Violacein Extracted from *Chromobacterium violaceum* Inhibits *Plasmodium* Growth In Vitro and In Vivo[▽]

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Violacein is a violet pigment extracted from the gram-negative bacterium *Chromobacterium violaceum*. It presents bactericidal, tumoricidal, trypanocidal, and antileishmanial activities. We show that micromolar concentrations efficiently killed chloroquine-sensitive and -resistant *Plasmodium falciparum* strains in vitro; inhibited parasitemia in vivo, even after parasite establishment; and protected *Plasmodium chabaudi chabaudi*-infected mice from a lethal challenge.

Violacein is a violet pigment isolated from *Chromobacterium violaceum*, a gram-negative betaproteobacterium found in the Amazon River in Brazil. It has been reported to kill bacteria (4) and induces apoptosis in various types of cancer cells (1, 5, 7, 8, 10, 11). Moderate activity against *Trypanosoma cruzi* and *Leishmania amazonensis* promastigotes has also been observed (3, 9). Due to the widespread presence of drug resistance in the malaria parasite, resulting in dramatically decreased efficacy of available antimalarial drugs (15), and the fact that immunoprotection achieved by the most successful malaria vaccine is only partial and short-lived (14), we evaluated the in vitro and in vivo effects of violacein on human and murine blood stage forms of *Plasmodium* parasites.

Isolation and purification of violacein, 3-[1,2-dihydro-5-(5-hydroxy-1*H*-indol-3-yl)-2-oxo-3*H*-pyrrol-3-ylideno]-1,3-dihydro-2*H*-indol-2-one (Fig. 1), from *C. violaceum* (CCT3496) were performed as previously described (12). Toxicity was measured as the concentration-dependent lysis of normal erythrocytes (NE) by counting red blood cells per milliliter with the aid of a Neubauer chamber. After 48 h of exposure to various concentrations of violacein, the percent red blood cell density (RBCD) relative to that of the control (without violacein) was monitored and calculated according to the formula (RBCD per milliliter in the presence of violacein/RBCD per milliliter without violacein) × 100. As shown in Fig. 2A, a slight reduction in the RBCD percentage at violacein concentrations of

>8.0 μM was observed. Significant (Mann-Whitney U test, $P < 0.05$) toxicity to NE occurred at a concentration of 14.0 μM.

Next, we performed dose-response assays to obtain the 50% inhibitory concentrations (IC_{50}s) of violacein against erythrocytes infected with chloroquine-sensitive or -resistant strains of *P. falciparum* (3D7 [16] or S20 [2], respectively) at 1% parasitemia and a 2% final hematocrit. We used [^{3}H]hypoxanthine (Amersham Biosciences, Amersham, United Kingdom) incorporation to assess parasite growth according to a protocol described elsewhere (13). Violacein was tested in triplicate at least three times with different batches and cells, and parasite growth was compared to that in nontreated infected erythrocytes (IE), which represented 100% parasite growth. Percent parasite growth inhibition was calculated according to the formula [1 − (cpm of treated IE − cpm of NE/cpm of nontreated IE − cpm of NE)] × 100. After a 48-h incubation, violacein inhibited parasite development even at the lowest tested concentration of 0.06 μM and completely abrogated parasite viability at concentrations of >1.0 μM (Fig. 2B).

The IC_{50} of violacein against *P. falciparum* strain 3D7 was calculated as 0.85 ± 0.11 μM. We then tested whether the effect of violacein was directed against young (rings, 0 to 24 h) or mature (trophozoites and schizonts, 24 to 48 h) blood forms by using synchronized parasites ($\pm$ 6 h) obtained by repeated 5% sorbitol treatment as previously described (6). After a 24-h incubation, inhibition of the different parasite stages by violacein was measured. As shown in Fig. 2C, there was no statistically significant difference (Mann-Whitney U test, $P > 0.05$) between the inhibitory values of violacein in either parasite stage. We then asked if susceptibility or resistance to chloroquine also predicts parasite sensitivity to violacein. As shown in Fig. 2D, the violacein IC_{50}s for strains 3D7 and S20 did not differ significantly (0.85 ± 0.11 and 0.63 ± 0.13 μM, respectively; Mann-Whitney U test, $P > 0.05$).

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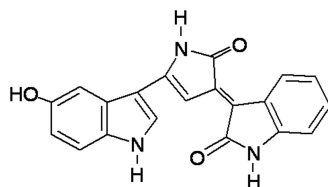▽ Published ahead of print on 9 March 2009.

FIG. 1. Chemical structure of violacein.

