European Journal of Medicinal Chemistry 44 (2009) 3388–3393

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Antimalarial pharmacodynamics of chalcone derivatives in combination with artemisinin against *Plasmodium falciparum* in vitro

Amit Bhattacharya [a], Lokesh C. Mishra [a], Manish Sharma [a], Satish K. Awasthi [b,*], Virendra K. Bhasin [a,**]

[a] Department of Zoology, North Campus, University of Delhi, New Delhi 110007, India
[b] Chemical Biology Laboratory, Department of Chemistry, University of Delhi, Delhi 110007, India

ARTICLE INFO

Article history:
Received 6 December 2008
Received in revised form
2 February 2009
Accepted 12 February 2009
Available online 21 February 2009

Keywords:
Artemisinin
Antimalarial chalcone combination
Chalcone derivatives
In vitro interaction

ABSTRACT

Use of artemisinin based combination therapies (ACTs) is increasing in treatment of malaria. Their extensive and indiscriminate deployment will ultimately lead to selection of resistance. Thus, alternate ACTs are needed. We reported in vitro antimalarial potential of chalcone derivatives. A few potent chalcones were selected for their antimalarial interaction in combination with artemisinin in vitro. Combinations evaluated show synergistic or additive interactions. Chalcones act on broad range of asexual stages of the parasite. The synergistic combinations decrease hemozoin formation in parasitized erythrocytes. These combinations do not affect new permeation pathways induced in the host cells. This is the first report showing antiplasmodial interactions between artemisinin and synthetic chalcone azole derivatives. Thus, chalcones and artemisinin combinations open the possibility of novel ACTs.

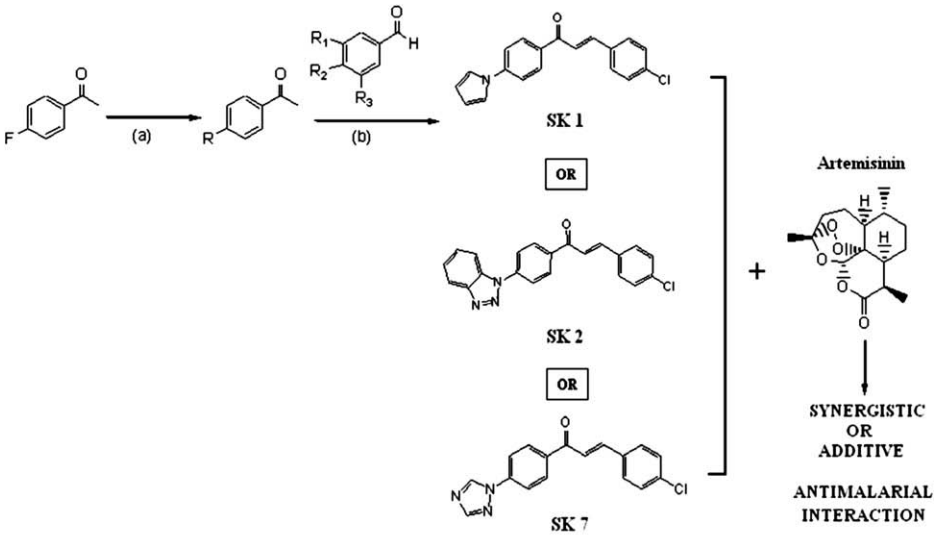
1. Introduction

Many modern medicines developed over the recent past have been derived from or inspired by particular natural products [1]. Natural products have also been the rich source of antimalarial drugs. Artemisinin was isolated and identified in 1970s as the principal antimalarial compound from extracts of a shrub *Artemisia annua*. The foliage decoctions of *A. annua*, though, have been used in China for over 2000 years for fever resolution [2]. Artemisinins (artemisinin and its derivatives) now represent the most powerful antimalarial drugs: resounding the success of quinine in the treatment of malaria, discovered some 350 years ago. These plant products have made a lasting impact in malaria treatment for long periods – spanning centuries – whereas; therapeutic life span of most synthetic antimalarial drugs has often been short-lived, lasting at the most for decades. Today artemisinins are the only drugs that cure chloroquine-resistant *Plasmodium falciparum* infections, and clinical resistance to these compounds has not yet been reported. To avert quick emergence of resistance to this vital class of compound, the malariologists have agreed not to deploy artemisinins as monotherapy but in combination with other antimalarial(s). WHO now recommends treating even uncomplicated malaria with artemisinin based combination therapies (ACTs) [3] in areas of chloroquine resistance, leading to increase in demand of artemisinin. Artemisinin being a natural product is at times in short supply [4]. Synthesis of artemisinin is difficult and expensive. Mounting demand and use of the few available ACTs is likely to result in selection of the resistant-parasites sooner than expected. Thus, there is a need for alternate ACTs. Continuous in vitro cultures of *P. falciparum* provide a good initial screen system for new ACTs. Natural products or their synthetic derivatives as partner drugs which decrease dependence on artemisinin form an attractive search option for novel ACTs.

