Two novel assays for the detection of haemin-binding properties of antimalarials evaluated with compounds isolated from medicinal plants

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Forty-two compounds isolated from nine plants used within South America for the treatment of malaria were tested for haemin binding using two novel, rapid screening methods. The data obtained were analysed with respect to IC50 values for in vitro toxicity to Plasmodium falciparum trophozoites. One method, a multiwell assay based on the inhibition of the interaction of haemin with glutathione (GSH), is sensitive in the 10 µM range, takes c. 1 h and is suitable for either a high throughput screen or rapid assay during natural product isolation. Of 19 compounds showing antiplasmodial activity (IC50 < 40 µM), 16 (84%) showed >40% inhibition of GSH–haemin reaction. The sensitivity and specificity of the assay were 0.85 and 0.82, respectively. The positive predictive value was 0.81 and the negative predictive value 0.86. A more sensitive assay (0.1 µM range) is based on the reversal by haemin-binding compounds of the haemin inhibition of the L-dopachrome-methyl ester tautomerase activity of human macrophage migration inhibitory factor. This assay gives a better idea of the affinity of interaction and uses very small amounts of test compound. The log[RI50] of eight of the compounds that tested positive in the above assays together with those of quinine and chloroquine showed a positive correlation with log[antiplasmodial IC50] for strain T9-96 (r = 0.824) and strain K1 (r = 0.904). Several of the antimalarial compounds that bind haemin are isoquinolines, a class not shown previously to interact with haemin.

Introduction

The blood-stage of the malarial parasite Plasmodium falciparum obtains the majority of the required essential amino acids by enzymic hydrolysis of haemoglobin, digesting up to 80% of the host cell’s protein in the acidic lysosomal food vacuole.1 The by-product of this digestion is the release of toxic haemin [Fe(III) protoporphyrin IX], which is detoxified in the lysosomal vacuole of the parasite via formation of an insoluble polymer, ‘malarial pigment’ or ‘haemozoin’, which is thought to be identical to synthetic β-haematin.2–4 In the presence of preformed haemozoin, polymerization of haemin occurs spontaneously in vitro;5 however, the involvement of a haemin polymerase enzyme6 has not been disproven. Quinoline antimalarials, e.g. quinine and chloroquine, are generally thought to act by inhibiting the formation of haemozoin, either by binding to free haemin or by capping the ends of the growing complex.2–3 As a result haemin accumulates and kills the parasite. Recently, an alternative haemin detoxication mechanism has been described in the parasitized erythrocyte involving reactions of haemin with glutathione (GSH),7 akin to the physiological mechanism for preventing damage by the low levels of free haemin in normal red blood cells.8 Remarkably, quinoline antimalarial compounds can also inhibit this route of detoxication9–11 or that dependent on peroxidation reactions.12

In order to test the ability of compounds to interfere with haemin polymerization, two in vitro polymerization assays have been developed previously. One utilizes the formation of ‘haemozoin’ from concentrated haemin solution in aqueous dimethylsulphoxide (DMSO)/Na acetate (pH 5), the polymer being quantified spectrophotometrically.13 The method is a good predictor of antimalarial activity, but takes 18 h and involves compound–haemin interaction at millimolar concent-
centration, which is not useful where there are limited quantities of test compound. A second method measures polymerized haemin radiochemically.\textsuperscript{14} This method allows compound–haemin interaction with 0.1 mM [\(^{14}\)C]haemin, and has been used with advanced robotic workstations to screen large numbers of compounds from a library.\textsuperscript{15} This method is suitable for a commercial operation, but is expensive and slow for academic studies and studies of natural products in short supply. Furthermore, the conditions of haemin concentration are still far above those able to differentiate a high-affinity interaction with haemin, which is likely to be in the sub-micromolar range, from many which occur in the high-affinity interaction with haemin, which is likely to be in malaria,\textsuperscript{16,17} both novel drug targets and structurally diverse drugs, active at validated targets like haemozoin formation, are urgently needed. In order to facilitate screening for novel haemin-binding antimalarials, two new assays are presented: one is based on the inhibition of the reaction(s) of GSH with haemin;\textsuperscript{9} the other on the reversal of the inhibition by haemin of the dopachrome tautomerase activity of human macrophage migration inhibitory factor (MIF).\textsuperscript{18} The assays were tested successfully using a series of 42 compounds isolated from antimalarial plant extracts.\textsuperscript{19} The methods offer improvements in sensitivity, speed and cost that should prove useful in antimalarial discovery.