We then investigated whether violacein antimalarial activity could be sustained in a mouse model, where other characteristics such as bioavailability and pharmacokinetics have to be taken in account. For the in vivo assays, C57BL/6 mice (7 to 10 mice per group, aged 7 to 10 weeks, and with a body weight of 20 ± 3 g) were infected with a nonlethal (AS) or a lethal (AJ) strain of *Plasmodium chabaudi chabaudi* by intraperitoneal (i.p.) injection with 10^6 IE. Parasitemia levels were determined daily by counting the IE among at least 1,000 erythrocytes in Giemsa-stained blood smears. As shown in Fig. 3A, daily ad-

ministration of violacein i.p. for 11 consecutive days (0 to 10 days postinfection [p.i.]) reduced the parasitemia of *P. chabaudi chabaudi* AS-infected mice. Thirty-nine percent inhibition was observed on day 7 p.i. (parasitemia peak), in comparison to nontreated mice (control; Table 1), even at a low dose of 0.75 mg/kg/day. Moreover, the two highest doses of violacein (3.75 and 7.5 mg/kg/day) almost completely abolished parasitemia on day 7 p.i., corresponding, respectively, to 82 and 87% inhibition of parasite development (Table 1). In addition, violacein doses of 0.75 to 7.5 mg/kg/day were able to inhibit the peak parasitemia in a dose-dependent manner (Table 1).

Since violacein did not completely abrogate parasitemia early in infection and drug pressure was removed by day 10 p.i., we monitored the parasitemia levels of *P. chabaudi chabaudi* AS-infected mice treated with the highest dose of violacein daily until day 22 p.i. Notably, on day 16 p.i., which represents the sixth day after the end of violacein treatment, parasite development was still significantly (Mann-Whitney U test, $P <$
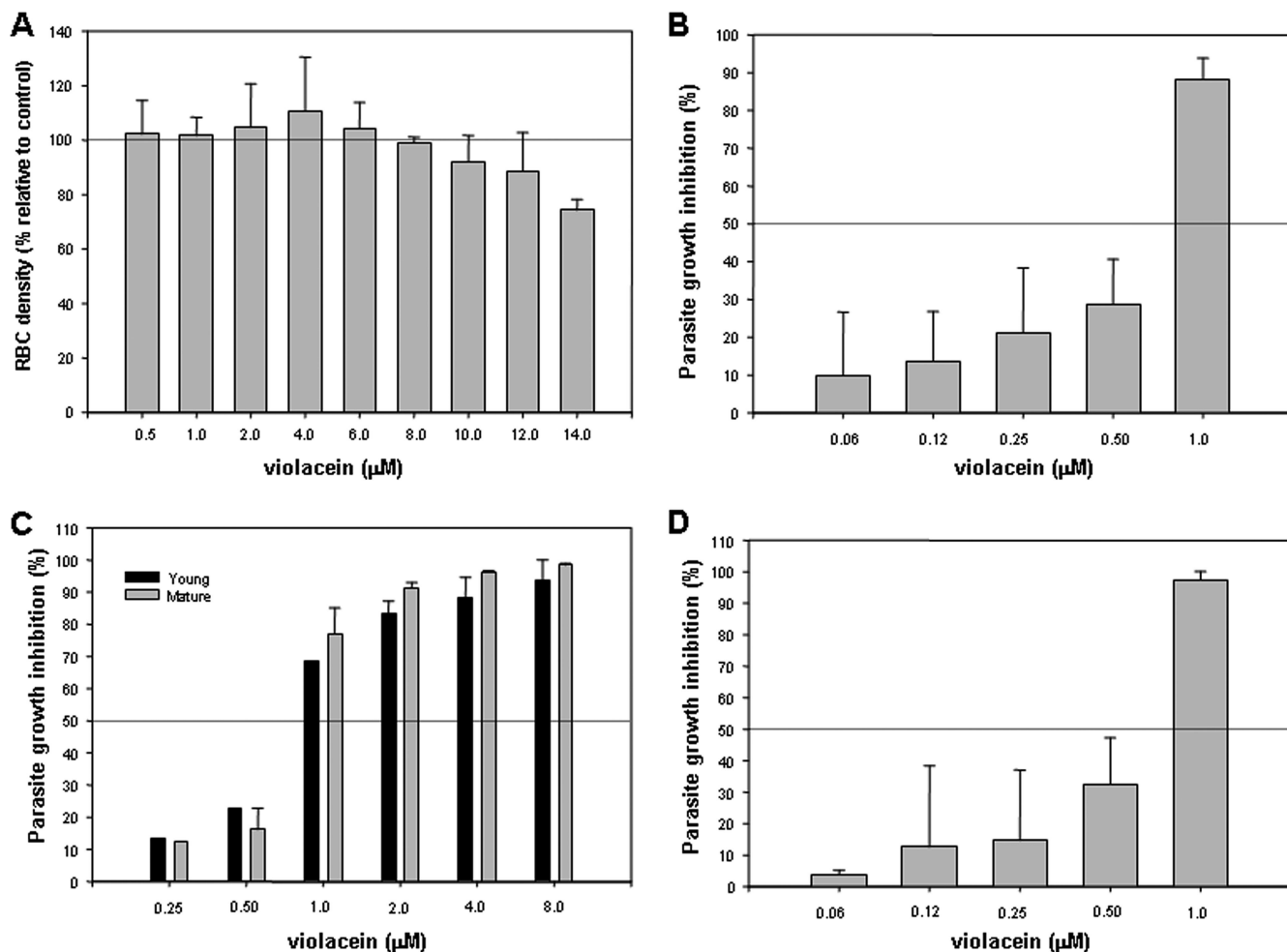

