Cycloprodigiosin Hydrochloride Obtained from *Pseudoalteromonas denitrificans* Is a Potent Antimalarial Agent

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Cycloprodigiosin hydrochloride (cPrG·HCl) is a stable fluorescent red pigment obtained from the marine bacterium *Pseudoalteromonas denitrificans*. It was found that the compound was incorporated into *Plasmodium falciparum* cells upon incubation and exhibited a potent antimalarial activity with the concentration required for 50% of the activity being 11 nM, which is stronger than that of chloroquine, a well-known antimalarial agent. The compound did not affect growth rate of mammalian cells. Antimalarial activity of cPrG·HCl was also observed *in vivo*. These results indicate that cPrG·HCl is a potent antimalarial drug.

Key words cycloprodigiosin; *Plasmodium falciparum*; malaria parasite; antimalarial drug; *Pseudoalteromonas denitrificans*; chloroquine

Worldwide emergence of a chloroquine-resistant variant of *Plasmodium falciparum* has stimulated development of a new type of drug effective against malaria.[1] We have looked for a candidate as an antimalarial agent from various natural products from marine organisms, since they have unique structure and a variety of biological activities.[2] Here, we report that cycloprodigiosin hydrochloride (cPrG·HCl) obtained from the marine bacterium, *Pseudoalteromonas denitrificans*,[3] shows potent antimalarial activity. Evidence is presented for the possible participation of vacuolar H^+-ATPase in the cPrG·HCl action.

MATERIALS AND METHODS

Preparation of cPrG·HCl cPrG·HCl was prepared as described previously.[3] In brief, packed cells (10 g wet weight), obtained from 600 ml of culture as described above, were extracted by shaking with a mixture of acetone and diethyl ether (4 : 1). The extract was centrifuged at 12000 rpm for 10 min to give a deep red supernatant, which was dried over anhydrous magnesium sulfate and then concentrated *in vacuo*. The red pigment thus obtained was found to be composed largely of a single component with an *Rf*-value of 0.20 (Kieselgel 60F254; solvent: benzene–diethylether=1 : 1). The free base of cPrG·HCl was obtained by either passing a dichloromethane solution of cPrG·HCl through a SiO_2 column (Wako gel, C-200) or the basic treatment of cPrG·HCl with a sodium bicarbonate solution.

***P. falciparum* Culture and Drug Treatment** *P. falciparum* strain FCR-3 (ATCC 30932) cells were cultured in 10% heat-inactivated A^+ human erythrocytes, then suspended at a hematocrit of 5% in RPMI 1640 medium (Gibco, NY) containing 50 mg/l gentamycin, and maintained at 37 °C under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 gas.[4,5] Various concentrations of cPrG·HCl or listed antimalarial agents (10 μl) were added to the individual wells of a 24-well plate. Erythrocytes exhibiting 0.3% parasitemia were added to each well in 990 μl of culture medium to give a final hematocrit of 3%. The plates were incubated at 37 °C under 5% CO_2, 5% O_2 and 90% N_2 gas for 72 h. Then, the parasite morphology in drug-treated culture was examined by staining with Giemsa, the number of parasitized cells per 10000 erythrocytes was determined and the growth rate was calculated as described previously.[6,7] The experiments were performed in duplicate for each concentration. The 50% inhibitory concentration (IC_{50}) was determined by comparison with drug-free controls incubated under the same conditions.

Antimalarial Activity *in Vivo* *Plasmodium berghei* (strain NK-65) was maintained by serial blood passaging in mice. The care and treatment of mice were performed in accordance with guidelines of the Science and International Affairs Bureau of the Japanese Ministry of Education, Science, Sports and Culture, No. 141, 1987. Male mice (ICR) weighing 20 to 25 g were obtained in sterile containers from Charles River Breeding Laboratories, Inc. They were housed under a natural day–night cycle at 25 °C. In each experiment, blood sampling from a donor mouse previously injected with *P. berghei* was performed when the parasitemia reached 10 to 15%.[6,7]

Accumulation of cPrG·HCl in *P. falciparum* Cells cPrG·HCl at 0.1 μM was incubated with infected blood cells at 37 °C for 30 min. Then, the cells were washed with the medium and observed under a confocal laser microscope (Meridian TR650) with an argon laser at 488 nm.

