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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Oxygenated chalcones and bischalcones as a new class of inhibitors of DNA topoisomerase II of malarial parasites

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Abstract The DNA topoisomerase (topo) II of chloroquine-sensitive and chloroquine-resistant strains of the rodent malaria parasite *P. berghei* were utilized as a target for testing of antimalarial compounds. Compounds belonging to the bischalcone and chalcone series significantly inhibited enzyme activity and percentage parasitaemia of chloroquine-sensitive and chloroquine-resistant strains of *P. berghei*. Compounds **1a**, **1b**, **2a**, **2b**, and **2c** showed 100% inhibition while compounds **2h** and **2i** showed 60% and 63% inhibition of topoisomerase II activity of the chloroquine-sensitive strain, respectively. Compounds **2a**, **2b**, and **2d** significantly inhibited the topo II activity of chloroquine-resistant strain. Compounds **2g** and **2e** specifically inhibited the topo II activity of the chloroquine-resistant strain of *P. berghei* with no effect on the chloroquine-sensitive strain. The in vitro topo II inhibition by chalcone and bischalcone analogs can be correlated with their in vivo antimalarial activity, as compounds **2c** and **2h** inhibited both in vitro activity of topo II and in vivo parasitaemia of the chloroquine-sensitive strain of *P. berghei*. In the chloroquine-resistant strain, compounds **2c**, **2e**, **2g**, and **2i** inhibited activity against both in vitro topo II and parasitaemia in vivo. The significant inhibition of topo II in the chloroquine-resistant strain by some of the analogs suggests the utilization of these structures for the synthesis of compounds active against chloroquine-resistant malarial parasites.

Keywords DNA · Topoisomerases · Chalcones · Bischalcones · *Plasmodium berghei* · Topoisomerase inhibitors · Chloroquine-resistant strain

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Introduction

Topoisomerases are ubiquitous enzymes involved in solving topological problems arising during the processes of DNA metabolism including transcription, recombination, replication, and chromosome partitioning during cell divisions (Wang 1985;Austin and Fisher, 1990; Osheroff *et al*.,1991; Gasser *et al*., 1992). As a result of performing this role, topoisomerases are essential for the viability of all organisms from unicellular bacteria to humans. According to their catalytic mechanism two categories of enzymes have been found in a variety of organisms. Type I enzymes transiently cut a single DNA strand, pass an intact single strand of DNA through the broken strand and then reseal the break. Type II enzymes make transient double-strand breaks in DNA and pass an intact duplex through the broken DNA before resealing the break. It has been found that the level of topoisomerase II activity is increased during rat liver regeneration after partial hepatectomy (Duguet *et al*., 1983) and in rapidly proliferating cells such as tumor cells (Riou *et al*., 1985).