Chalcones are aromatic ketones and key biosynthetic intermediates for combinatorial assembly of different heterocyclic scaffolds. They form an important group of natural compounds which are easy to synthesize, and some of them show wide range of biological activities. Licochalcone A, an oxygenated chalcone, isolated from the roots of Chinese licorice was reported to have antiplasmodial activity [5]. This prompted researchers to design and synthesize a variety of chalcone derivatives, and to evaluate their antimalarial potential [6,7]. Likewise we first reported the antiplasmodial activity of chalcones with azoles on acetophenone ring [8]. Three of these azole derivatives inhibited the parasite multiplication rate to 50% (IC_{50}) at concentrations of less than 3 μg/ml, indicating the potential to be developed as an antimalarial [7]. We considered it interesting to investigate the antiplasmodial interactions of artemisinin in combination with each of these three synthesized novel chalcone derivatives (Scheme 1). These chalcone derivatives alone or each in fixed-ratio combinations with artemisinin were employed to assess

* Corresponding author. Tel.: +91 11 27662104.
** Corresponding author. Tel.: +91 11 27667989; fax: +91 11 27667524.
E-mail addresses: awasthisatish@yahoo.com (S.K. Awasthi), virendrabhasin@hotmail.com (V.K. Bhasin).

0223-5234/$ – see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2009.02.008



SK 1 = 1-(4-(1H-pyrrol-1-yl)phenyl)-3-(4-chlorophenyl)prop-2-en-1-one

SK 2 = 1-(4-(1H-benzo[d][1,2,3] triazol-1-yl)-phenyl)-3-(4-chlorophenyl)-prop-2-en-1-one

SK 7 = 1-(4-(1H-1,2,4-triazole-1-lyl)-phenyl)-3-(4-chlorophenyl)-prop-2-en-1-one

Scheme 1.

their antiplasmodial activity in vitro. An isobologram method [9] was used to analyze the synergistic, antagonistic or additive interactions of the combinations. We also determined the combination effect of these compounds on hemozoin formation by the parasites; sorbitol-induced hemolysis of the parasitized erythrocytes and compared stage-specific inhibition of parasites by chalcone derivatives, with an aim to obtain clues to the mode of action of these combinations.

2. Results

2.1. In vitro sensitivity of parasites

The concentrations required to achieve IC_{50} with each of the compound evaluated is given in Table 1. Artemisinin was found to be the most effective followed by chloroquine, licochalcone A, SK7, SK2 or SK1 as shown by molar IC_{50}. These concentrations were in nM range for known antimalarials like artemisinin and chloroquine, whereas in μM range for the chalcone derivatives assayed.

2.2. In vitro stage-specific inhibition

Chalcone derivatives were found to be toxic to different asexual stages of *P. falciparum*. They are more toxic to trophozoite stages followed by ring and schizont stages (Table 2).

2.3. Antiplasmodial interactions between artemisinin and chalcone derivatives

In every combination assay IC_{50} was determined from two sets of drug response curves obtained from each replicate. Each set representing four combination curves and a curve of drug/compound alone. Mean FIC_{50} values derived from these curves are tabulated in Tables 3–5, for each chalcone derivative combination with artemisinin. Sum of FICs are presented in three isobolograms for each chalcone derivative SK1, SK2 or SK7 in combination with artemisinin (Fig. 1A,B,C). The isobolograms show that antimalarial interaction of the chalcone derivatives in vitro with artemisinin is not antagonistic. Compound SK1 in combination with artemisinin shows synergistic antiplasmodial interaction in three of the four fixed-ratio combinations evaluated and additive in the other. Similarly the combination of SK2 and artemisinin displays synergistic interaction in two combinations and additive in rest of the two combinations. Interaction of artemisinin with SK7 is consistently found to be additive.

2.4. Effect of drugs on hemozoin formation

Amount of hemozoin formed reflects the extent of hemoglobin digestion. Table 6 shows the percentage hemozoin formation by the parasites in the presence of artemisinin or chalcone derivatives and some synergistic combinations of these. Hemozoin formation in pyrimethamine treated parasites serve as a negative control. Hemozoin formation in parasites treated with IC_{50} concentration of artemisinin shows less than expected 50% pigment formation, so also treatment with the chalcone derivatives and some synergistic combinations evaluated. The results indicate that chalcone derivatives or artemisinin treatment affected the hemoglobin digestion in the parasites leading to reduced amount of hemozoin than expected.

Table 1
In vitro sensitivity of *P. falciparum* strains 3D7 to artemisinin, licochalcone A, chloroquine and chalcone derivatives.

Compounds	Mean $IC_{50} \pm SE$[a]
Artemisinin	4 nM ± 0.1
Licochalcone A	4.2 μM ± 0.2
Chloroquine	0.03 μM ± 0.01
SK1	9.5 μM ± 0.3
SK2	7 μM ± 0.8
SK7	4.9 μM ± 0.1

[a] Standard error ($n = 3$).

Table 2
Stage-specific effect of artemisinin and chalcone derivatives was determined by exposing ring, trophozoite and schizont stages to IC_{50} of each of the compounds for a period of 18 h for ring and trophozoite stages and 12 h for schizont, their respective residence time in each of these stages. Percent inhibition of parasitemia in relation to control was determined at the end of 48 h erythrocytic cycle.