RESULTS AND DISCUSSION

P. falciparum cells were found to be highly sensitive to cPrG·HCl when it was added to culture medium (Fig. 1). IC_{50} was 11 nM, and complete killing was attained at 0.1 μM. This killing potency was slightly stronger than those of chloroquine and its derivatives (Table 1). On the other hand, cPrG·HCl was not harmful to various cultured mammalian clonal cells including rat pheochromocytoma PC 12 cells even at more than 1 μM (Fig. 1). Other cell lines including BNL C1.2, mouse liver cells, U937, human monocytoma, U373, human astrocytoma, and HeLa were also insensitive to the compound up to 2 μM, but became sensitive at higher

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concentrations with IC_{50} values being 7.1, 6.0, 6.0, and 6.2 μM, respectively. It is noteworthy that the killing activity is stable: the activity did not change even after cPrG·HCl at 10 μM was incubated for a week at room temperature.

Figure 2 indicates that cPrG·HCl exhibited antimalarial activity *in vivo*: intraperitoneal injection of cPrG·HCl at 25 mg/kg completely prevented parasitemia from day 1 to day 10, while the malaria organisms appeared in the bloodstream on day 3 in control mice. Taken together, these results indicate that cPrG·HCl is a potent antimalarial drug.

Table 1. Comparison of Antimalarial Activity of cPrG·HCl with Known Antimalarial Agents

Agent	IC_{50} (nM)
cPrG·HCl	11
Chloroquine	18
Quinine	110
Mefloquine	32
Artesunate	17

Antimalarial activities of the listed compounds were measured as described in the text and expressed as IC_{50}.

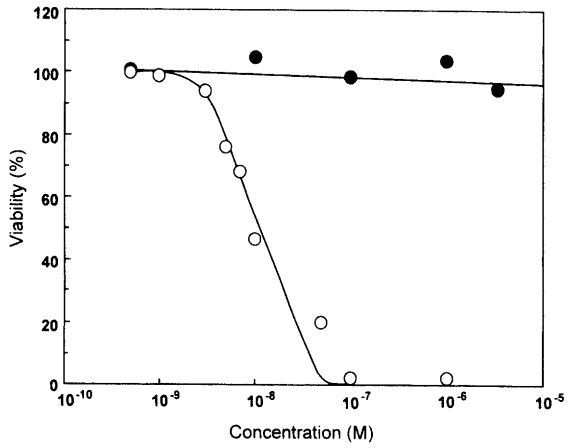

Fig. 1. Dose-Dependence of the Antimalarial Activity of cPrG·HCl (Open Circles), and Comparison with the Viability of PC12 Cells (Closed Circles)

Rat pheochromocytoma PC12 cells (1×10^6 cells) were cultured in the presence or absence of listed concentration of cPrG·HCl for 72 h, and the number of the living cells was determined by vital staining of trypane blue.

Since cPrG·HCl is a fluorescent compound, it is possible to detect its incorporation into *P. falciparum* cells under either fluorescence or laser microscope (Fig. 3). Upon incubation of infected blood cells with cPrG·HCl, the compound was significantly accumulated in the malaria parasites (Fig. 3 B, arrows), while no cPrG·HCl was accumulated in normal blood cells. The incorporation of cPrG·HCl into the malaria parasites was also evident on direct measurement of cPrG·HCl uptake into the malaria parasites; blood cells infected by *P. falciparum* cells were incubated with cPrG·HCl as described in Fig. 3, and washed three times with phosphate buffer saline . Then, the *P. falciparum* cells that had taken up cPrG·HCl were rapidly isolated by 0.075% (w/v) saponin treatment for 5 min,[6,7] and the cPrG·HCl content of the organisms was determined photometrically after solubilization with 0.5% sodium dodecylsulfate. More than 95% of cPrG·HCl incorporated in the blood fraction was recovered in the organisms. Pretreatment with both concanamycin A at 1 μM, a compound known to inhibit vacuolar H^+-ATPase[8,9] and