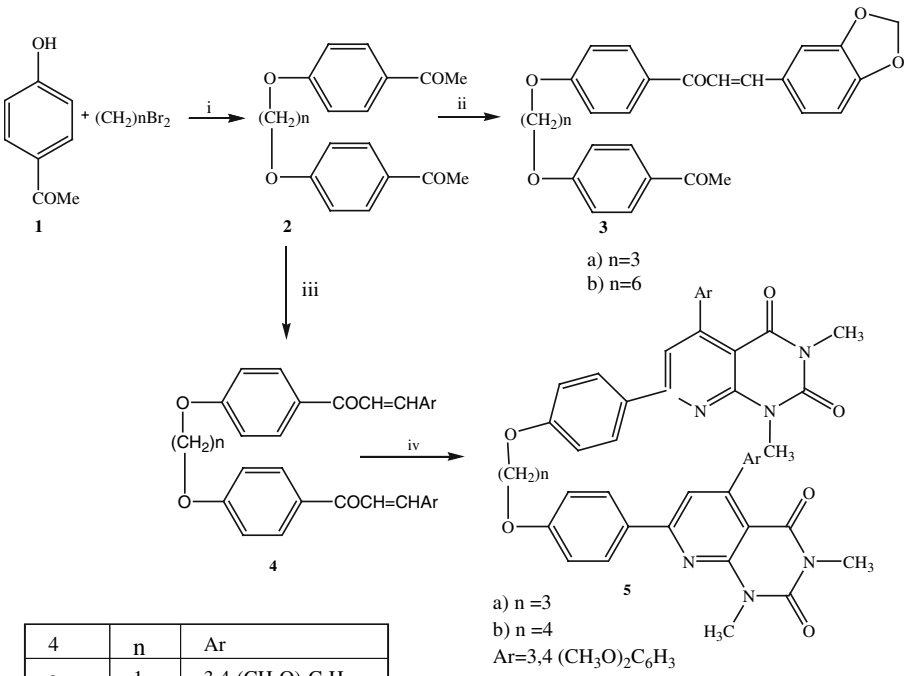
In recent years, DNA topoisomerases from parasites have been the focus of considerable study, not only because they are intrinsically interesting but also because they may provide a target for much needed new antiparasitic chemotherapy (Nenortas *et al*., 1998).The protozoan parasites of the genus *Plasmodium*, the causative agent of malaria in human beings and other vertebrates, undergo a complex evaluative cycle inside the red blood cells (RBC) of their host and proliferate rapidly, with more than 70% of red blood cells infested within a few days. The quinolones are the most widely used inhibitors of bacterial topoisomerases. It has also been reported that reactions catalyzed by topoisomerase extracted from rat liver and trypanosomes are inhibited by various drugs at low concentrations (Douc-Rasy *et al*., 1983; DoucRasy *et al*., 1984). Fluoroquinolone antibiotics are cytotoxic to malaria parasites in vitro (Krishna *et al*., 1988; Divo *et al*., 1988) and have efficacy in patients with malaria (Sarma, 1989). An assortment of novel DNA intercalating agents has been synthesized and tested for activity against malarial parasites. These include the 9-anilinoacridines, which stabilize cleavable complex formation in vivo in *L. chagazi* and are more cytotoxic to *Plasmodium falciparum* than to mammalian cells (Werbovetz *et al*., 1992; Werbovetz, 1993; Chavalitshewinkoon *et al*., 1993; Gamage *et al*., 1994).

Naturally occurring chalcones such as licochalcone A, isolated from Chinese licorice root, are known to have antimalarial activity against chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) isolates of *Plasmodium falciparum* (Kharazmi *et al*., 1997). Recently an analog of the licochalcones, 2,4-dimethoxy-4-butylchalcone was found to exhibit potent activity against the human malaria parasite *Plasmodium falciparum* in vitro and the rodent parasites *P. berghei* and *P. yoelii* in vivo (Chen *et al*., 1997). Structurally, bischalcones have the same pharmacophore as that of flavones and isoflavones, which are potent inhibitors of DNA topoisomerase II and are therefore considered to be good antitumor and anti-HIV agents (Wang *et al*.,1997). These findings prompted us to design and synthesize a new series of oxygenated chalcones and bischalcones for the inhibition of the DNA topoisomerase II of the chloroquine-sensitive and chloroquine-resistant strains and also the malaria parasite in vivo.

Materials and methods

Chemistry

Bischalcones were synthesized by base-catalyzed condensation of bis(4-acetylphenoxy) alkanes and the appropriate aldehyde (Fig. 1). To our surprise, a condensation of (4-acetylphenoxy) alkanes with 3,4-methylenedioxy benzaldehyde under similar conditions always yielded monochalcone structure 1 (Ram *et al.*, 2000). All the synthesized compounds were purified by column chromatography and characterized by spectroscopic and elemental analysis.



