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Antimalarial and β-hematin formation inhibitory activities of chromone derivatives

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ABSTRACT: A series of chromone compounds were evaluated as new potential antimalarial agents using in vitro antimalarial activity assay. The most potent compound 36 with an IC_{50} of 0.95 μM was shown to be more potent than primaquine and tafenoquine (IC_{50} 2.41 and 1.95 μM, respectively). β-Hematin formation inhibitory activity test and stoichiometry determination have also been performed to investigate the preliminary mechanism of antimalarial activity of the studied compounds. Compounds 23–28 (IC_{50} 1.41, 1.76, 2.30, 2.54, 4.60, and 3.69 μM, respectively) displayed greater β-hematin formation inhibitory activity than chloroquine, dihydroartemisinin, and mefloquine (IC_{50} 25.04, 18.04, 15.78 μM, respectively). Compounds 3, 4, 23, 24, 27, 36, 38, and 43 showed high potency in both antimalarial and β-hematin formation assays. Job's plots indicated that compounds showing high β-hematin formation inhibitory activity formed stable complexes with the same stoichiometric ratio of chromone:heme = 1:2 as chloroquine. This study opens up the possibility of development of chromone derivatives as new antimalarials targeting β-hematin formation.

KEYWORDS: chromone derivatives, antimalarial activity, β-hematin formation inhibitory activity

INTRODUCTION

Despite the efforts to eradicate and control malaria, this disease remains the major causes of ailment and mortality, threatening and killing millions of people each year[1,2]. The causative agents of malaria in human are five different species of Plasmodium, i.e., *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. Among these, *P. falciparum* is the most virulent parasite with high mortality rate. Malaria has become more difficult to treat because of the increasing prevalence of multi-drug resistance of malaria parasites and unavailability of a successful vaccine[3]. Discovery and design of new chemical compounds acting on novel targets are necessary to overcome the emergence of resistance to the clinical currently used drugs.

During the intraerythrocytic stages of malaria infection, the parasite consumes hemoglobin of the infected red blood cell to serve as amino acids sources. This process of hemoglobin degradation occurs within the acidic food vacuole of the parasite and subsequently toxic free heme is released. The plasmodium parasites detoxify the free heme by converting into a malaria pigment known as hemozoin[4,5]. Since hemozoin formation is essential for the survival of these parasites, inhibiting hemozoin formation leads to parasite death. X-ray crystallography and spectroscopic analyses indicate that hemozoin has the same structure as the synthetic analog, β-hematin[6,7]. β-Hematin is a heme dimer formed via reciprocal covalent bonds between carboxylic acid groups on the protoporphyrin-IX ring and the iron atoms of two heme molecules. These dimers interact via hydrogen bonds to produce crystals of hemozoin. The hemozoin formation pathway is unique to the malarial parasite, therefore, it serves as an attractive target for the new antimalarial drug discovery.

Several types of antimalarial drugs have been reported to exhibit antimalarial activity by enhancing free heme toxicity through the inhibition of β-hematin formation. The quinoline antimalarials, i.e., chloroquine, quinine, and amodiaquine were found to form complexes with dimeric hematin and preventing the formation of β-hematin[8–11]. 9-Anilinoacridine derivatives have been reported to target at two different sites within the malaria parasite, i.e., DNA topoisomerase II and β-hematin formation[12]. Xanthone derivatives, a novel antimalarial agent, have been found to exert their activity by complexation with heme and inhibition

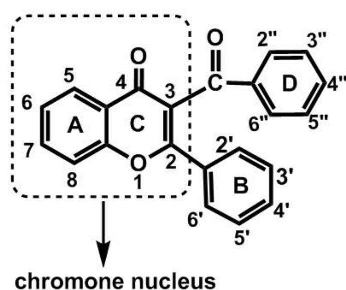


Fig. 1 General structure of chromone compounds.

of β-hematin formation[13,14].

