daily.[21] The percentage parasitemia and stages were assessed daily by microscopic examination of thin blood smears stained with Giemsa.

Preparation of Mitochondria Fraction

Mitochondria were prepared as described previously.[20] The cells were harvested and treated with 0.075% (w/v) saponin in AIM (120 mM KCl, 20 mM NaCl, 10 mM Pipes, 1 mM $MgCl_2$, and 5 mM glucose; pH 6.7). After washing with AIM, the cells were disrupted with the N_2 cavitation method using cell disruption Bomb (4639, Parr, USA) at 1,200 psi for 20 min at 4°C in MSE buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA, and 3 mM Tris HCl; pH 7.4) in the presence of 1 mM phenylmethylsulfonyl fluoride. Unbroken cells and cell debris were removed by centrifugation at 800× *g* for 5 min at 4°C. The mitochondrial fraction was then recovered as a precipitate by centrifugation at 23,000 × *g* for 20 min at 4°C.

Enzyme Assay

Succinate-ubiquinone reductase (SQR) activity and succinate dehydrogenase (SDH) activity were assayed as described previously.[20] Dihydroorotate dehydrogenase (DHOD) activity was measured by recording the absorbance change of dichlorophenol indophenol (DCIP) at 600 nm (Shimadzu spectrophotometer UV-3000). The reduction was started by the addition of 500 μM DHO to the reaction mixture containing 30 mM This-HCl (pH 8.0), 74 μg/ml DCIP, 100 μM UQ_2, and 2 mM KCN. DHO-cytochrome *c* reductase activity was measured by recording the absorbance change of cytochrome *c* at 550 nm, following the addition of 500 μM DHO to 30 mM This-HCl (pH 8.0), 20 μM cytochrome *c*, and 2 mM KCN. The effect of inhibitors to *Plasmodium* mitochondria was assayed in the presence or the absence of chalcone at 25°C. Licochalcone A was dissolved in DMSO and a final concentration of DMSO was fixed at 0.5% (v/v). Each reaction was initiated by the addition of DHO.

RESULTS

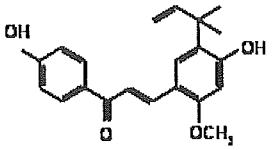
Inhibition of Complex II by Licochalcone A

Among the chalcones, licochalcone A (FIG. 2) was used in this study because it was demonstrated to exhibit potent antimalarial activity in the previous report.[12] First, we investigated the antimalarial activity of licochalcone A on *in vitro* culture of *P. falciparum*. The 50% inhibition concentration of growth (IC_{50} value) was 7.7 M, and this value was almost the same as that in the previous report (IC_{50} = 2 μM).

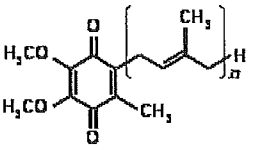
To investigate the effect of licochalcone A on complex II, we examined the effect of licochalcone A on SQR and SDH activity. The SQR activity of *Plasmodium* was sensitive to licochalcone A, and its IC_{50} value was 1.30 μM (TABLE 1). Rat mitochondrial SQR activity was also sensitive to licochalcone A, but its sensitivity was 10 times lower than that of *Plasmodium* complex II (TABLE 1). The SDH activity, on the other hand, was not affected even by 100 μM licochalcone A for both *Plasmodium* and rat liver (TABLE 1). The SDH activity is a partial reaction of SQR catalyzed by the electron transfer from succinate to the water-soluble electron acceptor. This

TABLE 1. Inhibitory effects of chalcones against *P. falciparum* and rat liver complex II

Species	Complex II activity (IC_{50})	
	SUC-UQ-DCIP	SUC-PMS-MTT
P. falciparum	1.30 μM	>100 μM
Rat	16.5 μM	>100 μM



Iicochalcone A



Ubiquinone

FIGURE 2. Structure of licochalcone A and ubiquinone.

activity does not require the membrane anchor subunits, which are essential components for electron transfer to ubiquinone (UQ).[6] Therefore, this finding indicates that the licochalcone A binding site is localized at membrane anchor subunits.

