Research Article

Tetramethylpiperidine-Substituted Phenazines as Novel Anti-Plasmodial Agents

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Strategy, Management and Health Policy				
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ABSTRACT Two novel derivatives of clofazimine [3-(p-chloroanilino)-10-(p-chlorophenyl)-2,10-dihydro-2-isopropylimino)phenazine] and the tetramethylpiperidine (TMP)-substituted phenazines, B4119 [3-(3-chloro-4-fluoroanilino)-10-(3-chloro-4-fluorophenyl)-2,10-dihydro-2(2,2,6,6-tetramethylpiper-4-ylimino)phenazine] and B4158 [3-(4-isopropylanilino)-10-(4-isopropylphenyl)-2,10-dihydro-2-(2,2,6,6-tetramethylpiper-4-ylimino)phenazine] (1–8 μM), were evaluated for activity against chloroquin-, quinine-, and sulfadoxine/pyrimethamine-sensitive and -resistant strains of *Plasmodium falciparum* in vitro, as well as for their effects on polymerisation of haeme to β-hematin. By using microscopic and flow cytometric methods, it was found that B4119 and B4158, but not clofazimine, inhibited the growth of sensitive, as well as resistant strains of *P. falciparum* with IC_{50} values between 0.22 and 0.7 μM, indicating lack of cross-resistance. Augmentation of anti-plasmodial activity was observed when B4119 and B4158 were used in combination with chloroquin or mefloquine. Inhibition of the growth of *P. falciparum* was associated with interference with haeme polymerisation to β-hematin in vitro, while administration of B4119 to *P. berghei*-infected mice was accompanied by a significant reduction in parasitemia and improved therapeutic activity was observed when this agent was combined with chloroquin. The data presented in this study demonstrate that the TMP-substitution at position 2 on the phenazine nucleus of riminophenazines confers anti-plasmodial activity on these compounds. These may prove to be useful forerunners in the design of novel anti-plasmodial pharmacologic agents. Drug Dev. Res. 50:195–202, 2000. © 2000 Wiley-Liss, Inc.

Key words: chloroquin-resistance; clofazimine; malaria; *Plasmodium falciparum*; TMP-substituted phenazines

INTRODUCTION

The World Health Organisation has estimated the global incidence of malaria to be around 110 million cases annually, with about 270 million people being infected and 1–2 million people dying from infections [WHO, 1990]. *Plasmodium falciparum* is the most pathogenic human malaria parasite, being responsible for the majority of malaria-related morbidity and mortality. Most cases occur in Africa, where approximately 1 million children die yearly. In adults, pregnancy and absence of specific immunity are recognised risk factors [Schapira et al., 1993]. This situation is complicated by the emergence of multidrug-resistance (MDR) in many parts of the world where malaria is endemic [Newbold, 1990; Schuster and Milhous, 1993]. There is clearly an urgent requirement

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for the development of novel, effective, and safe drugs for the prophylaxis and treatment of malaria. Innovative chemotherapeutic strategies may be based on the design of novel agents with reduced toxicity and enhanced activity, or by developing agents that potentiate the activities of existing antimalarial drugs.

Clofazimine is the prototype riminophenazine compound and was first described in 1957 as an anti-tuberculosis agent [Barry et al., 1957]. It has since been used as a component of the multidrug chemotherapy of both leprosy and *Mycobacterium avium* complex (MAC) [Brown and Hogerzeil, 1962; Garrelts, 1991]. Recently, a new class of phenazines has been described, which differs from clofazimine by substitution of the isopropyl group on the imino nitrogen at position 2 of the phenazine nucleus with a tetramethylpiperidine (TMP) group. In addition to potent anti-mycobacterial activity [Franzblau et al., 1989; Van Ladingham et al., 1993], these TMP-substituted phenazines have also been reported to inhibit P-glycoprotein activity in a MDR cancer cell line [Van Rensburg et al., 1997]. Unlike clofazimine, these new phenazine analogues are insoluble in body fat, do not crystallise inside macrophages [Reddy et al., 1999], and also show decreased direct toxicity in animal models of mycobacterial infection [Van Ladingham et al., 1993].

The current study was designed to investigate the anti-plasmodial potential of the novel TMP-substituted phenazines in vitro and in vivo, as well as the effects of these agents on drug-resistant strains of *P. falciparum* and on the polymerisation of haeme to β-hematin.