The in vitro studies have shown that flavonoid compounds, natural phenyl substituted chromones, exhibited remarkable antimalarial activity[15–18]. For example, dehydrosilybin and 8-(1;1)-DMA-kaempferide showed a direct antimalarial activity on several multidrug-resistant strains of culture-adapted *P. falciparum*[16] and the diprenylated flavonoids showed a notable antimalarial activity[18]. The aim of this study was to investigate the in vitro antimalarial activity against *P. falciparum* of compounds in chromone series (general structures as shown in Fig. 1). β-Hematin formation inhibitory activity test and stoichiometry determination have also been performed in order to explore the preliminary mechanism of antimalarial activity of compounds in this series.

MATERIALS AND METHODS

The chromone compounds used in this study were available from previous works and were prepared as described in the report of those studies[19,20].

In vitro antimalarial activity assay

The evaluation of antimalarial activity against *P. falciparum* using microculture radioisotope assay was performed by the Bioassay Laboratory, National Center of Genetic Engineering and Biotechnology (BIOTEC), Thailand. In vitro cultivation of *P. falciparum* (K1, multi-drug resistant strain) was performed according to the method previously described by Trager et al[21]. The parasite was cultivated in RPMI-1640 medium containing 25 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 25 mM $NaHCO_3$, 10% heat-activated human serum, and 3% erythrocytes. The culture was incubated at 37 °C in a humidified incubator with 3% CO_2-enriched atmosphere (3% CO_2, 17% O_2 and 80% N_2). Daily passaged to fresh medium containing erythrocyte in order to maintain parasite growth was performed. Prior to the assay, the parasite at an early ring-stage growth was collected and prepared to a parasite mixture of 1% parasitemia in 1.5% erythrocytes.

The protocol for the assay was a modification of the method described by Desjardins et al[22]. The uptake of [^{3}H] hypoxanthine by parasites was used as an indicator of viability. The assay was performed in duplicate wells in 96-well plate. In each well, 200 µl of parasite mixture (1% parasitemia and 1.5% erythrocytes) was pre-exposed with 25 µl of the medium containing a test sample dissolved in 1% DMSO for 24 hours. Medium (25 µl) containing 0.5 µCi [^{3}H]-hypoxanthine (Perkin Elmer, USA) was added to each well. The plates were incubated for an additional 24 hours. Levels of incorporated radioactive labeled hypoxanthine indicating parasite growth were determined using the TopCount NXT Microplate Scintillation and Luminescence Counters (Perkin Elmer, USA). All chromone derivatives were tested at concentration of 10 µg/ml (except for compound 36, 1 µg/ml was used due to the solubility limitation of this compound). Dihydroartemisinin and mefloquine (purchased from Tokyo chemical industry, Japan) were used as the positive control. The negative control was 0.1% DMSO. The percentage of parasite inhibition was calculated from the signal count per minute of treated (CPM_T) and untreated samples (CPM_U) using the following equation:

$$\text{Parasite inhibition}\,(\%) = \left(1 - \frac{CPM_T}{CPM_U}\right) \times 100.$$

If % parasite inhibition was $< 50\%$, the activity was reported as inactive, and as active if $\geqslant 50\%$.

All compounds reported as active were selected for the IC_{50} (the concentration of sample required to inhibit 50% parasite growth) determination. The IC_{50} was derived from the plot between six concentrations (0.0412, 0.123, 0.370, 1.11, 3.33, and 10.00 µM) and % parasite inhibition using curve-fitting method with SOFTMax Pro software (Molecular Devices, USA). The IC_{50} values reported herein were the average values of the three separated experiments.

β-Hematin formation inhibition assay

The β-hematin formation inhibitory activity was determined based on the method of Baelmans et al[23]. Forty two chromone compounds were tested at the concentration of 30 µM. Hemin chloride (purchased from Tokyo chemical industry, Japan) was used

as source of heme in this study. A solution containing 50 µl of 4 mM hemin chloride and 50 µl of chromone compound dissolved in DMSO was distributed in a microcentrifuge tube. β-Hematin formation was initiated by adding 100 µl of 0.2 M acetate buffer pH 4.4. The mixture was incubated at 37 °C for 48 hours to allow completion of the reaction. After centrifuging at 4000*g* for 15 minutes, the supernatant was discarded. The pellets (the generated β-hematin appeared as dark brown pellet) were washed 3 times with DMSO (300 µl) to remove the unreacted hemin chloride from β-hematin which was insoluble in DMSO. The pellets were dissolved in 0.2 N NaOH (200 µl) and the obtained solubilized aggregates (free heme in NaOH) were prepared for spectroscopic quantification. The absorbance was recorded at 386 nm using UV-microplate reader. The amount of free monomeric heme was calculated from the standard curve of hemin chloride. The results were expressed as percentage of inhibition of β-hematin formation using the following formula:

$$\text{Inhibition}\,(\%) = \left(1 - \frac{[\text{Heme}]_{\text{drug}}}{[\text{Heme}]_{\text{control}}}\right) \times 100$$

where $[\text{Heme}]_{\text{drug}}$ = amount of heme in sample and $[\text{Heme}]_{\text{control}}$ = amount of heme in control.

Chloroquine diphosphate, dihydroartemisinin, and mefloquine HCl (all purchased from Tokyo chemical industry, Japan) were used as positive control and DMSO as a negative control. The assay was performed in triplicate in each of the three separated experiments. The twelve most potent chromone compounds as well as chloroquine diphosphate, dihydroartemisinin, and mefloquine HCl were determined for the IC_{50} values. The IC_{50} value was calculated from the log dose-response curve using Graphpad Prism 5. The reported IC_{50} values were the average values of the three independent experiments.

Drug-heme interaction assay

An aqueous DMSO (40% v/v) solution of 10 µM hemin chloride (pH 7.4) was freshly prepared by mixing 25 µl of 4 mM hemin chloride in 0.1 M NaOH with 4 ml of DMSO and 1 ml of 0.02 M sodium phosphate buffer (pH 6.0). The solution was adjusted to the volume of 10 ml with deionized water (hemin chloride was monomeric form in this solution). Solutions of chromone compounds, dihydroartemisinin and chloroquine diphosphate were prepared as described for hemin chloride.

Interaction of chromone compounds with hemin chloride was investigated by monitoring their effect on the absorption profile of hemin chloride. The spectral changes were determined by a continuous variation technique (Job's plot). Solutions containing 14 molar ratios of chromone compounds and hemin chloride were prepared as follows: compounds 28, 38, 42, and dihydroartemisinin, 0:1, 1:9, 1:4, 3:7, 2:3, 7:9, 1:1, 52:47, 9:7, 3:2, 7:3, 4:1, 9:1, and 1:0; compounds 3, 4, 23–27, 43, chloroquine, and mefloquine, 0:1, 1:9, 1:4, 3:7, 13:27, 7:13, 3:5, 2:3, 1:1, 3:2, 7:3, 4:1, 9:1, and 1:0; compound 36, 0:1, 1:9, 7:33, 1:4, 1:3, 11:29, 3:7, 2:3, 1:1, 3:2, 7:3, 4:1, 9:1, and 1:0. The final combined concentration of hemin chloride and chromone compound in the mixture was 10 µM. Absorbance spectra between 200 and 700 nm were recorded by a Shimadzu UV-Visible spectrophotometer. The absorbance change was calculated using the absorbance value from each molar ratio at the maximum wavelength 400 nm. Plots were constructed between the absorbance change versus the molar ratio of compound and heme.

RESULTS AND DISCUSSION

In vitro antimalarial activity against *P. falciparum*

In our previous study, we have reported the antimalarial activity against *P. falciparum* (K1, multidrug resistant strain) of twenty-one chromone compounds using microculture radioisotope assay[20]. In this study, a series of more than 21 compounds were tested using the same method. Table 1 summarizes the antimalarial activity and IC_{50} values of the studied compounds whose structures were classified as 3-unsubstituted and 3-substituted chromone derivatives. Compounds with inhibitory activity higher than 50% were subjected to IC_{50} determination. The three most potent compounds, 34, 36 and 39, belonging to 3-substituted series, displayed the IC_{50} 3.82, 0.95, and 4.87 µM, respectively. Preliminary structure-activity relationship could be deduced from the results that most of the 3-substituted chromone compounds (compounds containing R_2CO at position 3 of the chromone nucleus) showed the higher potency (IC_{50} 0.95–12.40 µM, Table 1) than the 3-unsubstituted derivatives (IC_{50} 9.15–19.66 µM). Comparison of the activity for compounds with same OH and R_2 substituted patterns showed that the 3-substituted compounds displayed better activity than the corresponding 3-unsubstituted compounds, for examples, compounds 39 (IC_{50} 4.87 µM) versus 11 (IC_{50} 14.69

Table 1 Structures and antimalarial activity (% inhibition and IC_{50} (μM)) of the 3-unsubstituted and 3-substituted chromones tested at the concentration 10 μg/ml.