Compound	Percentage Inhibition ± S.E		
	Ring stage	Trophozoite stage	Schizont stage
SK1	51.4 ± 0.4	**61.5 ± 1.1**	48.9 ± 1.5
SK2	47.9 ± 1.3	**70.9 ± 0.9**	41 ± 0.7
SK7	48.7 ± 1.0	**65.8 ± 0.8**	40.1 ± 0.8

2.5. Effect of drugs on sorbitol-induced hemolysis

New permeation pathways are produced in the host erythrocytes by the developing intracellular parasites. These novel channels are blocked to the extent of about 50% by 10 μM frusemide resulting in reduced hemolysis. The results presented in Table 6 show that neither of the drugs used alone or in combination with chalcone derivatives reduced the hemolysis following sorbitol treatment, except for SK7 treated parasites. The results indicate that SK7 blocks some sorbitol-induced hemolysis.

3. Discussion

Artemisinin and quinine continue to retain their therapeutic efficacy against malaria for centuries [10]. Probably for most part of the history these two natural products had been used as multicomponent extracts of plant parts for therapeutics. There is consensus now that appropriate antimalarial combination therapies are capable to postpone the emergence of resistance [11]. Today ACTs are regarded as drugs of choice to cure any malaria, even chloroquine-resistant infections. Mounting deployment of the few available ACTs is likely to select resistance to these combinations sooner than expected. Thus, there is a huge unmet need for novel ACTs. Accordingly, it is an attractive proposition to search for alternate combinations which could decrease dependence on artemisinin without compromising on the potency to cure malaria.

Blood stage cultures *P. falciparum* provide a good initial screen for identifying schizontocidal action of a compound. Using this in vitro system we measured the effect of compounds on parasite multiplication by counting the 'live' parasites from a Giemsa stained thin blood films at the end of 48 h erythrocytic cycle. This morphological method, though tedious, was found to be more reproducible compared to the [^{3}H]-hypoxanthine incorporation method [12], in our lab as well. Antimalarials and chalcone derivatives studied for their inhibitory effect on parasites showed concentration dependent growth inhibition. IC_{50} values of licochalcone A and other antimalarials evaluated have been found to be comparable to those reported by others [13,14]. The stage-specific toxicity of three chalcone derivatives showed that they all inhibit R, T and S-stages at their respective IC_{50} values implying broad range inhibition of asexual stages. Similar observations have been made for ART [10]. Relatively, however, chalcone derivatives are more toxic to T-stages. ART is also known to be toxic to sexual stages [15].

We further employed erythrocyte cultures to study interactions of ART in combination with a chalcone derivative to determine schizontocidal activity of the combination on *P. falciparum* in vitro. Chalcones constitute an important group of natural products with selective inhibition against *P. falciparum*, first reported for licochalcone A [5], and low cost of production; thus making them promising compounds for evaluation in combination with artemisinin. Antiplasmodial activity of chalcone derivatives in combination with artemisinin has not been previously reported. ART in combination with SK1 or SK2 showed synergistic or additive interactions. SK7 being most effective chalcone derivative evaluated in inhibiting the parasite growth showed additive interaction in combination with ART. We recently reported that licochalcone A and ART combination shows synergistic interaction in vitro [16]. Interactions between ART and chalcone derivatives evaluated and analyzed using the fixed-ratio isobolograms demonstrate that none of the interactions are antagonistic.

Ideally components of the antimalarial combinations should target different metabolic pathways. Exact mode of antiplasmodial action of ART as well as chalcone derivatives is not known. There are varied views regarding ARTs' mode of action on *P. falciparum*. ART is a sesquiterpene lactone, with an endoperoxide bridge, which is believed to interact with the iron in the parasitized red blood cells to form free radicals that then destroy proteins in nanodomain vicinity [17], and exert pleiotropic ultrastructural damage to the parasite, leading to total disintegration of the parasite [18]. It has also been demonstrated that ART and its derivatives inhibit an essential calcium adenosine triphosphatase (PfATPase) enzyme [19], disrupting calcium homeostasis. Inhibition of this vital enzyme causes global release of calcium stored in endoplasmic reticulum into the cytoplasm, causing parasite death. Artemisinin has also been reported to be an effective inhibitor of heme polymerization activity mediated by *P. falciparum* histidine-rich protein II [20]. It has also been suggested that artemisinin react covalently

Table 3
Interaction between artemisinin and SK1 chalcone derivative against *P. falciparum* (3D7 strain) at six different preparations.

Combination preparation	Compound A (Artemisinin) Mean FIC_{50}[a]	Compound B (SK 1) Mean FIC_{50}[a]	∑FICs, interaction[b]
1	1.0 ± 0.02	0	1.0, ADD
2	0.5 ± 0.1	0.1 ± 0.02	0.6, SYN
3	0.3 ± 0.05	0.2 ± 0.04	0.5, SYN
4	0.3 ± 0.01	0.4 ± 0.01	0.7, SYN
5	0.2 ± 0.002	0.9 ± 0.01	1.1, ADD
6	0	1.0 ± 0.01	1.0, ADD

[a] Standard error ($n = 3$).
[b] SYN, synergy; ADD, additive.