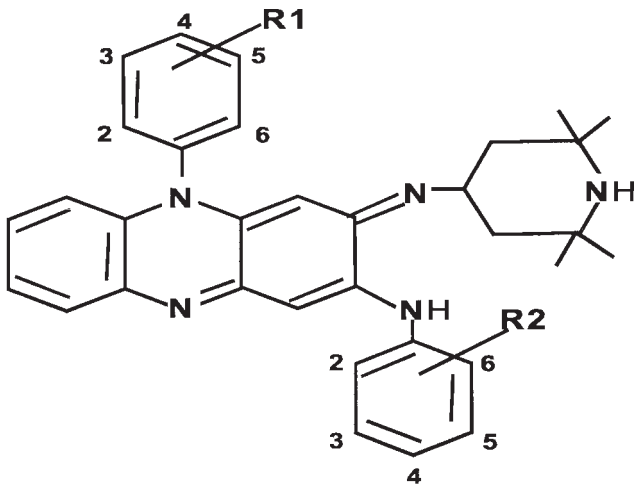
MATERIALS AND METHODS

Drugs

Clofazimine [3-(p-chloroanilino)-10-(p-chlorophenyl)-2,10-dihydro-2-(isopropylimino) phenazine; B663] [Van Rensburg et al., 1993] and the TMP-substituted phenazines were synthesised by Dr. J.F. O'Sullivan (Department of Chemistry, University College Dublin, Republic of Ireland). Their structures are shown in Figure 1. The agents were dissolved in ethanol containing 10 mM acetic acid, resulting in 3 mM stock solutions. Further dilutions were made in RPMI culture medium (Highveld Biological Products, Johannesburg, South Africa). Chloroquin diphosphate salt (Sigma Chemical Co., St. Louis, MO) and mefloquine hydrochloride (F. Hoffmann-La Roche, Basel, Switzerland) were dissolved in distilled water and 70% methanol, respectively, with further dilutions being made in the parasite culture medium. Appropriate solvent control systems were included for each drug concentration tested.

Culture-adapted strains of *P. falciparum*

Five culture-adapted strains of *P. falciparum* were used in this study. Strains RSA9, RSA16, and RSA17 were kindly provided by Dr. B. Sharp of the National Malaria Research Program of the South African Medical Research Council, while strains PfUP10 and RSA8 were provided by Prof. A. Louw, Department of Biochemistry, University of Pretoria, South Africa. The parasites were cultured in group O-positive human erythrocytes and suspended at 5% haematocrit in RPMI 1640 culture medium, which contained 50 μg of gentamicin per ml and 10% human serum. The suspension was maintained at 37°C in a gas mixture of 5% O_2/5% CO_2/90% N_2 [Trager and Jensen, 1976]. The cultures were monitored daily by microscopic analysis of parasitemia by using Giemsa-stained thin blood smears. Parasite cultures were synchronised by sorbitol lysis before use in all the assays [Lambros and Vandeberg, 1979].



COMPOUND	R_1 AND R_2
B4119	3-Cl, 4-F,
B4158	4-CH $(CH_3)_2$
B4112	3-Cl
B4103	4-CF_3
B4100	3,4-di-Cl
B4121	3,5-di-Cl
B4169	3,4,5-tri-Cl

Fig. 1. Chemical structures of the TMP-substituted phenazines.

Assays of parasite growth

These were performed using 96-well microtiter plates. To each well was added 20 μl of a parasite-infected erythrocyte suspension (2% parasitaemia and 0.5% final

haematocrit) and 180 µl of complete medium with or without the test agents B4100, B4103, B4112, B4119, B4121, B4158, and B4169 (1–8 µM). For drug combination studies, 20 µl of chloroquin diphosphate (0.044–0.5 µM) or mefloquine hydrochloride (0.01–0.065 µM) were added to the wells individually or in combination with 20 µl of B4119 (0.4 µM) or B4158 (0.8 µM). The total volume in the wells was 200 µl.

After 48 h of incubation at 37°C parasitemia was determined by using microscopic evaluation of Giemsa-stained slides, as well as by a flow cytometric procedure [Schulze et al., 1997]. Briefly, in the case of the latter, 25 µl of cell suspension were transferred into polypropylene tubes and mixed with 0.5 ml of thiazole orange (0.8 µg/ml), a fluorescent dye that binds to DNA. The tubes were incubated in the dark for 1 h and kept on ice until flow cytometric analysis of parasite DNA was performed using a Coulter Epics II flow cytometer (Coulter Electronics, Hialeah, FL). This method has been compared with the well-established procedures to determine parasitaemia, i.e., the [^{3}H]hypoxanthine uptake method, as well as the microscopic (Giemsa) assay and was found to be suitable for the in vitro evaluation of agents with anti-malaria potential [Schulze et al., 1997].

Stage-specific parasite growth inhibition

The PfUP10 (chloroquin-sensitive) laboratory isolate of *P. falciparum* was used to identify those stages of the growth cycle at which the parasite is most sensitive to the TMP-substituted phenazines. Stringently synchronised ring and late trophozoite parasite suspensions (5% parasitemia and 0.5% final haematocrit) were incubated with either B4119 or B4158 (1–8 µM) for 12 h and parasite development was monitored by using microscopy and flow cytometry.