3-Unsubstituted chromones				
Cp	Structure	R_2	% Inhibit	IC_{50}
1		Phenyl	−3.82	–
3		Benzyl	96.90[a]	9.43
4		Phenyl	71.90[a]	19.66
5		CH_3	−0.69	–
11		3′-(CF_3)-Phenyl	56.80	14.69
12		4′-(F)-Phenyl	17.49	–
13		3′, 5′-(diNO_2)-Phenyl	95.62	10.30
14		3′-(Cl)-Phenyl	23.18	–
15		3′, 4′-(diCl)-Phenyl	6.51	–
16		4′-(t-butyl)-Phenyl	82.30[a]	11.41
17		3′-(CF_3)-Phenyl	84.10[a]	11.07
18		4′-(F)-Phenyl	37.50[a]	–
19		3′, 4′-(diF)-Phenyl	24.30[a]	–
20		4′-(t-butyl)-Phenyl	95.20[a]	9.15
22		3′, 5′-(diNO_2)-Phenyl	−2.81	–
23		3′-(Cl)-Phenyl	76.20[a]	13.83
24		3′, 4′-(diCl)-Phenyl	99.50[a]	11.25
25		4′-(OCH_3)-Phenyl	27.50[a]	–
26		3′-(OCH_3)-Phenyl	28.00[a]	–
27		3′-(OCH_3)-Phenyl	68.00[a]	13.23
28		3′-(Cl)-Phenyl	39.52	–
29		4′-(F)-Phenyl	49.12	–
31		4′-(t-butyl)-Phenyl	8.40	–
49		3′-(OCH_3)-Phenyl	84.70[a]	13.94

3-Substituted chromones				
Cp	Structure	R_2	% Inhibit	IC_{50}
32		3′-(CF_3)-Phenyl	8.40[a]	–
33		3′-(Cl)-Phenyl	24.20	–
34		3′-(OCH_3)-Phenyl	98.38	3.82
35		4′-(F)-Phenyl	7.50[a]	–
36		4′-(NO_2)-Phenyl	77.50[a,b]	0.95
37		4′-(OCH_3)-Phenyl	66.19	9.32
38		3′, 4′-(diF)-Phenyl	65.10[a]	12.40
39		3′-(CF_3)-Phenyl	98.70[a]	4.87
40		3′-(Cl)-Phenyl	23.40	–
41		3′-(OCH_3)-Phenyl	-1.50	–
42		4′-(F)-Phenyl	-0.08	–
43		4′-(NO_2)-Phenyl	71.30[a]	9.85
44		4′-(OCH_3)-Phenyl	93.17	11.73
45		4′-(t-butyl)-Phenyl	98.50[a]	5.46
46		3′-(OCH_3)-Phenyl	65.93	10.47
47		4′-(NO_2)-Phenyl	99.50[a]	5.91
48		4′-(t-butyl)-Phenyl	89.07	9.33
50		3′-(OCH_3)-Phenyl	97.24	6.07
	Chloroquine			0.42
	Dihydroartemisinin			2.2×10^{-3}
	Mefloquine			5.7×10^{-2}
	Primaquine			2.41
	Tafenoquine			1.95

[a] results obtained from the previous study[20]; [b] activity tested at 1 μg/ml; Cp = compound,

μM), 45 (IC_{50} 5.46 μM) versus 16 (IC_{50} 11.41 μM), 46 (IC_{50} 10.47 μM) versus 26 (inactive), 48 (IC_{50} 9.33 μM) versus 31 (inactive), and 50 (IC_{50} 6.07 μM) versus 49 (IC_{50} 13.94 μM). With the exception of compounds 40 and 14, both showed a similar low-potency.