Materials and methods

Haemin, GSH, L-dihydroxyphenylalanine-methyl ester, Na m-periodate, EDTA, diethylenetriamine-penta-acetic acid (DETEPAC), NaCl (ultrapure) and DMSO (molecular biology grade) were obtained from Sigma. Analytical grade Na phosphates, NaOH, ethane-1,2-diol, ethanol and methanol were obtained from BDH/Merck. Recombinant human MIF was obtained from BioHit d.o.o (Ljubljana, Slovenia). Water used was of milli-Q grade. Flat-bottomed 96-well polystyrene plates were obtained from Life Technologies–Nunc.

Test compounds were extracted and purified from selected species of plant from six families used in traditional medicine for fever in South America: family Menispermaceae [\textit{Abuta grandifolia} (Martius) Sandwith, \textit{Abuta rufescens} Aublet, \textit{Cissampelos ovalifolia} Candolle (DC)]; family Apocynaceae [\textit{Aspidosperma excelsum} Bentham, \textit{Geissospermum sericeum} (Sagot) Bentham & Hooker]; family Myrtaceae [\textit{Myrciaria dubia} (Kunth) McVaugh]; family Simaroubaceae [\textit{Simarouba amara} Aublet]; family Lamiaceae [\textit{Plectranthus barbatus} Andrews]; and family Rutaceae [\textit{Zanthoxylum pentandrum} (Aublet) R. A. Howard].\textsuperscript{19} The compounds were structurally characterized\textsuperscript{19} and tested for the ability to inhibit the proliferation, according to incorporation of [\(^{3}\)H]hypoxanthine into nucleic acids, of a chloroquine-resistant (K1) and a sensitive (T9-96) strain of \textit{P. falciparum} cultured in vitro.\textsuperscript{20}

Scanning spectrophotometry

The effect of GSH upon haemin was analysed by repetitive scanning spectrophotometry (Perkin-Elmer \(\lambda5\)) in the absence or presence of known haemin-binding antimalarial compounds. A solution (0.8 mL) of 10 \(\mu\)M haemin in 1 mM DETE PAC, 0.05 M Na phosphate pH 7.0 at 25° C in a cuvette of 1 cm light-path, was scanned from 480 to 340 nm. Neutralized GSH was added to 2 mM (using a 100 mM stock solution) and the sample scanned at 3 min intervals. The experiment was repeated in the presence of 18 \(\mu\)M chloroquine, mefloquine, quinine, bi-desethyl-amodiaquine, desbutyl-halofantrine or artemisinin. In each case the drug was added after the haemin scan, and an extra scan was made before the addition of GSH to show formation of a drug–haemin complex.

Multiwell plate GSH–haemin interaction assay

Based upon the spectrophotometric data, a multiwell assay for the inhibition of GSH–haemin interaction was developed. The following stock solutions were prepared: 1 mM DETE PAC in 10 mM Na phosphate pH 7.0; 2 mM haemin in DMSO; and 100 mM GSH, 1 mM DETE PAC, 10 mM Na phosphate pH 6.8. Drug stock solutions (generally 2 mM) were prepared in water, methanol or DMSO as required. All stocks were stored frozen except the haemin which was prepared fresh daily. Working solutions were as follows: ‘A’, DETE PAC/phosphate stock 4 vol + 1 vol ethanol; ‘B’, 5 \(\mu\)L haemin stock solution per mL of solution A; and ‘C’, 0.15 mL GSH stock solution per mL solution ‘A’.