Effects of B4119 and B4158 on the susceptibility of erythrocytes to infection

Erythrocyte suspensions at 0.5% haematocrit in complete culture medium were incubated with or without B4119 and B4158 (1–8 µM) for 24 h. The erythrocytes were then washed twice with fresh culture medium and exposed to a highly-parasitized schizont suspension (65% parasitemia and 0.5% final haematocrit). This was obtained from a carefully synchronised ring stage suspension (15%) cultured over two growth cycles in 500 ml culture medium and low haematocrit. Parasite invasion and growth of control and drug pre-treated erythrocytes were then monitored for the following 48 h using microscopy.

Haeme polymerisation

Haeme polymerisation was measured as described previously [Egan et al., 1994]. Haemin (15 mg) was added to 3 ml of 0.1 M NaOH, 0.3 ml of 1 M HCl solutions with or without the powdered forms of the test agents (42 mg or 3 mole). The solutions were heated at 60°C until equilibrated and 1.74 ml of 12.9 M acetic acid was added. The control solution was immediately placed on ice to stop β-hematin formation, while solutions with or without test agents were incubated for a further 30 minute period. At the end of the incubation period, the reaction mixture was cooled on ice for 5 min. and then filtered using an 8 µm cellulose acetate/nitrate Millipore® filter type SC and extensively washed with water. The solid precipitate was dried over silica gel and phosphorous pentoxide at room temperature for 48 h. The precipitate (2 mg) and potassium bromide (200 mg) were ground into a fine powder, which was then compressed under 10 tons to prepare discs, which were analysed by infrared spectroscopy using a Perkin-Elmer 983 infrared spectrometer. β-hematin has characteristic peaks at 1,210 and 1,660 cm^{-1}.

Cytotoxic effects on erythrocytes

The cytotoxic effects of B4119 and B4158 (1–8 µM) on uninfected red cells were assayed using a haemolytic procedure, as well as by investigating the effects of these agents on glycolytic activity by measuring intracellular lactate and ATP levels. By using the haemolytic assay, the erythrocytes (0.5% haematocrit) in serum-supplemented, indicator-free RPMI, were exposed to the test agents for 30 min and 48 h at 37°C in an atmosphere of 5% CO_2. Following incubation, haemoglobin in the cell-free supernatants was measured spectrophotometrically at a wavelength of 405 nm.

To measure the effects of the test agents on intracellular lactate and ATP, a similar experimental set-up was used. However, for these assays the cells were washed twice with indicator- and serum-free RPMI following incubation and the cells lysed and protein precipitated by addition of 1 ml 0.6 N perchloric acid to the pellets. The tubes were then centrifuged at 3,000 rpm for 10 min and the protein-free supernatants removed and restored to neutral pH using 10 N NaOH. Lactate was assayed by using an enzymatic procedure based on the lactate dehydrogenase (from hog muscle, 220 milliunits/assay, Boehringer Mannheim, Marburg, Germany)-mediated conversion of lactate to pyruvate in the presence of 7 mM NAD. The conversion of NAD to NADH was monitored spectrophotometrically at 340 nm [Wahlefeld, 1974].

Erythrocyte ATP concentrations were measured in the extracts of control and drug-treated cells by using a luciferin/luciferase chemiluminescence method [Holmsen et al., 1972].

Murine model of experimental chemotherapy

Ethics Committee approval was obtained for all animal experiments. Scientific Procedures and the Code

of Practice for the Housing and Care of Animals Used in Scientific Procedures (Act 1986 and 1989, respectively) were strictly adhered to. Female, 6–8-weeks-old, BALB/c mice were divided into treatment groups with ten mice in each. B4119 was administered in the diet at a dosage of 30 mg/kg/day alone and in combination with chloroquin (1.25 μg/kg/day), intraperitoneally for 10 days, commencing on the day of infection with a chloroquin sensitive *Plasmodium berghei* strain (intraperitoneal injection with 2×10^6 parasitized erythrocytes). Parasitemia was evaluated by microscopic evaluation of Giemsa-stained thin smears every second day starting from day 4. The mice were terminated on day 10 and the results expressed as intensity of parasitemia.

Statistical Analysis

Results are expressed as the mean value ± SEM for each series of experiments. Levels of statistical significance were calculated using Student's t test (paired statistics).

RESULTS

Direct Antimalarial Activity of Riminophenazines In Vitro

The effects of the test agents on parasite growth, measured by flow cytometric and microscopic methods, are presented in Table 1. Of the eight compounds tested against the RSA8 strain, B4100, B44103, B4112, B4121, and B4169 inhibited parasite growth at concentrations of 1.2 μM and higher, while B4119 and B4158 showed significant activity ($P < 0.05$) at concentrations lower than 0.5 μM, with B4119 being the most active of the two. Clofazimine, the parent compound, did not show any activity at the concentrations tested (1–8 μM). Of the eight compounds tested, B4119 and B4158 were selected for assessment of their activities against additional strains of *P. falciparum* and the results are shown in Table 2 [Freese, 1993]. All of the strains tested, including those which were resistant to the conventional anti-malarial agents, were found to be sensitive to B4119 and B4158.