Table 4
Interaction between artemisinin and SK2 chalcone derivative against *P. falciparum* (3D7 strain) at six different preparations.

Combination preparation	Compound A (Artemisinin) Mean FIC_{50}[a]	Compound B (SK 2) Mean FIC_{50}[a]	∑FICs, interaction[b]
1	1.0 ± 0.01	0	1.0, ADD
2	0.9 ± 0.008	0.2 ± 0.01	1.1, ADD
3	0.4 ± 0.003	0.3 ± 0.01	0.7, SYN
4	0.3 ± 0.01	0.4 ± 0.004	0.7, SYN
5	0.3 ± 0.002	1.00 ± 0.01	1.3, ADD
6	0	1.0 ± 0.004	1.0, ADD

[a] Standard error ($n = 3$).
[b] SYN, synergy; ADD, additive.

Table 5
Interaction between artemisinin and SK7 chalcone derivative against *P. falciparum* (3D7 strain) at six different preparations.

Combination preparation	Compound A (Artemisinin) Mean FIC_{50}[a]	Compound B (SK 7) Mean FIC_{50}[a]	∑FICs, interaction[b]
1	1.0 ± 0.055	0	1.0, ADD
2	1.0 ± 0.1	0.2 ± 0.03	1.2, ADD
3	0.8 ± 0.004	0.5 ± 0.01	1.3, ADD
4	0.6 ± 0.4	0.9 ± 0.01	1.5, ADD
5	0.3 ± 0.01	1.1 ± 0.08	1.4, ADD
6	0	1.0 ± 0.03	1.0, ADD

[a] Standard error ($n = 3$).
[b] ADD, additive.

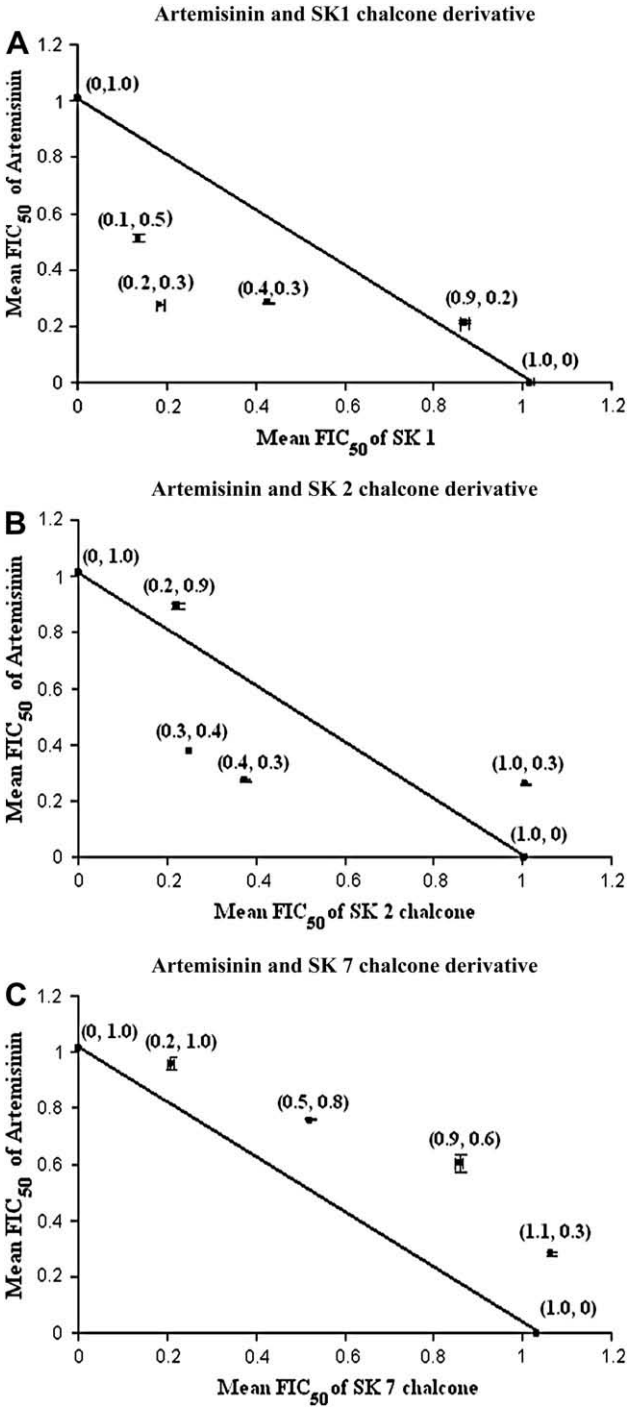


Fig. 1. Isobologram showing interaction between artemisinin and chalcone derivatives against *P. falciparum* 3D7 strain.

with heme of *P. falciparum* hemozoin both in vitro and in situ [21]. Thus multi-targeted attack on the parasite by ART may also be responsible for the non-emergence of resistance to this drug, despite its use for centuries. The available ACTs are safe and well tolerated [22]. The chalcone derivatives are believed to interact and inhibit the *P. falciparum* enzyme cysteine protease (falcipain), one of the key enzymes involved in the hemoglobin degradation within the acidic food vacuole of the intra-erythrocytic parasite [23]. The toxic heme (ferriprotoporphyrin IX) molecules released from hemoglobin catabolism is converted to non-toxic hemozoin (β-hematin) within the food vacuole [24] and inhibition of this

Table 6
In vitro sensitivity of artemisinin, chalcone derivatives and their synergistic combinations against *P. falciparum* (3D7 strain) to determine the percent hemozoin formation, and percent sorbitol-induced hemolysis.

