PLASMEPSIN II INHIBITORY ACTIVITY OF ALKOXYLATED AND HYDROXYLATED CHALCONES

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Abstract. Sixteen antimalarial alkoxylated and hydroxylated chalcones were tested for their ability to inhibit recombinant plasmepsin II *in vitro*. The best inhibitory compounds had either a chloro or dimethylamino group at the 4-position of phenyl ring A in the chalcone template. Combination of the chalcones with chloroquine showed additivity or mild antagonism of *Plasmodium falciparum* K1 growth in culture.

INTRODUCTION

The increasing resistance of *Plasmodium falciparum*, the most virulent of the four human malaria parasite species, to most available antimalarial drugs indicates the need for development of new antimalarial drugs and an understanding of their targets (Greenwood *et al*, 2005). There have been several approaches to antimalarial drug development, namely, using combinations of old and/or new compounds, modifications of existing compounds and discovery of natural products (Rosenthal, 2001).

Chalcones, composed of two aromatic rings linked by α, β-unsaturated alkenone (1, 3-diarylpropen-1-ones), are plant natural products belonging to a subgroup of flavonoids (Harborne *et al*, 1975). Screening of plant extracts identified licochalcone A from Chinese licorice roots as having the ability to inhibit *P. falciparum* growth in culture and to protect mice from lethal infection of *P. yoelii* (Chen *et al*, 1994). Molecular modeling studies resulted in the discovery of chalcone as a promising

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inhibitor of malarial cysteine protease (Li *et al*, 1995). Several analogs of chalcones were subsequently synthesized and demonstrated to be active against both chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *P. falciparum,* including chalcones sharing a common quinoline ring with chloroquine (Li *et al,* 1995). A series of quinolinyl chalcones were shown to be able to inhibit *in vitro P. falciparum* falcipain, a cysteine protease responsible for degrading hemoglobin in the parasite acidic food vacuole (Dominguez *et al,* 2001).

We describe here investigations of the ability of a series of previously synthesized antimalarial alkoxylated and hydroxylated chalcones (Liu *et al*, 2001) to inhibit *P. falciparum* plasmepsin II, one of four aspartic acidic proteases present in the parasite food vacuole that are also involved in hemoglobin degradation (Banerjee *et al*, 2002).

MATERIALS AND METHODS

Parasite culture

Chloroquine-resistant K1 strain of *P. falciparum* was continuously cultured under "candle jar" conditions as described by Trager and Jensen (1976) at 37ºC in human O[+] erythrocytes suspended in RPMI 1640 medium supplemented with 25 mM NaHCO$_3$, 25 mM

HEPES, 40-50 µg/l gentamicin sulfate and 10% human serum. The medium was changed every 24 hours and the parasite synchrony was maintained by sorbitol treatment (Lambros and Vanderberg, 1979).

Determination of *in vitro* antimalarial activity

Antiplasmodial activity of the chalcones was assessed using [³H]hypoxanthine incorporation assay as described by Desjardins *et al* (1979). Stock solutions of chloroquine (Sigma) and chalcones were prepared in sterile distilled water and dimethylsufoxide (DMSO) respectively, and were freshly diluted in medium without serum to give appropriate concentrations before use. A 25 µl aliquot of each drug dilution or medium without serum (control) and 200 µl of erythrocyte suspension of 1.5% hematocrit with 1% parasitemia were added to triplicate wells of a 96-well plate. After incubation at 37ºC in a candle jar for 24 hours, 25 µl of 10 µCi/ml [³H]hypoxanthine (specific activity of 17.4 Ci/mmol, Perkin Elmer) were added to each well. Cultures were further incubated for 18-24 hours, and the contents of each well were harvested onto glass filter papers using a cell harvester (Nunc). After drying and soaking the filter papers with liquid scintillation fluid, radioactivity incorporated into the parasites was then counted in a liquid scintillation counter (Beckman LS1801). IC_{50}, the drug concentration inhibiting ³H-incorporation by 50% compared to control, was estimated by plotting percent ³H-incorporation against log of drug concentration.

Evaluation of drug combinations was performed as follows. Each compound was diluted at double the concentration used in single drug test. An aliquot of 12.5 µl of chloroquine, 12.5 µl of chalcone and 200 µl of erythrocyte suspension of 1.5% hematocrit with 1% parasitemia were added to triplicate wells. The plates were then placed in a candle jar and incubated at 37ºC for 24 hours and processed as described above. Results were recorded as the mean sums of the fractional inhibitory concentrations (FIC), defined as (IC_{50} of chloroquine in mixture/ IC_{50} of chloroquine alone) + (IC_{50} of chalcone in mixture/ IC_{50} of chalcone alone) for each fixed concentration. Three types of drug interaction were defined as follows: additive, sum of FIC = 1; synergistic, sum of FIC < 0.5; antagonistic, sum of FIC > 4 .