4	n	Ar
a	1	3,4-$(CH_3O)_2C_6H_3$
b	1	2,4-$(CH_3O)_2C_6H_3$
c	3	3,4-$(CH_3O)_2C_6H_3$
d	3	2,4-$(CH_3O)_2C_6H_3$
e	3	3-$CH_3O.C_6H_4$
f	4	3,4-$(CH_3O)_2C_6H_3$
g	4	2,4-$(CH_3O)_2C_6H_3$
h	4	3-$CH_3O.C_6H_4$
i	6	3,4-$(CH_3O)_2C_6H_3$
j	6	3--$CH_3O.C_6H_4$

Fig. 1 Scheme for the synthesis of chalcones and bischalcones. Reagents and conditions were: (i) K_2CO_3/acetone/reflux; (ii) piperonal/alc. KOH/RT; (iii) aryl aldehyde/alc. KOH/RT; (iv) NaOEt/6-amino-1,3-dimethyluracil/reflux

Biology

DNA topoisomerase estimation

Supercoiled DNA pBR322 and all other chemicals used were obtained from Sigma Chemical Co., St. Louis, USA.

P. berghei chloroquine-sensitive and chloroquine-resistant strains were separated from the infected mice according to the method of Riou *et al*. (1986). DNA topoisomerase II from malarial parasites was partially purified according to the method of Pandya *et al*. (1999).

The enzyme activity was monitored by relaxation of supercoiled DNA pBR322 and separation of topological isomers of DNA by gel electrophoresis as reported earlier (Pandya *et al*., 1999). Briefly, for the activity measurements pBR322 DNA (0.25 μg) was incubated in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM Adenosine triphosphate (ATP), 0.1 mM Ethylene diamine tetra acetic acid (EDTA), 0.5 mM Dithiothreitol (DTT), 0.44 μM Bovine serum albumin (BSA), and enzyme protein. The reaction volume was 20 μl. Adding pBR322 DNA and incubating at 37°C for 30 min started the reaction. The reaction was stopped by adding 5 μl stop buffer. The samples were electrophoresed on 1% agarose gel in Tris-acetate buffer for 18 h at 20 volts. Gels were stained with ethidium bromide (0.5 μg/ml), visualized, and photographed on a UVP GDS 7500 ultraviolet (UV) transilluminator.

The effect of inhibitors on the enzyme activity was measured by incubating enzyme with inhibitor for 10 min at 37°C and starting the reaction by the addition of pBR322. The percentage relaxation was measured by microdensitometry of the gel by using the Gel Base/Gel Blot Pro gel analysis software programme.

In vivo screening

Maintenance of Plasmodium berghei infection (chloroquine-sensitive strain)

Blood from orbital plexus of heavily infected animals was drawn under aseptic conditions and mixed with 3.8 % sodium citrate to obtain a final dilution of approximately 5 million parasitized erythrocytes per milliliter of diluate. The counting of RBCs was done with the help of a hemocytometer. Each animal was then injected intraperitoneally with 0.2 ml of this suspension containing approximately 1×10^6 parasitized erythrocytes.

Development of the chloroquine-resistant strain of P. berghei

Chloroquine resistance was developed with a slight modification of the technique described by Warhurst and Folwell (1968). 1×10^6 parasitized RBCs were inoculated in a group of five animals and after 2 h these mice were given chloroquine diphosphate (15 mg/kg) for four consecutive days, when these animals

developed about 2% parasitaemia; the infected blood from these animals was used to infect healthy animals that were also given chloroquine. This process was repeated several times with *P. berghei* kept under continuous drug pressure. The line has approximately seven times higher chloroquine tolerance than the chloroquine-sensitive strain. This resistant line was used for the screening and biochemical studies.

Preparation of synthetic compounds

Synthetic compounds employed for screening were prepared by grinding accurately weighed compound with distilled water when the compounds were soluble in water. Poorly water-soluble compounds were ground up with dimethyl sulfoxide (DMSO). Finally, distilled water was added to obtain the desired concentration of the compound.