Drug	Concentration used (IC_{50})	% Hemozoin formation[a]	Percent sorbitol-induced hemolysis	
			Concentration used	% Hemolysis[a]
Artemisinin	4 nM	48.3 ± 1.4	5 nM	90.9 ± 0.2
SK 1	9.5 μM	48.8 ± 0.5	10 mM	85.9 ± 0.2
SK 2	7 μM	47.7 ± 0.2	10 mM	90.0 ± 0.2
SK 7	4.9 μM	47.8 ± 1.4	10 mM	36.1 ± 0.2
ART-SK1 3:2	19.2 nM: 30.8 μM	47.8 ± 0.5	3 nM:4 μM	84.7 ± 1.5
ART-SK1 2:3	12.8 nM: 46.2 μM	48.6 ± 0.4	2 nM: 6 μM	88.8 ± 1.7
ART-SK2 3:2	19.2 nM: 22.4 μM	47.6 ± 0.8	3 nM:4 μM	91 ± 1.1
ART-SK2 2:3	12.8 nM: 33.6 μM	47.1 ± 1.8	2 nM: 6 μM	88.6 ± 0.6
Pyrimethamine	3.5 nM	56.3 ± 1.0	10 nM	92.2 ± 0.4
Frusemide	ND	ND	10 μM	56.3 ± 0.2

ND, not determined.
[a] Standard error ($n = 3$).

process proves fatal for the parasite. Structure-based drug design of antimalarial chalcone derivatives predicts trophozoite cysteine protease as the most likely target enzyme [6,25]. Chalcones are α, β-unsaturated ketones [26] that assume linear or nearly planar structure. These compounds are stable in the presence of falcipain enzyme and adopt a unique folded conformation to fit into the long cleft of the active site of the cysteine protease enzyme [8]. We also assume that the protonation of triazolyl chalcone derivative under acidic condition may boost their interaction with cysteine protease His 67 amino acid present at the active site [8].

All the compounds showed inhibition of plasmodial hemozoin formation in culture and percent hemozoin formed in combination was comparable with concentration of either compound used alone, suggesting that both the compounds act on hemozoin formation pathways but may be at different parasite stages [27], considering our stage-specific inhibition results. Pyrimethamine, a known antimalarial did not inhibit hemozoin formation and was used as negative control.

Of the three novel chalcone derivatives, only SK7, a triazole substituted chalcone was found to inhibit parasite-induced permeation pathways to a significant extent. Few alkoxylated and hydroxylated chalcones have been previously reported to inhibit new permeation pathways induced by the parasite in the host erythrocyte membrane [28]. Artemisinin, SK1, SK2 or their synergistic combination ratios do not retard the parasite growth through inhibition of parasite-induced channels. Frusemide (Furosemide) [28] and artemisinin were used as positive and negative controls at their approximate IC_{50} values, respectively. These results suggest that different substituted chalcone analogs may exert their antimalarial activity through different pathways other than new permeation pathways in the parasitized erythrocytes.

In conclusion our results show that antiplasmodial activity of chalcone derivatives is exerted on all stages of asexual cycle predominantly acting on trophozoite stages by inhibiting the hemozoin formation and interaction of ART and chalcone derivative combination is not antagonistic thus hold a promise being an appropriate partner of alternate ACTs.

4. Experimental protocol

4.1. Estimation of in vitro antiplasmodial activity

4.1.1. Parasite culture

Stock culture of malaria parasite *P. falciparum* 3D7 strain (chloroquine sensitive) was continuously maintained in vitro using the candle jar method of Trager and Jensen [29]. *P. falciparum* parasites for normal growth and multiplication were incubated at low-oxygen, high carbon-di-oxide atmosphere – generated by the candle – at 37 °C. The parasites were maintained on B^+ human red blood cells suspended in a complete culture medium. Each liter of RPMI-1640 aqueous culture medium was prepared with 10.4 g of powdered RPMI-1640 (with glutamine but without bicarbonate), 5.94 g of HEPES buffer, 1 g of dextrose and 40 mg of gentamycin. Complete medium was constituted just before use by adding sterile 5% sodium bicarbonate at the rate of 4 ml per 96 ml, and supplemented with 10% (v/v) pooled B^+ human serum. Infected erythrocytes were suspended in these culture media at a starting hematocrit of 5% and parasitemia kept between 2 and 4% with sub-culturing done beyond 5%. Medium was changed once a day and percentage parasitemia was monitored by Giemsa stained slides.

4.1.2. Synthesis of substituted 1,3-diarylpropenone derivatives

The Claisen–Schmidt condensation method was employed for synthesis of chalcone derivatives (SK1, SK2, SK7 compounds) as reported earlier [8].