Inhibition of β-hematin formation

To preliminarily determine the possible the mechanism of antimalarial activity of the chromone compounds, the in vitro β-hematin formation inhibitory activity assay was performed. This assay closely paralleled the hemozoin formation within the parasite food vacuole. In this assay, heme (hemin chloride) was allowed to form β-hematin under acidic conditions (0.2 M acetate buffer, pH 4.4). In the presence of an inhibitor, the complexation of the inhibitor and heme led to the inhibition of β-hematin formation. The uncomplexed heme was allowed to generate β-hematin which was consequently converted into free monomeric heme. The amount of free heme was measured spectrophotometrically at 386 nm. The more the inhibitor-heme complex formed, the less the absorbance of free heme detected.

Forty-two chromone compounds were tested for their ability to inhibit β-hematin formation and the results obtained are summarized in Table 2. Compounds with more than 45% inhibition were considered as potent inhibitor (i.e., compounds 3, 4, 23–28, 36, 38, 42, and 43) and were subjected to IC_{50} determination. The six most potent compounds, 23–28 (IC_{50} values 1.41, 1.76, 2.30, 2.54, 4.60, and 3.69 μM, respectively) showed higher inhibitory activity than chloroquine, dihydroartemisinin, and mefloquine (IC_{50} 25.04, 18.04, and 15.78 μM, respectively). In contrast to antimalarial activ-

Table 2 The β-hematin formation inhibitory activity of the 3-unsubstituted and 3-substituted chromone derivatives tested at the concentration 30 μM.

3-Unsubstituted chromones		
Compound	% Inhibition	IC_{50} (μM)
1	14.55 ± 0.41	–
3	46.03 ± 0.99	30.56 ± 0.39
4	45.00 ± 0.79	27.72 ± 0.71
5	16.11 ± 0.38	–
11	37.19 ± 0.36	–
12	34.88 ± 1.01	–
13	16.19 ± 0.36	–
14	36.40 ± 0.71	–
15	14.18 ± 0.86	–
16	13.07 ± 0.20	–
17	18.91 ± 0.72	–
18	16.39 ± 0.52	–
19	25.64 ± 0.30	–
20	20.46 ± 0.33	–
22	23.92 ± 0.97	–
23	83.60 ± 0.21	1.41 ± 0.21
24	81.95 ± 0.60	1.76 ± 0.11
25	81.84 ± 1.06	2.30 ± 0.17
26	76.98 ± 0.35	2.54 ± 0.13
27	89.62 ± 0.28	4.60 ± 0.15
28	84.70 ± 0.42	3.69 ± 0.01
29	33.70 ± 0.26	–
31	38.43 ± 0.20	–
49	6.93 ± 0.16	–
3-Substituted chromones		
Compound	% Inhibition	IC_{50} (μM)
32	38.37 ± 0.23	–
33	31.34 ± 0.23	–
34	38.42 ± 0.35	–
35	39.90 ± 1.48	–
36	49.91 ± 1.02	21.65 ± 0.27
37	39.86 ± 0.50	–
38	50.90 ± 1.65	25.62 ± 0.39
39	9.18 ± 0.41	–
40	3.84 ± 0.21	–
41	2.40 ± 0.53	–
42	59.15 ± 0.93	22.54 ± 0.24
43	58.93 ± 0.41	24.01 ± 0.33
44	4.84 ± 0.19	–
45	10.48 ± 0.40	–
46	8.11 ± 0.20	–
47	9.63 ± 0.90	–
48	9.83 ± 1.06	–
50	6.93 ± 0.16	–
Chloroquine	43.13 ± 0.91	25.04 ± 0.26
Dihydroartemisinin	67.17 ± 0.92	18.04 ± 0.21
Mefloquine	77.74 ± 0.95	15.78 ± 0.12

Table 3 Classification of chromone compounds based upon the potency of antimalarial (AM) and β-hematin formation inhibitory (β-HFI) activities.