Method of screening

Chloroquine-sensitive strain

The suppressive method of screening of Peters (1965) was adopted. Male mice (Park strain) weighing 20 ± 2 g were infected intraperitoneally with standard inoculums of 1 million parasitized RBCs (1×10^6 PRBC). The animals were treated with the compounds 2-3 hrs post inoculation. For treatment the inoculated animals were divided into two groups with five animals in each group. All five animals in one group received the same dose of a compound intraperitoneally for four consecutive days, while infected animals without treatment served as control.

Chloroquine-resistant strain

In the resistant group the animals were infected as described for the sensitive strain with one group receiving chloroquine plus compound and the other group receiving the compound only; chloroquine was injected in the third group, and the fourth group of infected animals without any treatment served as control. The treatment was given to group one to three for four consecutive days.

Results and discussion

DNA topoisomerases are enzymes that alter the topological state viz., underwinding, knotting, and tangling of nucleic acids by generating transient breaks in DNA. In order to maintain the integrity of the genetic material during this process, topoisomerases form covalent bonds with the DNA termini created by their actions. Topoisomerase proteins characterized from lower eukaryotes appear to share many

characteristics associated with their human homologs, but striking differences, including different enzyme activity requirements, variable catalytic sites, and different sensitivities to topoisomerase poisons, provide insight for the development of topoisomerase-directed antiparasitic therapeutics. It has been established by several studies that the inhibitors of topoisomerases convert these essential enzymes into intracellular proliferating cell toxins and thereby provide a good tool for preferentially killing the highly replicative parasite cells within the host. Attempts have been made to evaluate topo II poisons as potential antimalarial agents viz., 9-anilinoacridines, which inhibited parasite metabolism and topo II activity (Chavalitshewinkoon *et al*., 1993). A related antimalarial agent with similar properties, pyronaridine (White and Kilbey, 1996), has been shown to be active against chloroquine-resistant strains of the parasite (Fu and Xiao, 1991).

Three compounds of the chalcone group and nine compounds of the bischalcone group were tested for their topoisomerase II inhibitory activity in chloroquine-sensitive (CQS) and chloroquine-resistant (CQR) strains of *P. berghei*. Compounds **1a**, **1b**, **2a**, **2b**, and **2c** showed 100% inhibition of topoisomerase II activity while compounds **2h** and **2i** produced 60% and 63% inhibition, respectively, in the chloroquine-sensitive strain of *P. berghei* (Table 1).

Compounds **2a** and **2c** when tested at 4 μg concentration did not inhibit topoisomerase II activity in chloroquine-sensitive strain (Fig. 2). In the chloroquine-resistant strain compounds **2a**, **2b,** and **2d** inhibited topoisomerase II activity by 71%, 98%, and 74%, respectively. Compounds **2b** and **2d** at the lower concentration of 4 μg (per 20 μl reaction volume) inhibited topo II activity by 70% and 50%,

Table 1 Effect of chalcones (compounds **1a–c**) and bischalcones (compounds **2a–i**) on DNA topoisomerase II of sensitive and resistant strains of *P. berghei*

No.	Struct. no.	Compd. no.	Concentration (μg/reaction mixture)	DNA topoisomerase II % inhibition	
				CQS strain	CQR strain
1	**1a**	98/260	40 μg	100	41*
2	**1b**	98/261	40 μg	100*	51*
3	**1c**	98/262	40 μg	Nil*	17
4	**2a**	98/371	40 μg	100*	71*
5	**2b**	98/372	40 μg	100	98
6	**2c**	98/257	40 μg	100*	50*
7	**2d**	98/373	40 μg	42	74
8	**2e**	98/263	40 μg	Nil*	54*
9	**2f**	98/258	40 μg	Nil	7*
10	**2g**	98/264	40 μg	Nil	66
11	**2h**	98/259	40 μg	60*	34*
12	**2i**	98/265	40 μg	63	50

The values are means of three experiments in duplicate. The enzymes from drug-sensitive and drug-resistant strains were incubated at 37°C for 10 min. The OD of relaxed DNA without any compound was 184. * Compound showed complex formation with DNA