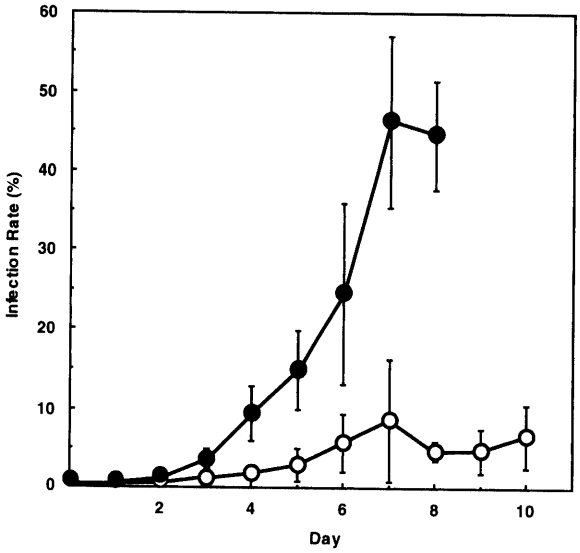

Fig. 2. cPrG·HCl Shows Antimalarial Activity *in Vivo*

Either cPrG·HCl (25 mg/kg body weight) (open circles) or saline as a control (closed circles) was injected intraperitoneally into mice. *P. bergheii* cells (5×10^6 parasites/0.2 ml saline) were then infected. Blood samples were taken daily and the parasitized blood cells were counted as described.[6,7]

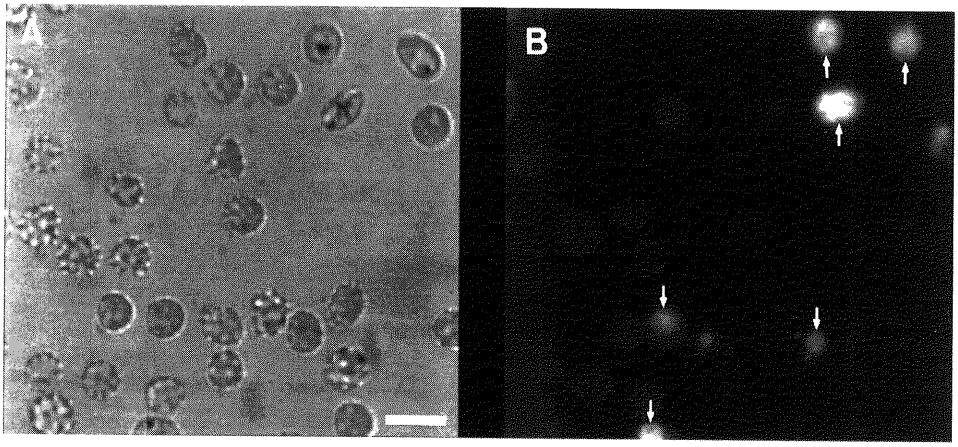

Fig. 3. Accumulation of cPrG·HCl in *P. falciparum*

Nomarski image of blood cells infected by *P. falciparum* after incubation with cPrG·HCl at 0.1 μM is shown in (A). Immediately after taking the Nomarski image, the same area was observed through a confocal laser microscope (B). cPrG·HCl was accumulated into a malaria parasite as indicated by arrows. Bar, 10 μm.

carbonylcyanide *m*-chlorophenylhydrazone (CCCP), a proton conductor, inhibited 78 and 89% of the accumulation of cPrG·HCl in malaria organisms, respectively.

In this study, we found that cPrG·HCl is a potent antimalarial drug and that it is accumulated in the malaria parasite. Accumulation of cPrG·HCl in malaria organisms may be the first event for its toxicity. We showed that the accumulation of cPrG·HCl in malaria organisms was inhibited by either a vacuolar H^+-ATPase inhibitor or a proton conductor, suggesting that the acidic pH environment established by vacuolar H^+-ATPase is somehow involved in the cPrG·HCl action. One plausible mechanism for the cPrG·HCl action is that the compound increases internal pH of food vacuoles. Since food vacuoles are counterparts of lysosomes of mammalian cells, an acidic internal pH plays an important role in degradation of exocytized haemoglobin, followed by energy acquisition for growth and cell division of malaria organisms.[10,11] Thus, it is possible that perturbation of internal acidic pH of food vacuoles by cPrG·HCl inhibits the degradation of hemoglobin, resulting in an energy crisis for the malaria parasite. More detailed studies will be necessary to elucidate the molecular mechanism of antimalarial activity by cPrG·HCl.

In conclusion, cPrG·HCl is a stable and highly fluorescent compound with low toxicity toward mammalian cells, and therefore it may be useful for studies on chemotherapy as well as the modes of action of antimalarial agents.

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