FIG. 2. Evaluation of violacein antimalarial activity against *P. falciparum*. (A) Noninfected erythrocytes (RBC) were cultivated for 48 h at 37°C in the presence of different concentrations of violacein. The RBCD was determined as a percentage of that the control (without drug). Inhibition of the growth of *P. falciparum* 3D7 IE cultivated for 48 h at 37°C with different concentrations of violacein (B) and of young or mature stages of this parasite (C) is also shown. (D) Effect of violacein against a chloroquine-resistant strain (S20) of *P. falciparum* in IE. Results are expressed as the mean of triplicate measurements ± the standard deviation.
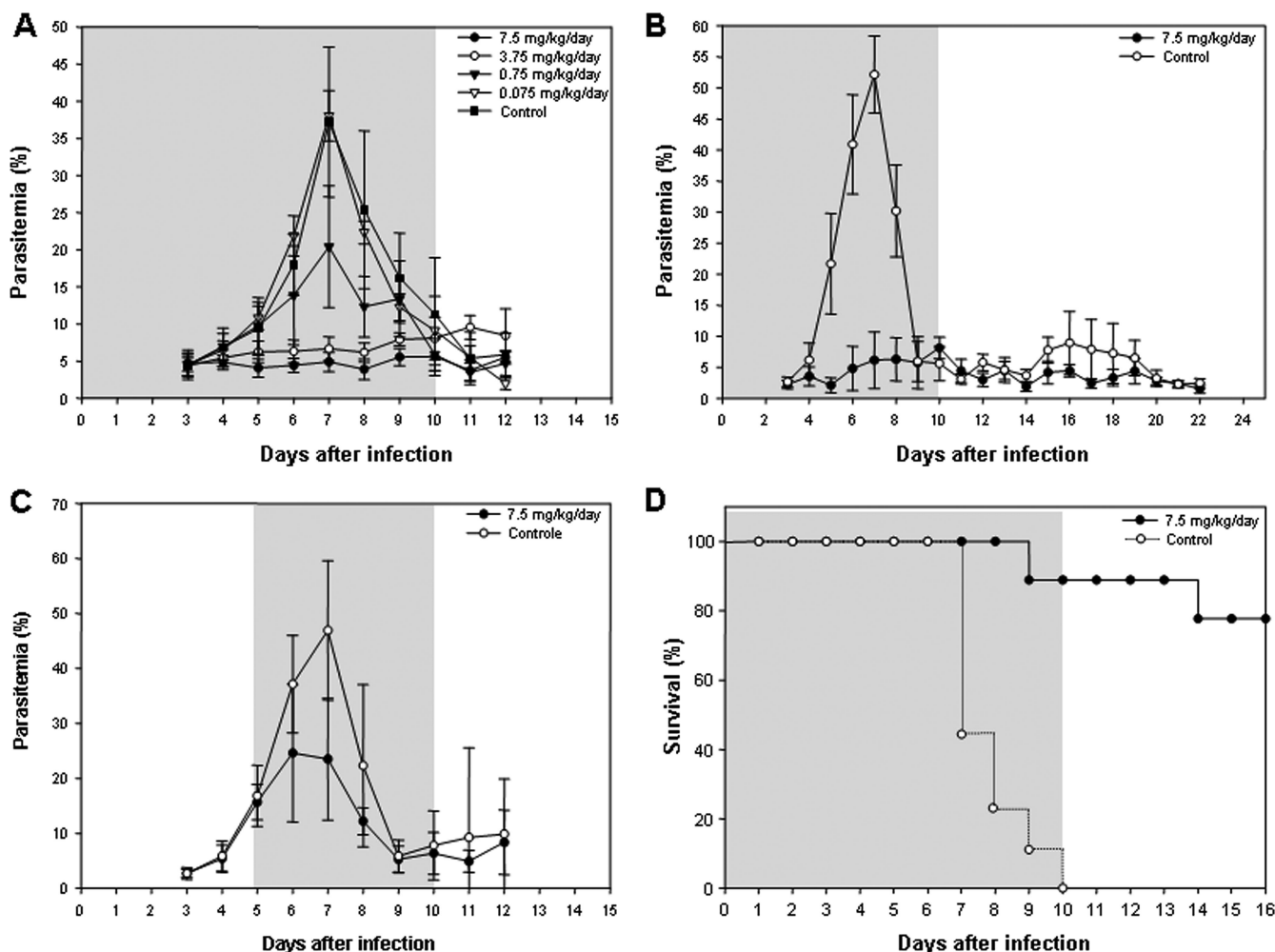
FIG. 3. Effect of violacein on mouse-derived *Plasmodium* parasites. Groups of 7 to 10 C57BL/6 mice were infected i.p. with 10^6 *P. chabaudi chabaudi* AS IE and then left untreated or treated with different doses of violacein administered i.p. for 11 consecutive days (days 0 to 10 p.i.), starting on day 0 at 1 h p.i. Parasitemia levels were determined daily until day 12 p.i. (A) or up to day 22 p.i. (B) after treatment with the highest dose (7.5 mg/kg/day). (C) Analysis of violacein antimalarial activity after parasite establishment and treatment for 6 consecutive days (days 5 to 10 p.i.) with the highest dose of violacein in *P. chabaudi chabaudi* AS-infected mice. Results are expressed as the mean of a group of mice ± the standard deviation. (D) Analysis of the survival of groups of 10 C57BL/6 mice infected with *P. chabaudi chabaudi* AJ (lethal strain) by i.p. injection of 10^6 IE and then treated i.p. with violacein at 7.5 mg/kg/day for 11 consecutive days (days 0 to 10 p.i.). The drug administration period is indicated by shading in each graph.