Group	AM	β-HFI	Compounds
I	high	high	3, 4, 23, 24, 27, 36, 38, 43
II	high	low	11, 13, 16, 17, 20, 34, 37, 39, 44–50
III	low	high	25, 26, 28, 42
IV	low	low	1, 5, 12, 14, 15, 18, 19, 22, 29, 31–33, 35, 40, 41

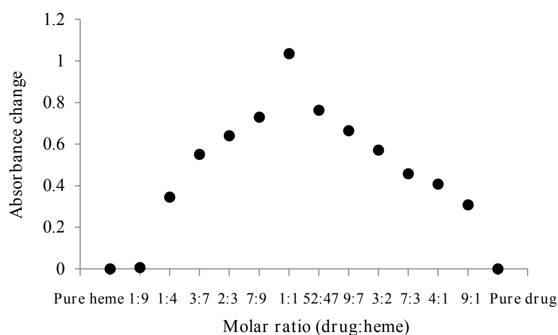
Table 4 The stoichiometric ratio (chromone:heme) of compounds in Groups I and III.

Stoichiometric ratio	Compounds
1:1	Group I: 38 Group III: 28, 42 Dihydroartemisinin
1:2	Group I: 3, 4, 23, 24, 27, 43 Group III: 25, 26 Chloroquine, Mefloquine
1:3	Group I: 36

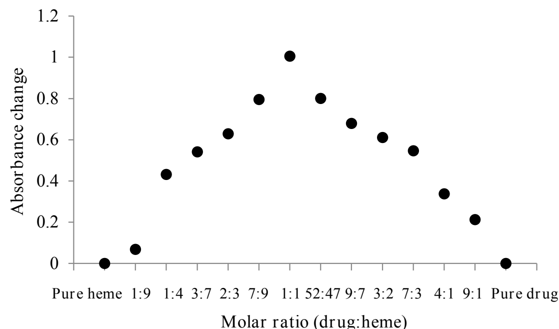
ity study, these potent compounds belonged to 3-unsubstituted chromones series. However, compounds in 3-substitued chromone series, 36, 38, 42, and 43 (IC_{50} 21.65, 25.62, 22.54, and 24.01 μM, respectively) were found to possess slightly higher activity than chloroquine.

Table 3 shows the classification of chromone compounds based upon the potency of antimalarial and β-hematin formation inhibitory activities. Compounds 3, 4, 23, 24, 27, 36, 38, and 43 in Group I displayed highly potent antimalarial activity (IC_{50} 0.95–19.66 μM) and β-hematin formation inhibitory activity (IC_{50} 1.41–30.56 μM). In group II, compounds 11, 13, 16, 17, 20, 34, 37, 39, 44–50 showed potent antimalarial activity (IC_{50} 3.82–14.69 μM) but they possessed low β-hematin formation inhibition (less than 45% inhibition). On the other hand, compounds 25, 26, 28, and 42 in Group III were potent β-hematin formation inhibitor (IC_{50} 2.30, 2.54, 3.69, and 24.01 μM, respectively) but weak as antimalarial drug (less than 40% inhibition). The rest of the compounds (i.e., 1, 5, 12, 14, 15, 18, 19, 22, 29, 31–33, 35, 40, and 41) in Group IV were inactive as antimalarial and showed low β-hematin formation inhibitory activity (less than 45% inhibition).