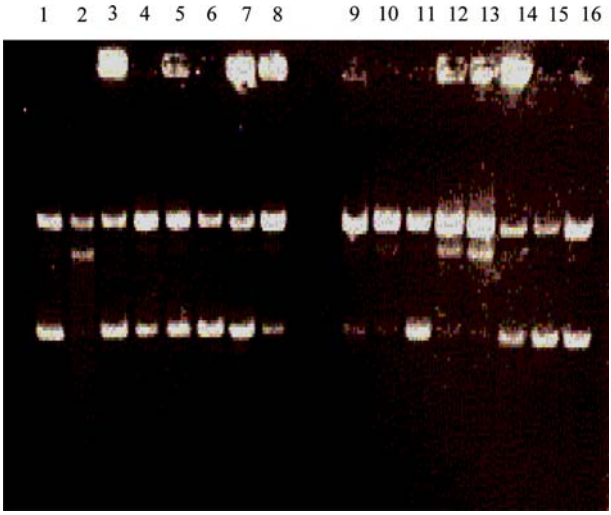


Fig. 2 Inhibition of DNA Topoisomerase II activity of CQS *P. berghei* by chalcones and bischalcones. Lane 1: pBR322 DNA alone; lane 2: topo II activity of CQS *P. berghei*; lane 3: identical to lane 2 + compound 98/257 (40 μg); lane 4: identical to lane 2 + compound 98/258 (40 μg); lane 5: identical to lane 2 + compound 98/259 (40 μg); lane 6: identical to lane 2 + compound 98/260 (40 μg); lane 7: identical to lane 2 + compound 98/261 (40 μg); lane 8: identical to lane 2 + compound 98/262 (40 μg); lane 9: identical to lane 2 + compound 98/263 (40 μg); lane 10: identical to lane 2 + compound 98/264 (40 μg); lane 11: identical to lane 2 + compound 98/265 (40 μg); lane 12: identical to lane 2 + compound 98/257 (4 μg); lane 13: identical to lane 2 + compound 98/371 (4 μg); lane 14: identical to lane 2 + compound 98/371 (40 μg); lane 15: identical to lane 2 + compound 98/372 (40 μg); lane 16: identical to lane 2 + compound 98/373 (40 μg)

respectively (Fig. 3). Compounds **2g** and **2e** caused 66% and 54% inhibition of topoisomerase II activity of the CQ-resistant strain of *P. berghei*, respectively. However, the same compounds (**2g** and **2e**) showed no inhibitory effects on the chloroquine-sensitive strain of *P. berghei*. Compounds **1a**, **1b**, **1c**, **2a**, **2c**, **2e**, **2f** and **2h** also showed complex formation with DNA (Figs. 2 and 3).

As reported earlier (Ram et al. 2000) compounds **2c**, **2e**, and **2f** showed 77%, 55%, and 75% inhibition of parasitaemia in the CQS strain of *P. berghei* up to day 11, respectively (Table 2). In a resistance reversal test chalcones and bischalcones showed better inhibition of parasitaemia than was observed with chloroquine-sensitive *P. berghei*. Compounds **2a**, **2i**, **2f**, **1c**, and **2e** showed 90%, 74%, 66%, 60%, and 53% inhibition of parasitaemia, respectively, up to day 11, while compounds **2g**, **2c,** and **1a** also inhibited parasitaemia by 57%, 52%, and 50%, respectively, until day 9 (Table 3).

Compounds **1a**, **1b**, **1c**, **2a**, **2c**, **2e**, **2f,** and **2h** also showed complex formation with the supercoiled DNA as indicated by the low level of migration of DNA in the treated lane as compared to the control. The stabilization of covalent complexes between topoisomerase II and DNA by these compounds might be responsible for the observed inhibitory effect.