Assays were carried out in 96-well (400 \(\mu\)L) flat-bottomed polystyrene plates. Solution A (100 \(\mu\)L) was added, followed by drug or solvent control (e.g. 2 \(\mu\)L of 2 mM drug stock) in triplicate assays. Solution B (haemin) (200 \(\mu\)L) was then added to all wells using a multichannel pipette followed by 50 \(\mu\)L solution C (GSH). Final concentrations of drug and haemin were 11 \(\mu\)M and 5.7 \(\mu\)M, respectively. The absorbance at 360 nm (\(A_{360}\)) was measured after c. 1 min using a plate reader (MCC Titertek, Thermo Life Sciences, Basingstoke, UK) set up to read at \(t = 0\) and \(t = 30\) min and to calculate the \(\Delta A_{360}\). The \(A_{360}\) of the controls (GSH + haemin + solvent) decreased by about 0.08. The effect of the haemin-binding compounds was evaluated as the percentage decrease compared with control absorbance. Mean and S.D. of the triplicates were calculated and significance determined by Student’s \(t\)-test.

MIF–haemin interaction assay

The following stock solutions were prepared: ‘D’, 40% (v/v) ethane-1,2-diol, 1 mM EDTA, 20 mM Na phosphate pH 6.8; ‘E’, 1 mM EDTA, 10 mM Na phosphate pH 6.4; ‘F’, 10 mM L-dihydroxyphenylalanine-methyl ester in water; and ‘G’, 20 mM Na m-periodate in water. Recombinant human MIF
was dissolved (0.5 mg/mL) in ‘D’. All solutions were stored at 4°C. A working solution of MIF (7.5 µg/mL) was prepared daily by diluting the stock with ‘D’, and kept on ice. Haemin (0.2 mM in DMSO) was also prepared daily. Tautomerase activity was assayed at 35°C in thermostatted 1 mL polystyrene cuvettes at 474 nm. To 740 µL solution E was added 32 µL solution F, stirred, then 24 µL solution G was added and stirred to form c. 0.4 mM L-dopachrome-methyl ester, all of the periodate (‘G’) being consumed. A control volume of DMSO (4 µL) was added and the rate of spontaneous dopachrome decarboxylation was measured by the decrease in A_{474} over 0.4 min. MIF (2 µL i.e. 15 ng) was added with stirring and the dopachrome tautomerization followed similarly to obtain an uninhibited control rate. In order to check for direct inhibition of tautomerization by the test compound, the assay was repeated using a maximal level of test compound (e.g. 2 µL of 2 mM in DMSO + 2 µL DMSO). If there was little or no inhibition, the original assay was repeated with c. 2 µL haemin, such that the activity due to MIF was inhibited by 75–95%. The test compound (maximal amount) and haemin were then both included in the assay to see whether there was reversal of the haemin-dependent inhibition of the MIF tautomerase activity. In positive cases, successive two-fold reductions in the concentration of test compound gave a ‘reversal of inhibition curve’ and the concentration causing 50% relief of the inhibition, ‘RI_{50}’, was obtained graphically.

**Results**

The reaction of haemin with GSH at pH 7.0 was monitored spectrophotometrically (Figure 1a). The broad absorption of 10 µM haemin from 360 to 390 nm was immediately changed to a peak at 364 nm upon addition of GSH. During the next few minutes this peak decreased with a slight increase at