Then, the mechanism of inhibition by licochalcone A was further studied with rat liver mitochondria, because specific activity of the *Plasmodium* enzyme was too low and mammalian complex II showed high enough activity for kinetic analysis. Double-reciprocal plots showed competitive inhibition of licochalcone A for UQ_2 (FIG. 3). This finding indicates that licochalcone A may block the electron transfer between the enzyme and UQ by binding to the UQ binding site.

Inhibition of the bc_1 Complex by Licochalcone A

From a study of the mechanism of complex II inhibition by licochalcone A, it was suggested that licochalcone A specifically inhibits the UQ binding site.Therefore, we examined the effect of licochalcone A on the enzymes possessing a UQ binding site such as the bc_1 complex and DHOD. To characterize the bc_1 complex, the ubiquinol-cytochrome *c* assay has generally been used.[22] However, we were unable to use this assay system because the activity of the *Plasmodium* bc_1 complex was too low to study detailed kinetics of the enzyme. Therefore, we measured the activity of DHO-cytochrome *c* reductase, which is much more reliable than the ubiquinol-cytochrome *c* assay. DHO-cytochrome *c* activity consists of two enzymes, DHOD and bc_1 complex (FIG. 4). Suitability of this system was confirmed by the specific and potent inhibitors of the bc_1 complex and DHOD, such as antimycin A and orotate, inhibiting DHO-cytochrome *c* activity (IC_{50} = 0.282 nM and 90 μM, respectively). To investigate the effect of licochalcone A on the bc_1 complex, the sensitivity of

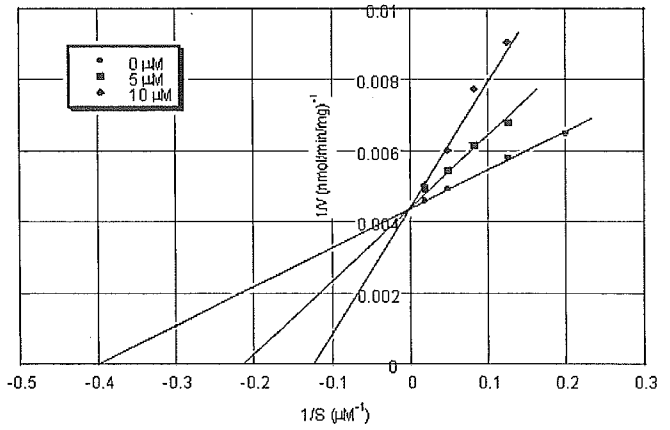


FIGURE 3. Double-reciprocal plots of licochalcone A inhibition of rat SQR activity. Inhibition of rat liver mitochondria by licochalcone A was measured in the presence of various concentrations of UQ_2. The assay was performed at 25°C in the presence 50 mM potassium phosphate buffer (pH 7.2), 74 μM DCIP, potassium succinate (10 mM), potassium cyanide (2 mM), and 10 μg of protein of rat liver mitochondria (298 nmol/min/mg). SQR activity was determined by the millimolar extinction coefficient for DCIP (21 $mM^{-1}cm^{-1}$ at 600 nm).

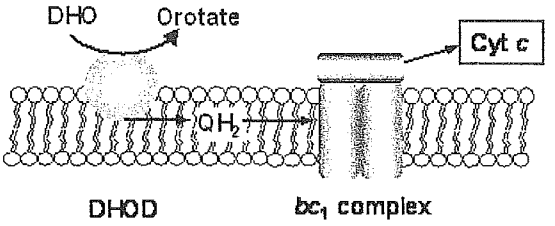


FIGURE 4. DHO-cytochrome *c* activity consists of two enzymes, DHOD and the bc_1 complex. DHO-cytochrome *c* reductase activity is determined by measuring the electron transfer from DHO to cytochrome *c* by monitoring the absorption change at 550 nm as described in the **Material and Methods** section.

TABLE 2. Mitochondrial enzyme activities of *P. falciparum* and inhibition by licochalcone A

Species	Activities	Enzyme	Specific activity (nmol/min/mg)	IC_{50}
P. falciparum	DHO-UQ-DCIP	DHOD[a]	7.10 ± 0.97	>100 μM
	DHO-cyt c	DHOD + bc_1 complex	2.56 ± 0.69	0.10 μM
Rat liver	DHO-UQ-DCIP	DHOD[a]	7.05 ± 0.62	1.42 μM
	DHO-cyt c	DHOD + bc_1 complex	10.8 ± 1.24	0.077 μM

[a]Dihydroorotate dehydrogenase.