SK 1. *1-(4-(1H-pyrrol-1-yl)phenyl)-3-(4-chlorophenyl)prop-2-en-1-one*: Yield 68%, R_f 0.67, off white crystals, m.p. 132–134 °C, MS *m*/*z* (M + 1) 309.15, ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.19–8.0 (m, 14H, Ar).

SK 2. *1-(4-(1H-benzo[d][1,2,3] triazol-1-yl)-phenyl)-3-(4-chloro phenyl)-prop-2-en-1-one*: Yield 60%, R_f 0.68, off white crystals, m.p. 154–155 °C, MS m/z 360 (M + 1), ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.52–8.15(m, 14H, Ar).

SK 7. *1-(4-(1H-1,2,4-triazole-1-lyl)-phenyl)-3-(4-chlorophenyl)-prop-2-en-1-one*: Yield 79%, R_f 0.55, yellow crystals, m.p. 160–162 °C, MS *m*/*z* 309.12 (M+•), ^{1}H NMR ($CDCl_3$, 300 MHz): δ, 7.31–7.90 (m, 12H, Ar).

4.1.2.1. Stock solution of compounds. Artemisinin (Sigma–Aldrich, USA), pyrimethamine (Sigma–Aldrich, USA) and licochalcone A (Calbiochem, Merck Bioscience, Pvt. Ltd., USA) were each prepared separately in DMSO to get the stock solution of 1 mg/ml strength. Compounds SK1, SK2 and SK7 were synthesized according to the procedure described by Mishra et al. [8] and each compound was made to strength of 1 mg/ml stock solution. Chloroquine phosphate ampoule (Ipca Laboratories, India) containing 40 mg/ml chloroquine aqueous solution drug was procured locally. The stock solution was diluted on the day of experiment to get the desired concentrations for each drug. The amount of DMSO in diluted concentrations used had no effect on parasite growth.

4.1.3. Compound (drug) concentration response assay

The concentration of an individual compound required to inhibit multiplication of parasites by 50% (IC_{50}) against *P. falciparum* was determined using concentration response assay in 24-well tissue culture plates in triplicates. Synchronous parasite [30] cultures were subjected to graded concentration of a compound, prepared in gentamycin-free RPMI culture medium, for 48 h at 37 °C in a candle jar [29]. Medium was changed in each well after 24 h with or without the compound. The results were expressed as the mean percentage inhibition ± standard error in relation to control; examined by thin smear Giemsa stained slides. IC_{50} values were computed from semi-log plots.

4.1.4. Slide preparation, staining and assessment

Thin blood smear slides were air dried, methanol fixed, and stained in Giemsa solution for 40 min. After staining slides were removed from coupling jar, washed in running tap water and air dried. The Giemsa stained slides were examined for counting the number of parasites in random adjacent microscopic fields, equivalent to about 4000 erythrocytes at 1000× magnification. Percent parasitemia was calculated. Reproducibility of counts was checked by two other readers to maintain the quality control.

4.2. In vitro stage-specific inhibition assay

Synchronized *P. falciparum* parasites (0.8–1.5% parasitemia and 2% hematocrit) were challenged with IC_{50} concentration of each compound at the ring (R) stage (between 0 and 18 h), trophozoite (T) stage (between 18 and 36 h) and schizont (S) stage (between 36 and 48 h) in triplicate along with control well (without compound). For each well 20 μl of parasitized blood and 480 μl of culture medium (with or without the compound) was used. Each test well containing R, T or S was loaded with drug (compound) for the specific time period (starting from 0 h); in a 48 h asexual life-cycle. The drug pressure was removed after desired time and was maintained in drug free-culture medium. Thin blood smears were prepared and processed for Giemsa staining at the end of 48 h asexual cycle. Stage (ring, trophozoite or schizont) specific percentage inhibition for each drug was calculated relative to control (without drug, 100% growth) by counting 3500 cells for each stage. This assay allows determining the sensitivity of each asexual stage to the compound evaluated.

4.3. Antiplasmodial interaction assay of artemisinin and chalcone derivative combinations

4.3.1. Preparation of fixed-ratio combinations

In each combination assays; two compounds (Compound A, artemisinin and Compound B, a chalcone derivative) were combined in four fixed ratios (4:1, 3:2, 2:3, and 1:4). Approximately eight-fold IC_{50} compound concentration of respective compound A or B was taken as 100%, so that IC_{50} of the individual compound falls in between third and fourth two-fold serial dilution. Six solutions in combination assay of ART (nM): SK1 (μM) used were 32:0, 25.6:15.4, 19.2:30.8, 12.8:46.2, 6.4:61.6, 0:72; respectively, for ART (nM): SK2 (μM) were 32:0, 25.6:11.2, 19.2:22.4, 12.8:33.6, 6.4:44.8, 0:56; respectively and for ART (nM): SK7 (μM) were 32:0, 25.6:8, 19.2:16, 12.8:24, 6.4:32, 0:40; respectively.