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**Figure 1.** Effects of quinoline drugs upon spectral changes occurring during the reaction of haemin with GSH. (a) The visible spectrum of 10 µM haemin in buffer A at 25°C was recorded. GSH was added to 1.8 mM and the spectral change recorded within 2 min. The sample was then rescanned at 3 min intervals. (b–f) The experiment was repeated in the presence of 18 µM of quinine, mefloquine, desbutyl-halofantrine, bi-desethyl-amodiaquine or chloroquine, respectively, the drugs being added before the addition of GSH.
450 nm and isosbestic points at 403 and 500 nm. Thence, further decreases at 364 nm were associated with loss of the isosbestic point at 403 nm and further small increases in the region of 450 nm, indicating that at least two processes had occurred. By 30 min, the $A_{364}$ was lowered to approximately half and there was little further decrease at 364 nm. Inclusion of antimalarial alkaloids (18 µM), added before the GSH addition (Figure 1b–f) resulted first in the alteration of the haeminspectrum due to the immediate formation of alkaloid–haemin complexes, and then to altered effects of GSH. In the cases of halofantrine, amodiaquine and chloroquine, the addition of GSH then failed to result in formation of the 364 nm peak (Figure 1d–f) and there was little change in their spectra over 30 min. In the presence of quinine (Figure 1b), the 364 nm peak was formed at a lower level than control (Figure 1a), and decreased less over time. Mefloquine (Figure 1c) showed effects intermediate between those of quinine and halofantrine/amodiaquine/chloroquine. The data extend earlier observations of changes at 400 nm,9 and indicate that it would be possible to detect haemin-binding antimalarials from the decreased loss of absorbance of GSH–haemin at 364 nm over 30 min, regardless of the spectrum of any preformed alkaloid–haemin complex. The $\Delta A_{364}/cm$ is 0.35 in the above assays at 10 µM haemin, so it was considered possible to use about half that amount of haemin and adapt the assay for a multiwell plate reader with optical path-length of c. 0.5 cm to give a control absorbance decrease of c. 0.1. Such an assay is shown in Figure 2 using a plate reader equipped with a 360 nm filter in kinetic mode. Triplicate assays were prepared in a 96-well plate. The $A_{360}$ was measured at 1 and 30 min after addition of GSH and the $\Delta A_{360}$ calculated. The method clearly distinguishes the haemin-binding antimalarials, which inhibit the loss of $A_{360}$ by >40%, from the other drugs. The method detects drug–haemin interaction in the 10 µM range, which should be capable of detecting both high- and moderate-affinity haemin-binding antimalarials. Artemisinin, which reacts more weakly with haemin than with haem,21,22 caused only 20% inhibition of the GSH-dependent loss of haemin in this assay. More detailed analysis of the effect of artemisinin by repetitive scanning spectrophotometry confirmed a low level of complexation and a 20% decrease in haemin–GSH reaction rate, which was incomplete at 30 min.

In order to test for haemin-binding compounds biochemically with a sensitivity that might correlate with antimalarial effects in cellular assays, we utilized the inhibition by haemin of the tautomerase activity of MIF ($I_{50}$ 0.15 µM18). As predicted, the haemin-binding alkaloids were able to reverse the inhibition of MIF tautomerase activity. A standardized assay was developed in which the control activity was inhibited by haemin to c. 85% (75–95% in practice) and various concentrations of drug were added to obtain a reversal of inhibition curve, and an ‘RI$_{50}$’. Several antimalarial drugs were tested and reversal of inhibition was detectable with as little as 70 nM chloroquine (Figure 3). In this assay, a maximum of 0.6% (v/v) DMSO + methanol was used to avoid solvent inhibition of MIF and this led to solubility problems with some drugs such as lumefantrine for which a complete relief of inhibition curve could not be obtained.

The novel assays were tested using a group of 42 compounds isolated from plants that are used for malarial therapy in South America.19 The structures of the compounds had been fully or partially characterized, and analysed for antiplasmodial activity in cellular assays using two strains of P. falciparum (K1 and T9-96) in comparison with chloro-

![Figure 2](https://example.com/figure2.png)

Figure 2. Test of multiwell plate GSH–haemin interaction assay. The effect of various drugs (11 µM) on the decrease in $A_{360}$ due to interaction of 1 mM GSH with 5.7 µM haemin was determined as described in the text using a plate reader. Values are the mean of triplicate assays, which differed by <5%.