It has been found that in CQS *P. berghei* the compounds **2c** and **2h** showed 100% and 60% topo II inhibition, respectively. These compounds also showed 77% and

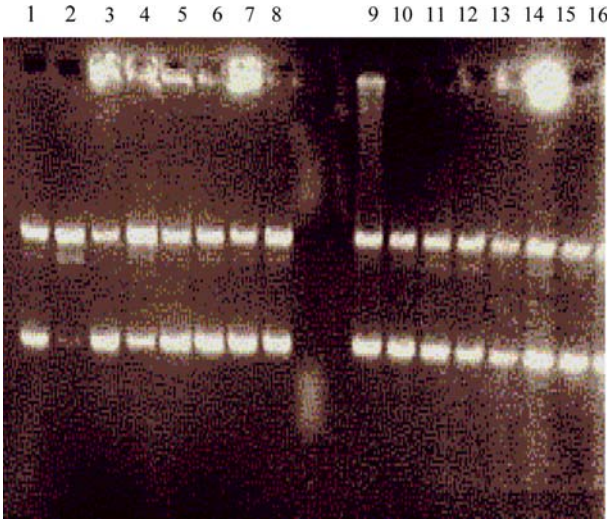


Fig. 3 Inhibition of DNA Topoisomerase II activity of CQR *P. berghei* by chalcones and bischalcones. Lane 1: pBR322 DNA alone; lane 2: topo II activity of CQR *P. berghei*; lane 3: identical to lane 2 + compound 98/257 (40 μg); lane 4: identical to lane 2 + compound 98/258 (40 μg); lane 5: Identical to lane 2 + compound 98/259 (40 μg); lane 6: identical to lane 2 + compound 98/260 (40 μg); lane 7: identical to lane 2 + compound 98/261 (40 μg); lane 8: identical to lane 2 + compound 98/262 (40 μg); lane 9: identical to lane 2 + compound 98/263 (40 μg); lane 10: identical to lane 2 + compound 98/264 (40 μg); lane 11: identical to lane 2 + compound 98/265 (40 μg); lane 12: identical to lane 2 + compound 98/373 (40 μg); lane 13: identical to lane 2 + compound 98/372 (40 μg); lane 14: identical to lane 2 + compound 98/371 (40 μg); lane 15: identical to lane 2 + compound 98/372 (4 μg); lane 16: identical to lane 2 + compound 98/373 (4 μg)

47% inhibition of parasitaemia in vivo in the CQS strain. In the resistance reversal test compounds **2e**, **2i**, and **2a** showed 53%, 74%, and 91% inhibition of parasitaemia until day 11, respectively. The same compounds showed 54%, 50%, and 71% topo II inhibition, respectively.

Pyronaridine has been shown to be an effective gametocytocidal against drug-resistant *P. falciparum* and exhibited both schizontocidal and gametocytocidal activity. Norfloxacin, amsacrine, and etoposide, the DNA topo inhibitors were effective against asexual but not sexual stages of the malaria parasite (Chavalitshewinkoon *et al*., 2000). Chloroquine, quinine, and related 4-aminoquinolines are effective drugs against malarial infection, and the ability of chloroquine to form complexes with DNA is well documented (Blodgett and Yielding, 1968; Yielding *et al*., 1971). Chloroquine, mepacrine, and other quinoline derivatives are potent inhibitors of DNA and RNA polymerase (O'Brien *et al*., 1966; Whichard *et al*., 1972), as well as DNA and RNA synthesis in *P. knowlesi* (Gutteridge *et al*., 1972). Chloroquine and quinine inhibited 50% of DNA topoisomerase II activity of drug-sensitive and drug-resistant *P. berghei* at a 40 μg/reaction mixture concentration.