DHO-cytochrome *c* activity was analyzed and compared with that of DHOD activity assayed using DCIP as an electron acceptor.

The DHO-cytochrome *c* activity of *P. falciparum* was sensitive to licochalcone A, and its IC_{50} was 0.10 μM (TABLE 2). The sensitivity was 10 times higher than that of complex II. DHOD, which is one of the components of DHO-cytochrome *c* activity, was not inhibited even by 100 μM licochalcone A, indicating that the target of licochalcone A is the bc_1 complex of *Plasmodium*. The bc_1 complex and DHOD of rat mitochondria were sensitive to licochalcone A and their IC_{50} was 0.077 and 1.42 μM (TABLE 2).

DISCUSSION

Licochalcone A is a characteristic chalcone of Xin-jiang licorice, which is the root of *Glycyrrhiza inflata* and its structure was established by one of the present authors as 3-a,a-dimethylallyl-4,4′-dihydroxy-6-methoxychalcone.[23] Licochalcone A has been reported to show anti-inflammatory activity, anti-tumor activity, and antimicrobial activity.[13] In this study, we showed that licochalcone A has multiple targets in *P. falciparum* mitochondria. Complex II and bc_1 complex were inhibited by licochalcone A at low concentrations (TABLE 1). Thus, energy metabolism is one of the indispensable systems for the survival of the parasite, and the enzymes of the energy-transducing pathway are promising targets of antiparasitic drugs as discussed previously.[1,24]

Considering the report of *Leishmania*,[19] it may be possible to speculate that the growth inhibition of *P. falciparum* by licochalcone A is due to the inhibition of complex II. The IC_{50} of *Plasmodium* SQR activity was very low, and its sensitivity was 10 times higher than that of rat mitochondrial complex II. The kinetics analysis of licochalcone A on rat SQR activity showed that licochalcone A was the competitive inhibiter of UQ. This finding indicates that the binding site of licochalcone A overlaps with the quinone-binding site. The important role of complex II in the erythrocytic stage of *Plasmodium* has been shown,[10,20,25] although more biochemical study is essential to understanding its physiological functions.

It should be noted that the *Plasodium* bc_1 complex was more sensitive to licochalcone A than was complex II. The bc_1 complex is an essential enzyme in the *Plasmodium* respiratory chain and a key molecule with regard to the chemotherapy of

Plasmodium. The bc_1 complex has two quinone binding sites, Qi site and Qo site. The Qo site of the *Plasmodium* bc_1 complex is the binding site of the antimalarial, atovaquone. Chalcone inhibits not only parasites, but also the bacterial bc_1 complex.[14] The low IC_{50} value (0.10 μM) of licochalcone A in the present study indicates that a major part of antimalarial activity may be explained by the inhibition of the *Plasmodium* bc_1 complex, although modification of the erythrocyte membrane has been suggested as a mechanism of the antiplasmodial activity of the compound.[26] To evaluate the effect of licochalcone A and its analogs on the *Plasmodium* bc_1 complex, the DHO-cytochrome *c* reductase assay system was useful. The ubiquinone- cytochrome *c* reductase assay was unable to perform because the artificial electron flow from ubiquinol to cytochrome *c* was very fast, and it could not be subtracted as a background. Since the DHO-cytochrome *c* reductase system is useful for the drug screening of the *Plasmodium* bc_1 complex inhibitor and the bc_1 complex has been suggested as a target of atovaquone and primaquine,[7,8,27] it would be interesting to investigate the effects of these compounds using this assay system.

In conclusion, we showed that licochalcone A inhibits the complex II and the bc_1 complex in the respiratory chain of *Plasmodium* mitochondria. As licochalcone A might have potential as a good lead compound, several analogs of licochalcone A have been analyzed for their efficacy. Interestingly, we found more effective derivatives, such as 3-hydroxy-3′-methylchalcone (3′Me-3C), on the parasite growth than licochalcone A among the compounds. Further study is now ongoing.