![Figure 3](https://example.com/figure3.png)

Figure 3. Test of MIF–haemin interaction assay. The reversal, by antimalarial drugs, of the haemin inhibition of human MIF dopachrome tautomerase activity was assayed spectrophotometrically at 35°C, pH 6.4, as described in the text. Relief of inhibition curves are shown for chloroquine (empty circle), mepracin (filled circle), bi-desethyl-amodiaquine (empty inverted triangle), quinidine (filled inverted triangle), quinine (filled diamond), desbutyl-halofantrine (filled square) and primaquine (empty square).
Novel screens for haemin-binding antimalarials

The group comprised 14 bis-benzyl-isoquinolines (BBIQs), one benzyl-isoquinoline, one quarter-nary-benzyl-isoquinoline, three β-carboline, nine indole-alkaloid, three aporphine, two protoberberine, six terpenoid, one quassinoid, one coumarin and one unknown.

The percentage of GSH-dependent loss of A_{360} in the presence of each of the 42 compounds was compared with the IC_{50} of antiplasmodial activity in the T9-96 (Figure 4a) and K1 (Figure 4b) strains of *P. falciparum*. Compounds that inhibited growth by <50% at 40 µM were grouped as not significantly growth inhibitory (IC_{50} > 40 µM). Twenty-one of the compounds showed ≥40% inhibition of the effect of GSH on haemin (13 bis-benzyl-isoquinoline, three β-carboline, two protoberberine, two indole-alkaloid and one aporphine). Four compounds (two indole alkaloids and two β-carboline) were positive in the GSH–haemin interaction assay but failed to show significant inhibitory activity against either strain of *P. falciparum* (IC_{50} > 40 µM). In addition one compound (aporphine) was inhibitory only towards the K1 strain, and another (protoberberine) was inhibitory only towards the T9-96 strain. Inhibition of the GSH–haemin reactions correlated well with antiplasmodial activity (Table 1). Thus, only 3/19 antiplasmodial compounds failed to inhibit GSH–haemin reaction by >40%: an indole alkaloid of relatively low antiplasmodial activity, the benzyl-isoquinoline and the least potent of the antimalarial BBIQs.

From the results summarized in Table 1, the sensitivity of the GSH–haemin interaction assay was 0.85, the specificity was 0.82, the positive predictive value was 0.81 and the negative predictive value 0.86.

Eight of the 17 compounds showing both antiplasmodial activity and inhibition of GSH–haemin reaction were chosen to represent a range of potency for testing in the MIF–haemin interaction assay (i.e. the reversal of haemin inhibition of MIF dopachrome tautomerase activity). The log[RI_{50}] of the test compounds, together with those of chloroquine and quinine were positively correlated with the log[antiplasmodial IC_{50}] for the T9-96 strain (r = 0.824) and the K1 strain (r = 0.904) (Figure 5).

**Table 1.** Correlation of GSH–haemin interaction inhibition with antiplasmodial IC_{50}

<table>
<thead>
<tr>
<th>GSH–haemin effect &lt;60% of control</th>
<th>GSH–haemin effect &gt;60% of control</th>
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<tr>
<td><em>P. falciparum</em> IC_{50} &lt; 40 µM in T9-96 or K1</td>
<td>17 (40.5%) true positive</td>
</tr>
<tr>
<td><em>P. falciparum</em> IC_{50} &gt; 40 µM in T9-96 and K1</td>
<td>4 (9.5%) false positive</td>
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</table>

The ability of 42 isolated compounds to inhibit the reaction of haemin with GSH, determined using the plate-reader assay as described in the text, is compared with their ability to inhibit the *in vitro* growth of two strains of *P. falciparum*.19
an inhibition of 40% as a significant effect on the GSH–haemin interaction, it failed to detect artesiminin, which gave only 20% inhibition. Such compounds could also be included in the positives if a higher level of false positives was acceptable. The GSH–haemin interaction assay is sensitive in the 10 μM range and is simple, cheap and fast.