TABLE 1. Sensitivity of the RSA8 Strain of *Plasmodium falciparum* to the Riminophenazine Agents In Vitro[a]

Compound	IC_{50} (μM)
Chloroquin	0.076
B663	>8.00
B4100	2.50
B4103	1.50
B4112	1.20
B4119	0.22
B4121	5.00
B4158	0.40
B4169	6.00

[a]IC_{50} concentrations are the means of three separate experiments.

TABLE 2. In Vitro Sensitivity of Four Strains of *Plasmodium falciparum* to B4119 and B4158 and to the Standard Antimalarial Compounds, Chloroquin, Quinine, and Sulfadoxine/pyrimethamine

	IC_{50} (μM) and *P. falciparum* strains			
Compound	PfUP10[b]	RSA17	RAS16	RSA9
Chloroquin	0.074	0.08[c]	0.06[c]	0.16[c]
Quinine	ND	0.59[c]	0.65	0.67[c]
Sulfadoxine/ pyrimethamine	ND	2.3[c]	2.1[c]	6.20
B4119	0.22	0.24	0.22	0.40
B4158	0.40	0.60	0.30	0.70

[a]IC_{50} concentrations represent the means of three separate experiments.
[b]ND, not determined
[c]Values obtained from Freese [1993].

In Vitro Activity of Drug Combinations

The effects of chloroquin and mefloquine on the growth of *P. falciparum* in the presence or absence of the TMP-substituted phenazines, B4119 and B4158 are shown in Figures 2 and 3, respectively. For these experiments, the PfUP10 laboratory strain of *P. falciparum* was passaged for 2 months under proper culture conditions without cryopreservation such that the IC_{50} concentrations for chloroquin increased from 0.075 μM to 0.38 μM in the absence of drug pressure. Combining the TMP-substituted phenazines with low concentrations of chloroquin (0.044–0.5 μM) or mefloquine (0.01–0.065 μM) resulted in inhibition of parasite growth, which was greater than that observed with the individual agents.

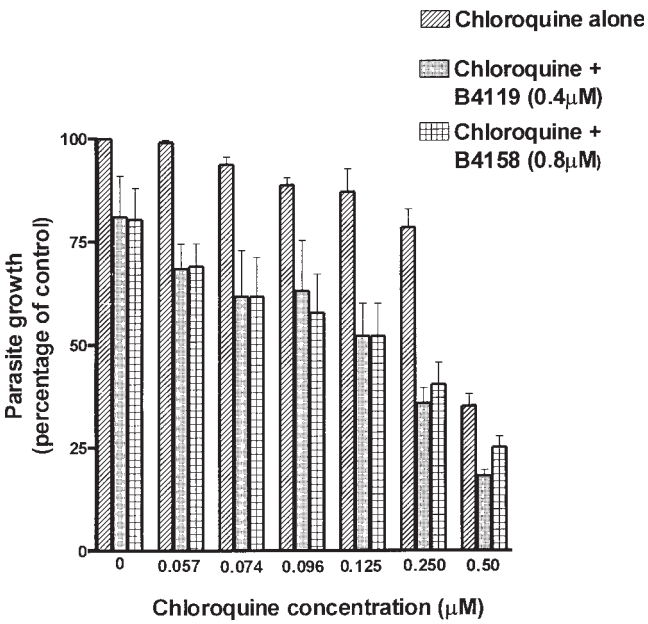


Fig. 2. Effects of chloroquin on the growth of the PfUP10 strain of *P. falciparum* in the presence and absence of B4119 and B4158. Data from three experiments performed in duplicate are presented as the mean percentage parasitemia of the corresponding control systems ± SEMs.

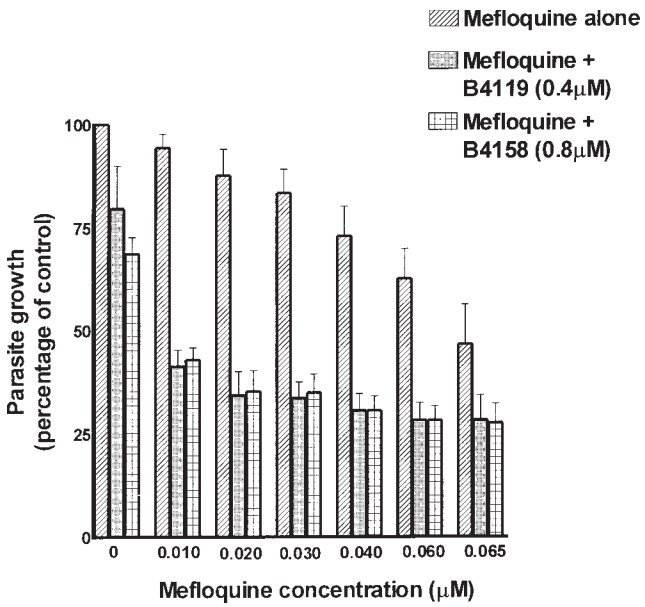


Fig. 3. Effects of mefloquine on the growth of the PfUP10 strain of *P. falciparum* strain in the presence and absence of B4119 (A) and B4158 (B). Data from three experiments performed in duplicate are presented as the mean percentage parasitemia of the corresponding control systems ± SEMs.