4.3.2. Plate preparation for antiplasmodial interaction assay

Compound dilutions of each combination solution were made in sterile flat-bottom 96-well tissue culture plates as described by Fivelman and others [9]. Six times two-fold serial dilution was done for each combination in triplicate. Each well contained a total volume of 200 μl of complete culture medium with or without compound and pre-synchronized infected RBCs (1% parasitemia at 2.5% hematocrit). Control cultures (without compound) were maintained on the same plate in triplicate. Two 96-well plates (for six combinations) were used for each combination experiment. The plates were stacked in a candle jar and gassed to increase carbon-di-oxide concentration [29]. The plates were incubated at 37 °C for 48 h with a brief interruption after 24 h to reconstitute gas mixture by burning the candle and change the medium.

4.3.3. Isobologram preparation and data analysis

For each combination assay, IC_{50} was calculated from two sets of concentration response graphs, each containing compound alone curve and four combination curves. Fractional inhibitory

concentration values of compound A (FIC_A) and compound B (FIC_B) were calculated separately in each combination. FIC values were calculated by the following equation to plot isobologram:

$$FIC = \frac{\text{Fraction of drug concentration required to produce } IC_{50} \text{when used in combination}}{\text{Fraction of drug concentration required to produce } IC_{50} \text{when used alone}}$$

The sum FIC for each combination ratio of two combined compounds shows that the drug–drug schizontocidal interaction between them [31,32] was determined by the following equation:

$$\text{SumFIC}\left(\sum \text{FIC}\right) = \frac{IC_{50}\text{of A in mixture}}{IC_{50}\text{of A alone}} + \frac{IC_{50}\text{of B in mixture}}{IC_{50}\text{of B alone}}$$

$\sum FIC < 1$ represents synergism, $\sum FIC \geq 1$ and <2 represents additive interaction, $\sum FIC \geq 2$ and <4 represents slight antagonism while $\sum FIC \geq 4$ represents marked antagonism [33–35].

4.4. Hemozoin inhibition assay

The hemozoin (β-hematin) inhibition by drugs in *P. falciparum* cultures was determined using drug concentrations in the vicinity of IC_{50} concentrations at the end of 48 h [35]. The test cultures were centrifuged at 1300 rpm for 5–10 min to remove the culture medium. Infected erythrocyte pellet (mixture of erythrocyte membrane and β-hematin) was subjected to 0.01% saponin lysis for 10 min at 25 °C to release parasites from erythrocyte. The parasites were washed three times with PBS, re-suspended in 2.5% sodium-dodecyl sulfate buffer solution (SDS in PBS) and subjected to spin at 20,000*g* for 1 h. The supernatant was discarded and the insoluble hemozoin pellet was washed in 2.5% SDS in PBS and then dissolved in 20 mM NaOH. The hemozoin content was estimated by determining the absorbance at 400 nm and using a standard curve prepared from β-hematin (Sigma) [36]. The amount of hemozoin formed in relation to control was calculated.

4.5. Sorbitol-induced hemolysis assay

Late trophozoite to schizont stage parasites (36–44 h old post invasion) when suspended in an iso-osmotic sorbitol solution; there is net uptake of sorbitol and water into the parasitized erythrocytes, resulting in cell swelling and hemolysis. Hemolysis is reduced in the presence of channel blockers.

The hemolysis was estimated spectrophotometrically by measuring absorbance at 540 nm with drug at a concentration near to the antiplasmodial IC_{50} [28] estimated earlier. Infected cell suspension was prepared by harvesting 36–44 h old synchronized culture (with 15% parasitemia), by 600*g* spin for 5 min. Pellet was washed two times with a 7.4 pH solution of NaCl (150 mM)–HEPES (20 nM) and finally re-suspended in this solution to give 50% hematocrit of infected cells. Stock solutions of drugs were diluted using sorbitol (300 mM)–HEPES (20 mM) buffer (pH 7.4) to make 0.1 mM buffered drug stock solutions. 100 μl of infected cell preparation (50% hematocrit) was mixed with desired concentration of buffered drug solution to make a total volume of 1 ml. The cell suspension was incubated at 37 °C for 15 min, centrifuged and aliquots of supernatant were used to estimate the amount of hemoglobin released by determining absorbance at 540 nm. The degree of hemolysis was compared with that of control, to observe the channel blocking effect of drugs. Compounds that inhibited sorbitol-induced lysis of parasitized erythrocytes to a significant extent (≤40% of control values) near to the IC_{50} concentration were considered as a good channel blocker [28].

Acknowledgements

Work was supported in part by the Department of Biotechnology, Ministry of Science and Technology, Government of India and University of Delhi, Delhi. AB and LCM received fellowship from the Council of Scientific and Industrial Research, New Delhi, India. MS received fellowship from University Grants Commission (UGC), New Delhi.