Plasmepsin II inhibition assay

Recombinant plasmepsin II was kindly provided by Dr Jirundon Yuvaniyama, Department of Biochemistry, Faculty of Science, Mahidol University. Stock solutions of chalcones and plasmepsin II substrate, Malaria-FRET (DABSYL-Glu-Arg-Nle-Phe-Leu-Ser-Phe-Pro-EDANS; AnaSpec Inc), were prepared in DMSO. Plasmepsin II activity was measured in a total volume of 1 ml containing 2 µM Malaria-FRET, 0.3 nM plasmepsin II, 100 mM sodium acetate buffer pH 5.0, 0.01% Tween 20 and 10% glycerol. Fluorescence was monitored at emission wavelength of 490 nm, excitation at 336 nm, for 3 minutes at 25ºC in a spectrofluorophotometer (Shimadzu RF-5301). For inhibition assay, 10 µM chalcone were preincubated in the mixture for 5 minutes before the addition of substrate to initiate the reaction. The final concentration of DMSO was 5%, which did not interfere with the assay. Results are reported as percent inhibition of the rate of fluorescence increase in the absence of the inhibitor.

RESULTS

Assay of the proteolytic activity of *P. falciparum* plasmepsin II was based on the use of a fluorogenic peptide substrate containing a fluorophor (fluorescence donor group EDANS, 5-[(2-aminoethyl) amino]naphthalene-1-sufonic acid) at one end and a quencher [fluorescence acceptor group DABCYL, 4-(4-dimethyl-amino-phenylazo) benzoic acid] at the other end of the molecule. In the absence of the enzyme, the two moieties are in close proximity

Table 1

Alkoxylated and hydroxylated chalcones inhibition of plasmepsin II and of *P. falciparum* growth in culture, either alone or in combination with chloroquine.
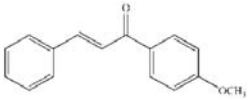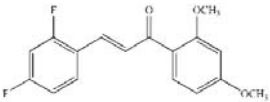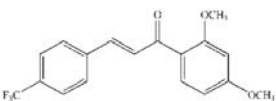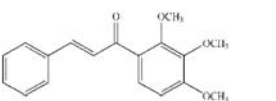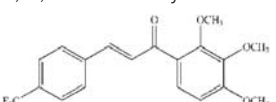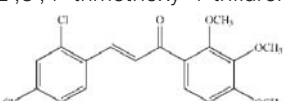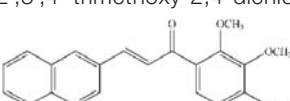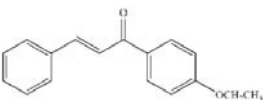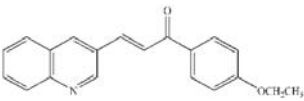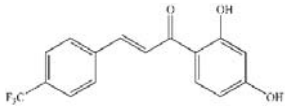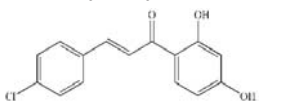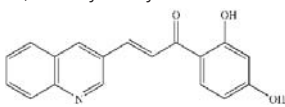
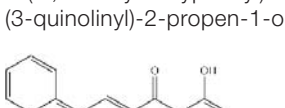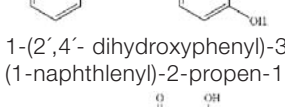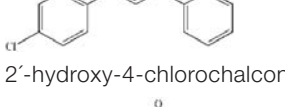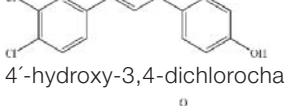| Chalcone	Code	Plasmepsin II activity	Inhibition of *P. falciparum* growth	
		(percent inhibition)	IC_{50} (μM) (alone)	Sum of FIC (combination)
4´-methoxychalcone	MC	24	84	0.91-1.55
2´,4´-dimethoxy-2,4-difluorochalcone	DMDF	20	6	ND
2´,4´-dimethoxy-4-trifluromethylchalcone	DMTFM	16	6	ND
2´,3´,4´-trimethoxychalcone	TM	15	16	0.92-1.76
2´,3´,4´-trimethoxy-4-trifluromethylchalcone	TMTFM	21	9	0.95-1.72
2´,3´,4´-trimethoxy-2,4-dichlorochalcone	TMDCl	17	23	0.97-1.41
1-(2´,3´,4´-trimethoxyphenyl)-3-(3-quinolinyl)-2-propen-1-one	TM3Q	18	8	0.94-1.63
4´-ethoxychalcone	EC	22	66	0.98-1.70
1-(4´-ethoxyphenyl)-3-(3-quinolinyl)-2-propen-1-one	E3Q	24	50	1.00-2.21

Table 1 (continued).