0.05) inhibited (up to 59%; Fig. 3B). To verify whether violacein had an effect on parasite growth after the establishment of infection, *P. chabaudi chabaudi* AS-infected mice received violacein from day 5 (16% parasitemia) to day 10 p.i. This can reflect the time point when malaria therapy is given to patients. As shown in Fig. 3C, violacein administration during patent parasitemia was able to reduce parasite growth significantly (Mann-Whitney U test, $P < 0.01$), by up to 50.1%, in compar-

TABLE 1. Inhibition of parasitemia in *P. chabaudi chabaudi* AS-infected mice under violacein treatment

Violacein dose (mg/kg/day)	Mean % inhibition[a] ± SD (P value)			
	Day 5	Day 6	Day 7	Day 8
7.5	56.48 ± 12.84 (<0.005)	75.40 ± 5.17 (<0.005)	86.90 ± 3.53 (<0.005)	84.67 ± 5.43 (<0.05)
3.75	33.23 ± 14.72 (NS)[b]	64.89 ± 8.00 (<0.005)	82.12 ± 4.33 (<0.005)	75.73 ± 13.93 (<0.05)
0.75	3.58 ± 33.80 (NS)	20.42 ± 36.30 (NS)	39.25 ± 14.15 (<0.05)	48.85 ± 15.08 (NS)
0.075	NI[c] (ND)[d]	NI (ND)	NI (ND)	12.04 ± 6.11 (NS)

[a] The data shown are percentages of the parasitemia of nontreated mice, which was defined as 100%. Values for groups of 7 to 10 mice are shown.
[b] NS, not statistically significant.
[c] NI, no inhibition.
[d] ND, not determined.

ison to nontreated animals on the seventh day of infection. Next, to determine the protective effect of violacein, mice were infected with the lethal AJ strain of *P. chabaudi chabaudi* and their survival rate was evaluated. As shown in Fig. 3D, 100% of the nontreated mice died by day 10 p.i., with 50% of the deaths occurring early on day 7 p.i. In contrast, animals treated with violacein at 7.5 mg/kg/day did not succumb to infection until days 9 (10%) and 14 (10%) p.i., reaching 80% survival on day 16; clearly demonstrating the significant (log rank test, $P <$ 0.0001) protective effect of violacein.

This study demonstrates for the first time the antimalarial activity of violacein by showing inhibition of the growth of human- and mouse-derived *Plasmodium* parasites. Also, violacein was effective against young and mature forms of the human parasite and its activity extended to chloroquine-sensitive and -resistant strains of *P. falciparum*. Our data call for new formulations based on violacein nanoparticles to improve solubility, bioavailability, and activity and to decrease drug toxicity.

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