ACKNOWLEDGMENTS

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REFERENCES

1. KITA, K., C. NIHEI & E. TOMITSUKA. 2003. Parasite mitochondria as a drug target: diversity and dynamic changes during the life cycle. Curr. Med. Chem. **10:** 1241–1253
2. OMURA, S., H. MIYADERA, H. UI, *et al.* 2001. An anthelmintic compound, nafuredin, shows selective inhibition of complex I in helminth mitochondria. Proc. Natl. Acad. Sci. USA **98:** 60–62.
3. NIHEI, C., Y. FUKAI & K. KITA. 2002. Trypanosome alternative oxidase as a target of chemotherapy. Biochim. Biophys. Acta **1587:** 234–239.
4. WHO World malaria situation in 1994. Weekly epidemiological record, 1997 WHO.
5. OLLIARIO, P. & P. TRIGG. 1995. Status of antimalarial drugs under development. Bull. WHO **73:** 565–571.
6. KITA, K., H. HIRAWAKE, H. MIYADERA, *et al.* 2002. Role of complex II in anaerobic respiration of the parasite mitochondria from *Ascaris suum* and *Plasmodium falciparum*. Biochim. Biophys. Acta **1553:**123–139.

7. SRIVASTAVA, I.K., H. ROTTENBERG & A.B. VAIDYA. 1997. Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. J. Biol. Chem. **272:** 3961–3966.
8. SRIVASTAVA, I.K., J.M. MORRISEY, E. DARROUZET, *et al.* 1999. Resistance mutations reveal the atovaquone-binding domain of cytochrome *b* in malaria parasites. Mol Microbiol **33:** 704–711.
9. LOOAREESUWAN, S., C. VIRAVAN, H.K. WEBSTER, *et al.* 1996. Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of acute uncomplicated malaria in Thailand. Am. J. Trop. Med. Hyg. **54:** 62–66.
10. MI-ICHI, F., S. TAKEO, E. TAKASHIMA, *et al.* 2003. Unique properties of respiratory chain in *Plasmodium falciparum* mitochondria. Adv. Exp. Med. Biol. **531**: 117–133
11. ELLIS, J.E. 1994. Coenzyme Q homologs in parasitic protozoa as targets of chemotherapeutic attack. Parasitol. Today. **10:** 296–301
12. CHEN, M., T.G. THEANDER, S.B. CHRISTENSEN, *et al.* 1994. licochalcone A, a new antimalarial agent, inhibits in vitro growth of the human malaria parasite *Plasmodium falciparum* and protects mice from *P. yoelii* infection. Antimicrob Agents Chemother. **38:** 1470–1475.
13. SHIBATA, S. 2000. A drug over the millennia: pharmacognosy, chemistry, and pharmacology of licorice. Yakugaku Zasshi **120:** 849–862.
14. HARAGUCHI, H., K. TANIMOTO, Y. TAMURA, *et al.* 1998. Mode of antibacterial action of retrochalcones from *Glycyrrhiza inflata*. Phytochemistry **48:** 125–129.
15. LIU, M., P. WILAIRAT & M.L. GO. 2001. Antimalarial alkoxylated and hydroxylated chalcones: structure-activity relationship analysis. J. Med. Chem. **44:** 4443–4452.
16. DOMINGUE, J.N., J.E. CHARRIS, G. LOBO, *et al.* 2001. Synthesis of quinolinyl chalcones and evaluation of their antimalarial activity. Eur. J. Med. Chem. **36:** 555–560.
17. RAM, V.J., A.S. SAXENA, S. SRIVASTAVA & S. CHANDRA. 2000. Oxygenated chalcones and bischalcones as potential antimalarial agents. Bioorg. Med. Chem. Lett. **10:** 2159–2161.
18. CHEN, M., B.S. CHRISTENSEN, L. ZHAI, *et al.* 1997. The novel oxygenated chalcone, 2,4-dimethoxy-4′-butoxychalcone, exhibits potent activity against human malaria parasite *Plasmodium falciparum in vitro* and rodent parasites *Plasmodium berghei* and *Plasmodium yoelii in vivo*. J. Infect. Dis. **176:** 1327–1333.
19. CHEN, M., L. ZHAI, S.B. CHRISTENSEN, *et al.* 2001. Inhibition of fumarate reductase in *Leishmania major* and *L. donovani* by chalcones. Antimicrob. Agents Chemother. **45:** 2023–2029.
20. TAKASHIMA, E., S. TAKAMIYA. F. TAKEO, *et al.* 2001. Isolation of mitochondria from *Plasmodium falciparum* showing dihydroorotate dependent respiration. Parasitol. Int. **50:** 273–278.
21. TRAGER, W. & J.B. JANSEN. 1976. Human malaria parasites in continuous culture. Science **193:** 673–675.
22. KRUNGKRAI, J., S.R. KRUNGKRAI, N. SURAVERATUM & P. PRAPUNWATTANA. 1997. Mitochondrial ubiquinol-cytochrome *c* reductase and cytochrome *c* oxidase: chemotherapeutic targets in malarial parasites. Biochem. Mol. Biol. Int. **42:** 1007–1014.
23. SAITOH, T. & S. SHIBATA. 1975. New type chalcones from licorice root. Tetrahed. Lett. **16:** 4461–4462
24. SHERMAN, I.W. 1979. Biochemistry of *Plasmodium* (malaria parasite). Microbiol. Rev. **43:** 453–495
25. TAKEO, S., A. KOKAZE, C.S. NG, *et al.* 2000. Succinate dehydrogenase in *Plasmodium falciparum* mitochondria: molecular characterization of the SDHA and SDHB genes for the catalytic subunits, the flavoprotein (Fp) and iron-sulfur (Ip) subunits. Mol. Biochem. Parasitol. **107:** 191–205.
26. ZIEGLER, H.L., H.S. HANSEN, D. STAERK, *et al.* 2004. The antiparasitic compound Licochalcone A is a potent echinocytogenic agent that modifies the erythrocyte membrane in the concentration range where antiplasmodial activity is observed. Antimicrob. Agents Chemother. **48:** 4067–4071.
27. VAIDYA, A.B., M.S. LASHGARI, L.G. POLOGE & J. MORRISEY. 1993. Structural features of *Plasmodium* cytochrome *b* that may underlie susceptibility to 8-aminoquinolines and hydroxynaphthoquinones. Mol. Biochem. Parasitol. **58:** 33–42.

