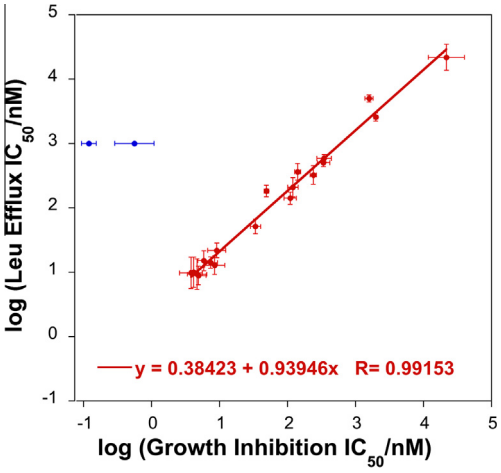


Figure 5. Inhibition of parasite growth and Leu efflux is closely correlated for MQ and 18 non-piperidine analogs (red points). These activities are not correlated for atovaquone and WR99210 (blue points). Data are taken from Table 1. Note that atovaquone and WR99210 did not measurably effect Leu efflux at 1000 nM; thus the Leu efflux IC_{50} values assigned for these compounds in the plot represent a lower bound. For atovaquone and WR99210, error is only available in log (Growth Inhibition IC_{50}/nM).

protease inhibitors (E64, ALLN) causes a significant increase in internalized undegraded hemoglobin, consistent with reduced hemoglobin catabolism.[26] In contrast, treatment with MQ under conditions similar to those of Figure 3 (60–156 nM, 5 h)[7c,26] significantly reduced hemoglobin within parasites. Furthermore, MQ antagonized the increase in internalized hemoglobin induced by protease inhibitors.[26] Thus at these concentrations MQ does not appear to directly inhibit hemoglobin catabolism, or to directly inhibit efflux of the products of hemoglobin catabolism from the food vacuole. Taken together, our data and these observations support the hypothesis first articulated by Famin and Ginsburg that MQ inhibits parasite growth through inhibition of hemoglobin endocytosis.[20a] Further development of chemical biological tools to test this hypothesis is in progress and will be reported in due course.

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Supplementary data

Supplementary data (includes synthetic procedures, analytical tabulations, and copies of the ^{1}H and ^{13}C NMR spectra for all tested compounds; biological assay procedures and biological data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.08.005.

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