Parasite Stage-Specific Inhibition

The results shown in Figures 4 and 5 indicate that both B4119 and B4158 significantly ($P < 0.05$) inhibited the growth of the first (ring forms to trophozoites) and particularly the last (trophozoites/schizonts to rings) stages of the parasite growth cycle in a dose-related manner.

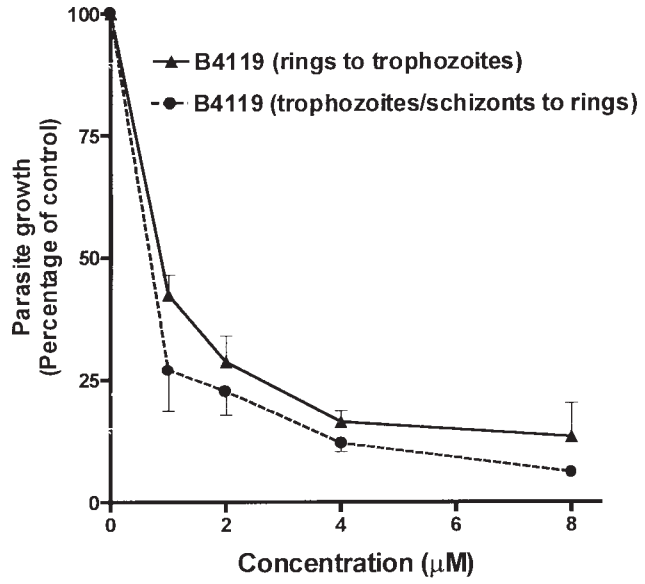


Fig. 4. Stage-specific antimalarial activity of B4119 at the first (rings to trophozoite) and last (trophozoite/schizont to rings) 24 h of the parasite life cycle. Data from three experiments conducted in duplicate are expressed as the mean percentage parasitemia of the control system ± SEMs.

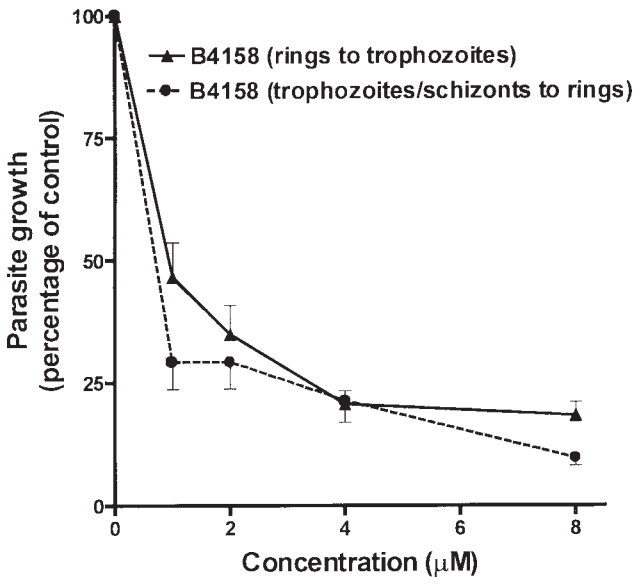


Fig. 5. Stage-specific antimalarial activity of B4158 at the first (rings to trophozoite) and last (trophozoite/schizont to rings) 24 h of the parasite life cycle. Data from three experiments conducted in duplicate are expressed as the mean percentage parasitemia of the control system ± SEMs.

Invasion Inhibition Potential

Since riminophenazines are membrane-active agents, their ability to alter the erythrocyte membrane and inhibit parasite invasion and maturation into trophozoites was also investigated. To promote merozoite invasion schizont-infected erythrocytes were coincubated with control and drug-pretreated red cells. Neither agent at the concentrations (1–8 μM) used inhibited invasion. However, both agents interfered with the maturation of ring forms into trophozoites at 4 and 8 μM as shown in Figures 6 and 7.

Haeme Polymerisation

The effects of B4119 and B4158 on haeme polymerisation are shown in Figure 8. Addition of acetic acid (to give a final concentration of 4.5 M, pH 4.5) to haemin at 60°C followed by 30 min incubation leads to the formation of β-hematin (hemozoin or malaria pigment) with a characteristic infrared spectrum which includes intense peaks at 1210 and 1661 cm^{-1} indicated by arrows in Figure 8A. When 3 mole equivalents of B4119 or B4158 were mixed with hematin (before addition of acetic acid), the formation of β-hematin was inhibited as indicated by the absence of strong peaks at the positions indicated by arrows in Figure 8B,C. Although the peak at 1,661 cm^{-1} may be obscured by drug peaks in both cases, the peak at 1,210 cm^{-1} is clearly absent.