PfPDE1, a novel cGMP-specific phosphodiesterase from the human malaria parasite *Plasmodium falciparum*

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This is the first report of molecular characterization of a novel cyclic nucleotide PDE (phosphodiesterase), isolated from the human malaria parasite *Plasmodium falciparum* and designated PfPDE1. *PfPDE1* cDNA encodes an 884-amino-acid protein, including six putative transmembrane domains in the N-terminus followed by a catalytic domain. The *PfPDE1* gene is a single-copy gene consisting of two exons and a 170 bp intron. PfPDE1 transcripts were abundant in the ring form of the asexual blood stages of the parasite. The C-terminal catalytic domain of PfPDE1, produced in *Escherichia coli*, specifically hydrolysed cGMP with a K_m value of 0.65 μM. Among the PDE inhibitors tested, a PDE5 inhibitor, zaprinast, was the most effective, having an IC_{50} value of 3.8 μM. The non-specific PDE inhibitors IBMX (3-isobutyl-1-methylxanthine), theophylline and the antimalarial chloroquine had IC_{50} values of over 100 μM. Membrane fractions prepared from *P. falciparum* at mixed asexual blood stages showed potent cGMP hydrolytic activity compared with cytosolic fractions. This hydrolytic activity was sensitive to zaprinast with an IC_{50} value of 4.1 μM, but insensitive to IBMX and theophylline. Furthermore, an *in vitro* antimalarial activity assay demonstrated that zaprinast inhibited the growth of the asexual blood parasites, with an ED_{50} value of 35 μM. The impact of cyclic nucleotide signalling on the cellular development of this parasite has previously been discussed. Thus this enzyme is suggested to be a novel potential target for the treatment of the disease malaria.

Key words: antimalarial drug, cGMP, malaria parasite, phosphodiesterase (PDE), *Plasmodium*, zaprinast.