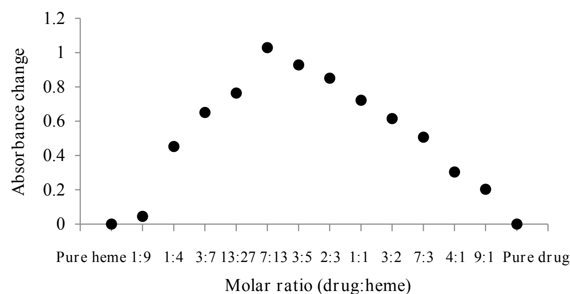
Dihydroartemisinin



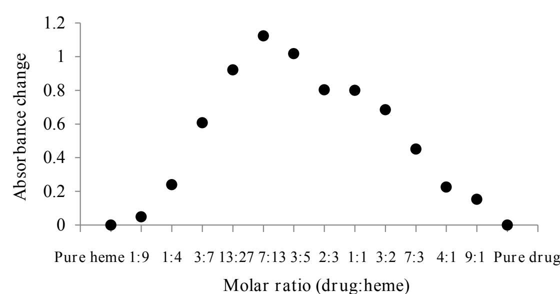
Compound 28



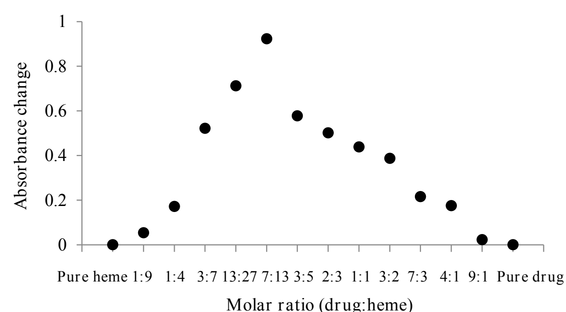
Chloroquine



Compound 23



Compound 25



Compound 36

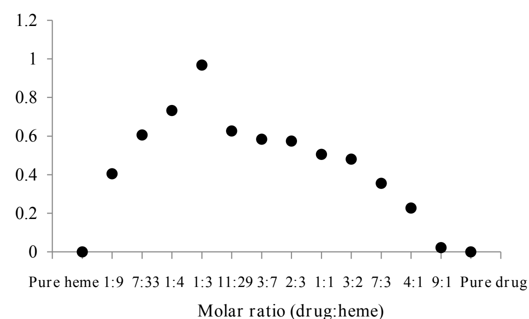


Fig. 2 Job's plots of dihydroartemisinin, chloroquine, and chromone compounds forming complex to heme with stoichiometric ratio of chromone:heme = 1:1 (dihydroartemisinin and compound 28), 1:2 (chloroquine and compounds 23 and 25), and 1:3 (compound 36).

Drug-heme interaction

Gorka et al have reported that the interactions between quinoline related drugs (including chloroquine) and heme leading to the inhibition of β-hematin formation which correlated with parasite growth inhibition[11]. In this study, the ability of chromone compounds to interact and form complex with heme was investigated by the continuous variation method (Job's plot)[24]. This method was based on the difference between UV spectral characteristics of free heme and drug-heme complex. A solution of hemin chloride showed a sharp peak at 400 nm, indicating that free heme predominated under the in vitro condition used (40% DMSO, sodium phosphate buffer at pH 6.0). The addition of potent chromone compounds (i.e., compounds 3,

4, 23, 24, 27, 36, 38, and 43 in Group I and compounds 25, 26, 28, and 42 in group III) resulted in decreasing of the absorption band of free heme, indicating that compounds formed complex with free heme. Plots between the difference in free heme absorbance at 400 nm versus the chromone:heme molar ratio were performed. Absorbance changes intensity were maximal when the molar fraction of compound 28 to heme was 1:1 (Fig. 2 for representative plots) indicating the stoichiometric ratio of chromone:heme = 1:1 as did dihydroartemisinin under the same experiment. The most potent compounds 23 (Group I) and 25 (Group III) showed the same stoichiometric ratio of 1:2 as chloroquine whereas compound 36 showed ratio of 1:3. Table 4 summarizes the stoichiometric ratios of compounds in Group I and III complexed with heme. These results indicated the ability of the chromone compounds to interact with free monomeric heme and confirmed the β-hematin formation inhibitory activity of the compounds.

CONCLUSIONS

The in vitro antimalarial activity against *P. falciparum* showed that most of the chromone compounds displayed moderate to high activity (IC_{50} 0.95–19.66 μM). β-Hematin formation inhibition assay has been performed to explore the preliminary mechanism of antimalarial activity of the studied compounds. Eight out of forty-two chromone compounds (compounds 3, 4, 23, 24, 27, 36, 38, and 43) were found to exhibit high potency in both antimalarial and β-hematin formation assays. Compounds 23–28 showed higher β-hematin formation inhibitory activity than chloroquine, dihydroartemisinin, and mefloquine. Job's plots revealed that compounds 23 and 24 strongly interacted with heme and formed stoichiometric ratio of chromone:heme = 1:2 same as chloroquine. Although further investigations are needed, the current data basically suggested that inhibiting β-hematin formation might be one of the mechanisms of antimalarial activity of compounds in chromone series.

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