Cytotoxic Effects of the Riminophenazines

Exposure of erythrocytes to 8 μM B4119 and B4158, but not at lower concentrations, resulted in release of

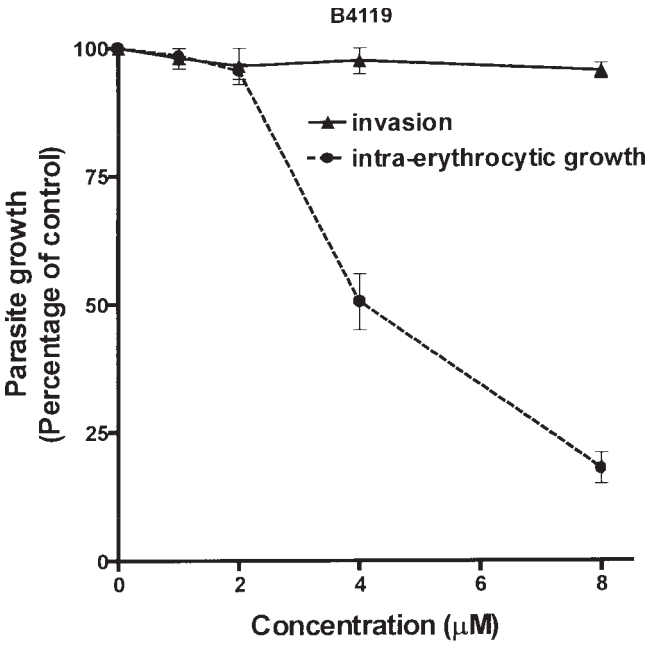


Fig. 6. Effects of pre-treatment of erythrocytes with B4119 on the invasion and intra-erythrocytic growth of *P. falciparum*. Data from three experiments conducted in duplicate are presented as the mean percentage parasitemia of the control system ± SEMs.

80% and 65% haemoglobin, respectively, after 48 h of incubation, while no significant ($P > 0.05$) haemolysis was detected at any concentration tested after a 45 min incubation period. There were no noticeable changes in intracellular levels of lactate and ATP after treatment with the two agents (1 and 2 μM) for 48 h (results not shown).

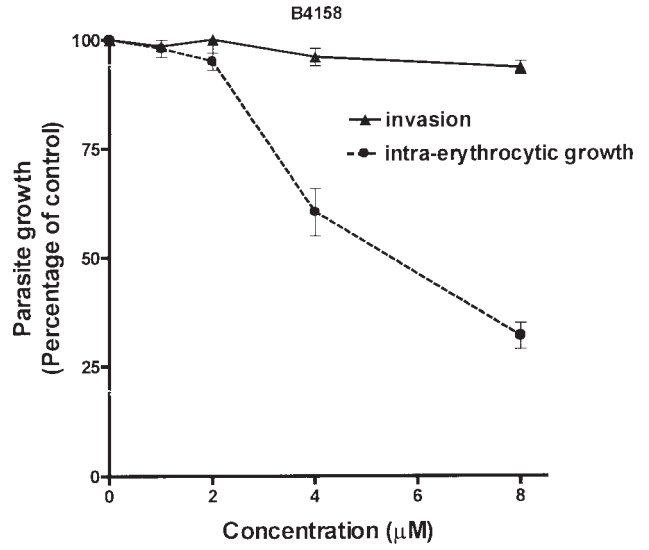


Fig. 7. Effects of pre-treatment of erythrocytes with B4158 on the invasion and intra-erythrocytic growth of *P. falciparum*. Data from three experiments conducted in duplicate are presented as mean percentage parasitemia of the control system ± SEMs.