Chalcone	Code	Plasmepsin II activity	Inhibition of *P. falciparum* growth	
		(percent inhibition)	IC$_{50}$ (μM) (alone)	Sum of FIC (combination)
2′,4′-dihydroxy-4-trifluromethylchalcone	DHTFM	12	13	1.07-1.79
2′,4′-dihydroxy-4-chlorochalcone	DH4Cl	29	12	ND
1-(2′,4′- dihydroxyphenyl)-3-(3-quinolinyl)-2-propen-1-one	DH3Q	16	28	0.89-1.43
1-(2′,4′- dihydroxyphenyl)-3-(1-naphthlenyl)-2-propen-1-one	DH1N	19	25	ND
2′-hydroxy-4-chlorochalcone	2HCl (4)	28	13	ND
4′-hydroxy-3,4-dichlorochalcone	4HDCl (3,4)	18	18	ND
4′-hydroxy-4-dimethylaminochalcone	4HDMA	30	18	ND

ND = not done

so that any photon emitted from the fluorophor is quenched by the acceptor group (a phenomenon known as fluorescence resonance energy transfer, FRET) (Gulnik *et al*, 1997). Upon cleavage of the substrate, the fluorophor is free from the influence of the quencher, and its emitted fluorescence can be detected. The peptide substrate, Malaria-FRET, contains a cleavage site (Phe-Leu) of plasmepsin II (Goldberg *et al*, 1991). The assay was conducted at pH 5.0 to mimic the acidic condition of the parasite food vacuole. Km for Malaria-FRET was 0.43 μM (data not shown).

Table 1 summarizes the inhibition studies

of 16 alkoxylated and hydoxylated chalcones, together with their IC_{50} values against *P. falciparum* K1 strain. Inhibition of plasmepsin II activity ranged from 12 to 30 %, the most potent chalcones being 2HCl (28%), DHCl (29%) and 4HDMA (30%). The IC_{50} values were in agreement with the previous report (Liu *et al*, 2001). A correlation between percent inhibition of plasmepsin II activity *in vitro* and IC_{50} value of *P. falciparum* K1 growth in culture was not discernable. A similar observation has been reported for chalcones that inhibit falcipain (Dominguez *et al,* 2001).

If the chalcone analogs target plasmepsin II *in situ*, they should antagonize the inhibitory effect of chloroquine on *P. falciparum* growth. Studies of 9 of the chalcone analogs in combination with chloroquine showed only an additive phenomenon or mildly antagonistic effect at best (Table 1).

DISCUSSION

Falcipain has been identified as a possible target of chalcones (Dominguez *et al* 2001). From structure-function studies, alkoxylated and hydroxylated chalcones possessing alkoxylated B rings and electron-deficient A rings have good antiplasmodial activity (Liu *et al*, 2001). We demonstrate here that alkoxylated and hydroxylated chalcones are able to inhibit recombinant plasmepsin II *in vitro*. Although a limited number of chalcones were tested, analogs having Cl or $N(CH_3)_2$ at the 4-position of the phenyl ring A were superior inhibitors of plasmepsin II.

To show that the chalcone analogs were able to target plasmepsin II *in situ*, the chalcones were tested in combination with chloroquine for their inhibition of *P. falciparum* growth in culture, on the notion that drugs that prevent hemoglobin degradation would antagonize the inhibitory effect of chloroquine, whose mechanism of action is to interfere with parasite heme detoxification (formation of hemozoin) within the acidic food vacuole by binding with heme released from the proteolytic degradation of host hemoglobin (Sullivan *et al*, 1996; Mungthin *et al*, 1998). Only additive or mildly antagonistic effects were observed (sum of FIC in the range of 0.87-2.21) suggesting that these alkoxylated and hydroxylated chalcones are unable to reach plasmepsin II *in situ* and/or that they have other mechanisms of action. We have previously shown that antiplasmodial chalcones with methoxy or dimethoxy (viz. DMDF) groups on ring B inhibit sorbitol-induced hemolysis of *P. falciparum*-infected erythrocytes (Go *et al*, 2004).

In summary, this study has demonstrated that alkoxylated and hydroxylated chalcones can inhibit *P. falciparum* aspartic protease plasmepsin II *in vitro*, but they may not be able to reach this target within the parasite acidic food vacuole.

ACKNOWLEDGEMENTS

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