In order to test the practical performance of the assay, 42 natural compounds isolated from species of antimalarial plants were screened. The assay successfully detected 16 of the 19 showing antiplasmodial activity in vitro. Among the three ‘false negatives’, one was a derivative of the indole alkaloid geissoschizoline, which was only weakly antimalarial. The other two, a benzyl-isoquinoline, a quinoline and two β-carbolines from S. amara, one of which was a glycoside. This test may clearly give positive results for various compounds with a hydrophilic moiety such as glycosides, which will be unlikely to have antiplasmodial activity in vitro. A group of 10 myricetin and quercetin glycosides were also isolated from the aqueous acetic acid-soluble fraction of methanol extracts (non-alkaloidal) of M. dubia. As expected, they showed no antiplasmodial activity in vitro; however, they were potent inhibitors of the GSH–haemin reaction, even at sub-stoichiometric levels of glycoside, which may be due to an antioxidant effect. Compounds of this type are expected to increase the numbers of ‘false positives’ in the GSH–haemin interaction assay, but could perhaps be detected by their sub-stoichiometric potency.

The sensitivity of the GSH–haemin interaction assay is not high enough to cover the range of association constant of most useful haemin-binding antimalarials. In order to obtain information on the relative haemin affinity of compounds detected in the above assay, a more sensitive test was devised, namely the relief of haemin inhibition of the dopachrome tautomerase activity of human MIF, or ‘MIF–haemin interaction assay’. This assay successfully detected the predicted haem binding of all the antimalarials tested (Figure 4) although the RI50 of lumefantrine could not be obtained due to its insolubility above 0.5 μM under these conditions. This assay is carried out in 99.5% aqueous solution generally in the range 50 nM to 5 μM of inhibitor with c. 200 nM haemin. The reversal of inhibition curve takes c. 1 h to obtain for each compound. The spectrophotometer is the only significant expense: only 150 ng MIF is needed per test compound at a cost of about £0.02.

Selecting eight of the compounds that gave a positive GSH–haemin interaction result for the MIF–haemin interaction assay, and adding chloroquine and quinine, RI50 values were obtained. The log10 of the RI50 for the compounds is reasonably proportional to the log10 of the antiplasmodial IC50 in the two strains (Figure 5) and is expected to be able to predict compounds capable of interacting with haemin at physiologically significant aqueous concentrations of haemin (sub-micromolar). The recombinant human MIF used in the assay is stable as a stock solution in 40% (v/v) ethan-1,2-diol, 10 mM Na phosphate for at least a year at 4°C. Although the antimalarial activity of BBIQ compounds has been observed previously, their mode of action has not until now been related to their interaction with haemin.

It was considered that the GSH–haemin interaction assay could be used as a rapid screen for compounds binding in the micromolar range, and the inhibition of the MIF–haemin interaction assay could be used to test for sub-micromolar potency of interaction in order to select compounds that would render worthwhile the more complex assays that are better predictors of antimalarial activity.

The MIF–haemin interaction assay, although not suitable for high-throughput screening, should be useful as an aid in the identification of the most potent haemin-binding natural or synthetic products, which could then be selected for structural characterization where needed, and further testing. The assay should also be most useful when a very small amount of test compound is available. The novel assays described are unlikely to predict antimalarial activity as well as those involving direct inhibition of haemin polymerization; however, they have important advantages in terms of sensitivity, speed, simplicity and cost.
Both assays depend on interactions thought to occur during plasmodial infection, and since MIF is present in human erythrocytes (c. 15 μg/mL, D. J. Meyer, unpublished) and may act as a proinflammatory cytokine, its release from parasitized or lysed erythrocytes could contribute to the symptoms of malaria. If this is the case, the interaction of MIF with haemin and its prevention by haemin-binding antimalariais might be of direct therapeutic significance.

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