INTRODUCTION

Malaria is a parasitic disease caused by four species of the protozoan parasites of the genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* in humans. *P. falciparum* is the most virulent form of the four. Several drugs, including chloroquine and quinine, are used for the treatment of malaria; however, *P. falciparum* has developed resistance to most antimalarial agents, such as chloroquine. Therefore in order to overcome malaria, the genome project of *P. falciparum* has been completed [1]. Potential drug target molecules are currently being discussed [2].

The life cycle of the malaria parasite is complex, with several stages [3]. Soon after a bite by an infected *Anopheles* mosquito, sporozoites invade hepatocytes. Intense asexual division, termed exoerythrocytic schizogony, occurs in the liver, and up to 40000 merozoites are generated after schizont rupture. Merozoites invade erythrocytes and additional rounds of asexual replication, a pathogenic phase of malaria, termed erythrocytic schizogony, takes place. Some merozoites arrest their cell cycle to differentiate into male or female gametocytes (gametogenesis), which are infectious to the *Anopheles* mosquitoes. Thus cellular differentiation is an important process in the life cycle of the malaria parasite. Intracellular cyclic nucleotides cAMP and cGMP play a pivotal role in the growth and differentiation of this organism, acting as second-messenger molecules. For example, cGMP signalling has been shown to be implicated in *Plasmodium* gametogenesis [4,5]. Consistent with this, expression of PfGC (guanylate cyclase) protein is found in gametocytes [6,7]. The presence of PfPKG (cGMP-dependent protein kinase) in the ring form of asexual blood stages, but not gametocytes, suggests a role of cGMP signalling in this cell form [8].

This cyclic nucleotide signalling is regulated through cyclic nucleotide production by adenylate and guanylate cyclases and hydrolysis by cyclic nucleotide PDEs (phosphodiesterases) [9,10]. In mammals, PDEs have been categorized into 11 families (PDEs 1–11) according to their amino acid sequence similarity, biochemical properties and inhibitor sensitivity [11,12]. Inhibitors of mammalian PDEs are used, or have been developed, for the treatment of diseases such as erectile dysfunction, thrombosis, asthma and chronic obstructive pulmonary disease [13–15]. PDEs from lower single-cell organisms have been reported and revealed to be distinct from the 11 mammalian PDE families. PDEs from a soil amoeba, *Dictyostelium*, and a protozoan parasite, *Trypanosoma*, have been isolated using their genome sequences and their characteristics successfully demonstrated. In *Trypanosoma brucei*, two class I PDEs (TbPDE1 and TbPDE2), which are specific for cAMP, have been cloned [16–19]. Interestingly PDE inhibitors against TbPDE2 block proliferation of bloodstream form trypanosomes [17], and inactivation of TbPDE2 by RNA interference induces growth inhibition of bloodstream-form *T. brucei*. This suggests that specific PDE inhibitors

Abbreviations used: BLAST, basic local alignment search tool; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; Ht, hematocrit; IBMX, 3-isobutyl-1-methylxanthine; IPTG, isopropyl β-D-thiogalactoside; PDE(s), phosphodiesterase(s); PfPDE, *P. falciparum* PDE; PfGC, *P. falciparum* guanylate cyclase; PfPKG, *P. falciparum* cGMP-dependent protein kinase; RT, reverse transcriptase; Tb, *Trypanosoma brucei*.

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The nucleotide sequence of *PfPDE1* has been submitted to DDBJ, EMBL, GenBank®/EBI (European Bioinformatics Institute) and GSDB Nucleotide Sequence Databases under the accession number AB100091.

may be useful for anti-protozoal chemotherapy [19]. Thus cyclic nucleotide signalling in lower organisms is essential for their survival, and disruption of this signalling would be expected to cause cell stress, leading to cell death.