Therapeutic Activity of B4119 in *P. berghei*-Infected Mice

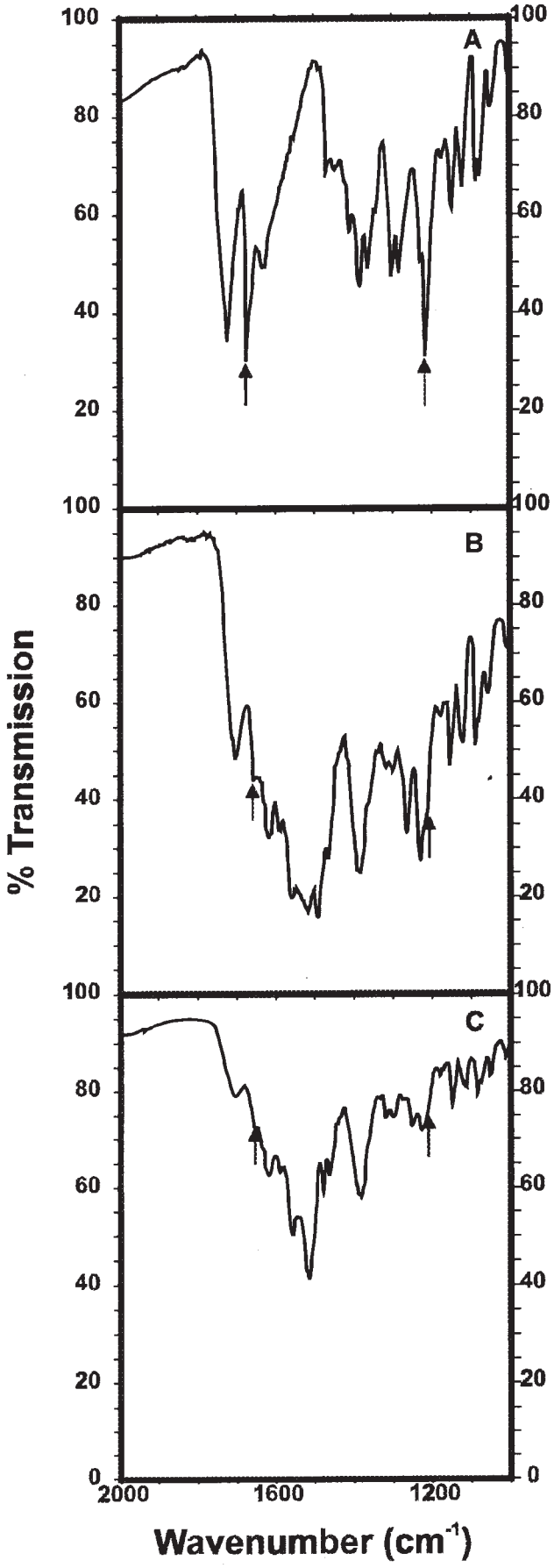
These experiments were undertaken to investigate the therapeutic efficacy of B4119 used as a single agent, as well as in combination with low-dose chloroquin, in the treatment of *P. berghei*-infected mice. The results are shown in Figure 9. When used as a single agent, B4119 significantly reduced the level of parasitemia in the experimentally infected mice. This effect was most evident when B4119 (30 mg/kg/day) was combined with low-dose chloroquin (1.25 μ/kg/day).

DISCUSSION

It has previously been reported that the prototype riminophenazine, clofazimine, which is primarily an antimycobacterial agent [Barry et al., 1957; Schaad-Lanyi et al., 1987], does not possess anti-plasmodial activity [Sheagren, 1968]. In the current study, we have observed that novel derivatives of clofazimine, the TMP-substituted phenazines, do, however, inhibit the growth of *P. falciparum* in vitro. The major structural difference between clofazimine and the TMP-substituted phenazines relates to the nature of the substitutent bound to the imino nitrogen functional group at position 2 on the phenazine nucleus. In the case of clofazimine, this position is occupied by an isopropyl group, and by a TMP group in the case of the novel compounds. All seven of the TMP-substituted phenazines varied according to the number and type of substitutents on the aniline and phenyl rings attached to positions 3 and 10 of the phenazine nucleus respectively. Two of these, B4119 and B4158, exhibited anti-plasmodial activity which was comparable with that of chloroquin. B4119, the most potent of the two agents, is halogenated, whereas B4158 is isopropylated at their respective aniline and phenyl rings. B4119- and B4158-mediated inhibition of the growth of the RB-1 laboratory strain of *P. falciparum* was observed at sub-micromolar concentrations of these agents. Importantly, serum concentrations of up to 8 μM have been reported in patients with leprosy following oral administration of clofazimine [Schaad-Lanyi et al., 1987].

Stringently synchronised cultures were used to investigate the stages of growth at which *P. falciparum* is most vulnerable to the TMP-substituted phenazines. In these experiments B4119 and B4158 were added to ring and late trophozoite cultures of the parasite. Both test agents were found to interfere with parasite development at both stages of the life cycle, with the late phase of parasite development being the most sensitive. These observations suggest that B4119 and B4158 affect metabolic events, which are essential for parasite survival throughout the life cycle.

B4119 and B4158 were also found to be active against quinine- and sulfadoxine/pyrimethamine-resistant strains of *P. falciparum*, with the levels of sensitivity be-



ing similar to those of the drug-sensitive strains of the parasite. These observations demonstrate that the sensitivity of *P. falciparum* to the TMP-phenazines is unaffected by the mechanisms, which confer resistance to the three conventional anti-malaria agents. Chloroquin and quinine are lysosomotropic weak bases, which accumulate within food vacuoles, killing the parasite by interfering with haeme polymerization [Wahlefeld, 1974; Ridely, 1997]. Chloroquin-mediated inhibition of the polymerisation of haeme to β-hematin is achieved through the formation of haeme-chloroquin complexes that are toxic for the parasite [McChesney and Fitch, 1984]. Sulfadoxine/pyrimethamine is an anti-metabolite combination which inhibits plasmodial folate metabolism, resulting in parasite death [Peterson et al., 1990].