Flavones and isoflavones are known to display significant topoisomerase II inhibitory activity due to oxygenated substituents and the presence of the CO–CH = CH pharmacophore. Chalcones and bischalcones both meet these requirements and thus are expected to display this type of activity. In vitro, topoisomerase

Table 2 In vivo activity of chalcones (**1a–c**) and bischalcones (**2a–i**) (50 mg/kg/day) against the CQS strain of *P. berghei* in mice

No.	Struct. no.	Code no.	Dose (mg/kg)	Inhibition of Parasitaemia (%) on days			
				5	7	9	11
1.	Control (Avg. % parasitaemia)			2.2	4.2	5.5	7.3
2.	1a	98/260	100	100	74	45	31
3.	1b	98/261	100	25	28	22	21
4.	1c	98/262	100	38	25	7	10
5.	2a	98/371	100	10	20	N.I.	D
6.	2b	98/372	100	28	79	D	
7.	2c	98/257	100	93	96	81	77
8.	2d	98/373	100	33	16	D	
9.	2e	98/263	100	100	67	60	55
10.	2f	98/258	100	100	100	77	75
11.	2g	98/264	100	7	28	9	8
12.	2h	98/259	100	100	88	58	47
13.	2i	98/265	100	25	15	3	N.I.

Route = ip (intraperitoneal) × 4 days

D: death of animal, N.I.: no inhibition

Table 3 In vivo effect of chloroquine (CQ, 15 mg/kg/day), chalcones (**1a–c**), bischalcones (**2a–i**) (50 mg/kg/day), and their combination against the CQR strain of *P. berghei* in mice

No.	Struct. no.	Code no.	Dose (mg/kg)	Inhibition of parasitaemia (%) on days			
				5	7	9	11
1. Control (avg. % parasit)			-	3.2	5.4	8.1	14.6
2. CQ (avg. % parasit)			15	0.2	1.3	3.3	5.7
3.	1a	98/260	50	27	40	45	N.I.
	1a + CQ	98/260 + CQ	50 + 15	100	99	50	34
4.	1b	98/261	50	45	39	43	30.
	1b + CQ	98/261 + CQ	50 + 15	99	66	42	33
5.	1c	98/262	50	42	44	43	46.
	1c + CQ	98/262 + CQ	50 + 15	99	65	62	60
6.	2a	98/371	50	40	28	16	N.I..
	2a + CQ	98/371 + CQ	50 + 15	100	100	96	90
7.	2c	98/257	50	68	20	50	13
	2c + CQ	98/257 + CQ	50 + 15	100	57	52	N.I.
8.	2d	98/373	50	9	57	23	49
	2d + CQ	98/373 + CQ	50 + 15	16	N.I.	22	23
9.	2e	98/263	50	42	57	38	43
	2e + CQ	98/263 + CQ	50 + 15	97	75	53	53
10.	2f	98/258	50	50	17	37	23
	2f + CQ	98/258 + CQ	50 + 15	91	90	55	66

Table 3 continued

No.	Struct. no.	Code no.	Dose (mg/kg)	Inhibition of parasitaemia (%) on days			
				5	7	9	11
11.	2g	98/264	50	37	52	16	30.
	2g + CQ	98/264 + CQ	50 + 15	73	27	57	47
12.	2h	98/259	50	77	50	50	35
	2h + CQ	98/259 + CQ	50 + 15	100	98	58	50
13.	2	98/265	50	28	N.I.	N.I.	N.I.
	2i + CQ	98/265 + CQ	50 + 15	100	100	98	74

Route = ip (intraperitoneal) × 4 days, N.I.: no inhibition

II inhibitory activity of bischalcones can be correlated with in vivo antimalarial activity. Methylene chain length in bischalcones seems to be crucial for exhibiting topoisomerase inhibitory activity. As the chain length increases from CH_2 to $(CH_2)_4$, inhibitory activity decreases. Secondly compounds with methoxy substituents at position 2 and 4 of the phenyl ring are more active than the compounds having methoxy substitution at positions 3 and 4 of the phenyl ring.

The results of the present study show that DNA topoisomerase II of *P. berghei* can be utilized as a target for the synthesis of new antimalarials based on the structure of bischalcones. The specific inhibition of topo II by some compounds in drug-resistant malarial parasites can also be utilized for the synthesis of compounds specifically active against drug-resistant malaria parasites.

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