References

[1] D.J. Newman, G.M. Cragg, J. Nat. Prod. 70 (2007) 461–477.
[2] C. Liu, Y. Zhao, Y. Wang, Appl. Microbiol. Biotechnol. 72 (2006) 11–20.
[3] WHO 1998, ed. World Health Organization, The use of artemisinin and its derivatives as antimalarial drugs, Report of a joint CTD/DMP/TDR informal consultation, WHO document WHO/MAL/98.1086.
[4] V.K. Bhasin, L. Nair, Lancet. Infectious. Dis. 3 (2003) 609.
[5] M. Chen, T.G. Theander, S.B. Christensen, L. Hviid, L. Zhai, A. Kharazmi, Antimicrob. Agents. Chemother. 38 (1994) 1470–1475.
[6] R. Li, G.L. Kenyon, F.E. Cohen, X. Chen, B. Gong, J.N. Dominguez, E. Davidson, G. Kurzban, R.E. Miller, E.O. Nuzum, P.J. Rosenthal, J.H. McKerrow, J. Med. Chem. 38 (1995) 5031–5037.
[7] M. Liu, P. Wilairat, S.L. Croft, A.L. Tan, M.L. Go, Bioorg. Med. Chem. 11 (2003) 2729–2738.
[8] N. Mishra, P. Arora, B. Kumar, L.C. Mishra, A. Bhattacharya, S.K. Awasthi, V.K. Bhasin, Eur. J. Med. Chem. 43 (2008) 1530–1535.
[9] Q.L. Fivelman, I.S. Adagu, D.C. Warhurt, Antimicrob. Agents. Chemother. 48 (2004) 4097–4102.
[10] N.J. White, Science 320 (2008) 330–334.
[11] World Health Organisation, Antimalarial Drug Combination Therapy. Report of WHO Technical Consultation. (WHO/CDS/RBM/2001.35), WHO, Geneva, 2001.
[12] R.E. Desjardins, C.J. Canfield, J.D. Haynes, J.D. Chulay, Antimicrob. Agents. Chemother. 16 (1979) 710–718.
[13] H.L. Ziegler, H.S. Hansen, D. Stærk, S.B. Christensen, H. Hägerstrand, J.W. Jaroszewski, Antimicrob. Agents. Chemother 48 (2004) 4067–4071.
[14] S.F. Nielsen, A. Kharazmi, S.B. Christensen, Bioorg. Med. Chem. 6 (1998) 937–945.
[15] P.Q. Chen, G.Q. Li, X.B. Guo, K.R. He, Y.X. Fu, L.C. Fu, Y.Z. Song, Chin. Med. J. (Engl.) 107 (1994) 709–711.
[16] L.C. Mishra, A. Bhattacharya, V.K. Bhasin, Acta Trop. 109 (2009) 194–198.
[17] D.L. Klayman, Science 228 (1985) 1049–1054.
[18] Y. Maeno, T. Toyoshima, H. Fujioka, Y. Ito, S.R. Meshnick, A. Benakis, W.K. Milhous, M. Aikawa, Am. J. Trop. Med. Hyg. 49 (1993) 485–491.
[19] U.E. Ludwig, R.J. Webb, I.D.A. van Goethem, J.M. East, A.G. Lee, M. Kimura, P.M. O'Neill, P.G. Bray, S.A. Ward, S. Krishna, Nature 424 (2003) 957–961.
[20] A.V. Pandey, B.L. Tekwani, R.L. Singh, V.S. Chauhan, J. Biol. Chem. 274 (1994) 19383–19388.
[21] Y.L. Hong, Y.Z. Yang, S.R. Meshnick, Mol. Biochem. Parasitol. 63 (1994) 121–128.
[22] I.R. Ribeiro, P. Olliaro, Med. Trop. (Mars) 58 (1998) 50–53.
[23] B.R. Shenai, P.S. Sijwali, A. Singh, P.J. Rosenthal, J. Biol. Chem. 275 (2000) 29000–29010.
[24] A.U. Orjih, Exp. Biol. Med. (Maywood) 226 (2001) 746–752.
[25] R. Li, X. Chen, B. Gong, P.M. Selzer, Z. Li, E. Davidson, G. Kurzban, R.E. Miller, E.O. Nuzum, J.H. McKerrow, R.J. Fletterick, S.A. Gillmor, C.S. Craik, I.D. Kuntz, F.E. Cohen, G.L. Kenyon, Bioorg. Med. Chem. 4 (1996) 1421–1422.
[26] Z. Nowakowska, Eur. J. Med. Chem. 42 (2007) 125–137.
[27] A.V. Pandey, B.L. Tekwani, Febs. Lett. 402 (1997) 236–240.
[28] M.L. Go, M. Liu, P. Wilairat, P.J. Rosenthal, K.J. Saliba, K. Kirk, Antimicrob. Agents. Chemother. 48 (2004) 3241–3245.
[29] W. Trager, J.B. Jensen, Science 193 (1976) 673–675.
[30] C. Lambros, J.P. Vanderberg, J. Parasitol. 65 (1979) 418–420.
[31] M.C. Berenbaum, J. Infect. Dis. 137 (1978) 122–130.
[32] S. Gupta, M.M. Thapar, W.H. Wernsdorfer, A. Bjorkman, Antimicrob. Agents. Chemother. 46 (2002) 1510–1515.
[33] F.C. Odds, J. Antimicrob. Chemother. 52 (2003) 1.
[34] L.C. Mishra, A. Bhattacharya, V.K. Bhasin, Am. J. Trop. Med. Hyg. 76 (2007) 497–501.
[35] A. Bhattacharya, L.C. Mishra, V.K. Bhasin, Am. J. Trop. Med. Hyg. 78 (2008) 721–728.
[36] T. Akompong, N. Ghori, K. Haldar, Antimicrob. Agents. Chemother. 44 (2000) 88–96.