Interestingly, B4119 and B4158 were also found to inhibit haeme polymerisation in vitro, suggesting that these TMP-substituted phenazines and chloroquin have a common mechanism of anti-plasmodial action. Inhibition of polymerisation of hematin to β-hematin may therefore be a shared property of lipophilic, cationic amphiphiles, which accumulate in acidic organelles.

Combining chloroquin or mefloquine, at sub-therapeutic concentrations, with sub-micromolar concentrations of either B4119 or B4158 in vitro resulted in anti-plasmodial activities, which were considerably higher than those of the individual agents. This is not entirely surprising since these different agents appear to have a common target in malarial parasites. Similar findings were obtained in a murine model of experimental chemotherapy. In these experiments B4119 (30 mg/kg) was found to be effective against *P. berghei* in vivo, while the combination of the TMP-substituted phenazine together with a sub-therapeutic dose of chloroquin exhibited therapeutic activity against this microbial pathogen, which was superior to that observed with the individual agents. These observations suggest that the TMP-phenazines may be particularly useful in combination with chloroquin or mefloquine in order to maximise the potency and minimise the toxicity of these conventional anti-plasmodial agents. Although the issue of TMP-substituted phenazine-mediated prevention of resistance or restoration of chemosensitivity to chloroquin has not been addressed in the present study, it is noteworthy that the PF UP10 strain of *P. falciparum* used in drug combination studies had developed intermediate resistance to chloroquin as a result of repeated culture in vitro. Exposure of this relatively resistant strain to minimally active con-

Fig. 8. Infrared spectra of β-hematin in the absence (**A**) and presence of either B4119 (**B**) or B4158 (**C**). The definitive peaks for β-hematin (malaria pigment) at 1,660 cm^{-1} and 1,210 cm^{-1} are marked with arrows in A. The peaks are clearly absent in the drug-treated samples as indicated by the arrows in B and C.

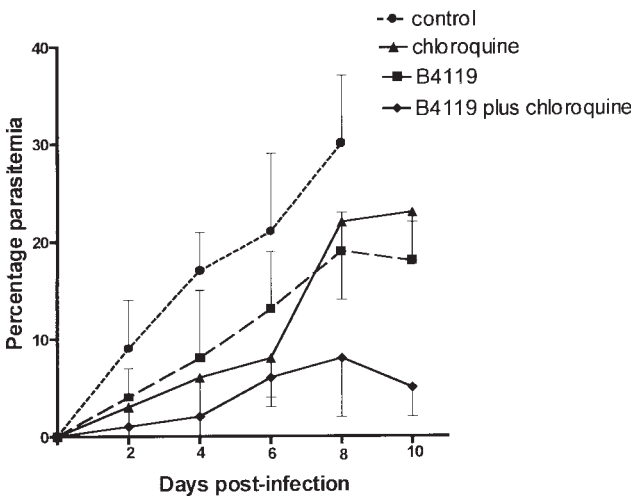


Fig. 9. Effects of B4119 (30 mg/kg/day) alone and in combination with chloroquin (1.25 μg/kg/day) on the level of parasitemia in *P. berghei* infected mice.

centrations of B4119 and B4158 appeared to increase its sensitivity to chloroquin and mefloquine.

To establish if the observed anti-plasmodial activity of B4119 and B4158 was indeed primary, or rather secondary to membrane-disruptive or cytotoxic effects on erythrocytes, the susceptibility to infection, viability, and metabolic activity of these cells was measured following exposure to the TMP-substituted phenazines for 48 h. Relative to control, untreated erythrocytes, pretreatment with the TMP-substituted phenazines followed by washing did not affect vulnerability to infection with *P. falciparum*. Drug-mediated cytotoxic effects on erythrocytes were investigated using a haemolytic procedure, as well as by measurement of glycolytic activity according to the production of intracellular lactate and ATP in control and drug-treated erythrocytes. At the highest concentration used (8 μM), both TMP-phenazines were cytolytic for erythrocytes. However, at lower concentrations, at which effective anti-plasmodial activity was evident, neither B4119 nor B4158 affected erythrocyte viability or metabolic activity. These observations demonstrate that at concentrations of 4 μM and lower, B4119 and B4158 are selectively active against *P. falciparum*.

In conclusion, we have demonstrated that TMP-substitution at position 2 on the phenazine nucleus of riminophenazines confers in vitro and in vivo to anti-plasmodial activity on these compounds. These may prove to be useful forerunners in the design of novel anti-plasmodial pharmacologic agents.

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