In *Plasmodium*, the presence, and some physiological roles, of PDEs have been predicted. Nevertheless, the molecular basis of *Plasmodium* PDEs has not yet been established, in spite of the genome project of *P. falciparum* having been completed. In the present paper, we report a novel PDE (PfPDE1) in *P. falciparum*. The *PfPDE1* cDNA was cloned and both the enzymatic characteristics and PDE inhibitor sensitivities of the gene product were investigated in detail. Endogenous PDE activities of this organism were also examined. The physiological effect of a PfPDE1 inhibitor on this organism was also demonstrated. The findings reported here provide us with fundamental knowledge of the cyclic nucleotide metabolism in the human parasite *P. falciparum*. *Plasmodium* PDE is suggested to be a novel therapeutic target for the disease malaria and discovery of inhibitors would lead to improved treatment of the disease.

EXPERIMENTAL

Materials

[^{3}H]cAMP, [^{3}H]cGMP and [α-^{32}P]dCTP were purchased from Amersham Biosciences. IBMX (3-Isobutyl-1-methylxanthine) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Vinpocetine, EHNA [*erythro*-9-(2-hydroxy-3-nonyl)adenine], milrinone, rolipram, zaprinast, dipyridamole, and chloroquine were purchased from Sigma–Aldrich. Theophylline was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Papaverine, E4021 and sildenafil were synthesized at Tanabe Seiyaku Co. Ltd. (Osaka, Japan).

Parasite culture and isolation of nucleic acids

P. falciparum 3D7 and Honduras-1 strains were cultivated in RPMI 1640 (Invitrogen) with 10% (v/v) inactivated type A human plasma with 5% (v/v) and 3% (v/v) Ht (haematocrit) type A human erythrocytes respectively [20]. The plate was placed in a CO_2 incubator [$CO_2/O_2/N_2$ (5:5:90)] at 37 °C. Erythrocytes infected with *P. falciparum 3D7* were harvested and treated with 0.075% (w/v) saponin in PBS to obtain the parasites. Chromosomal DNA was obtained from the parasites using QIAamp DNA Mini Kit (Qiagen). Total RNA was isolated using Isogen (Nippon Gene, Toyama, Japan) and first-strand cDNA was prepared according to the instructions with GeneAmp RNA PCR Kit (Applied Biosystems).

Cloning of chromosomal DNA and cDNA for *P. falciparum* PDE

The amino acid sequences of mammalian PDEs (PDEs 1–11) were used as queries to search the expressed sequence tag and genome databases. A BLAST (basic local alignment search tool) search [21] of the National Center for Biotechnology Information *P. falciparum* genome database identified putative *PfPDE* genes. PCR primers were designed based on the sequences retrieved from the database.

Chromosomal DNA fragments for the *PfPDE1* gene were generated by PCR amplification, using *P. falciparum* chromosomal DNA as a template. The 5′-region of the *PfPDE1* gene was amplified using the primer set (Pf1-F1: 5′-ATGGAATATTTTAATTGTGTTAATAATCTATGTTG-3′ and Pf1-R3: 5′-ATTTAATATATCTTCTATGGGCGATGTAGG-3′). To amplify the 3′-region of *PfPDE1* gene, the primer set (Pf1-F3: 5′-CCATCCTTTTATGAATATCTTATGTTTACGTTGATG-3′ and Pf1-R1: 5′-TTATTCAAATTTGATGAGCTCAAGTTTGCTTAG-3′) was used. PCR amplification was carried out through 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 3 min. PCR products were cloned into the TA-cloning vector pGEM-T Easy (Promega) and then sequenced.

The cDNA fragments encoding *PfPDE1* were obtained by PCR using *P. falciparum* cDNA as a template. N-terminal (nt 1–1329) and central to C-terminal (nt 367–2650) regions of *PfPDE1* were amplified using the above primer sets, Pf1-F1 plus Pf1-R3 and Pf1-F3 plus Pf1-R1 respectively. PCR amplification was carried out using the conditions described above and the amplified products were cloned into pGEM-T Easy. Five independent PCR clones encoding each region were sequenced to verify that a correct whole cDNA sequence had been cloned. One of the clones that included the C-terminal catalytic domain of PfPDE1, pGEM-PfPDE1c, was used for further experiments.

Nucleotide sequences were determined by an automated DNA sequencer ABI PRISM™ 310 using BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems). Nucleotide and amino acid sequence data were analysed using the computer program GENETYX (Software Development Co., Tokyo, Japan). The deduced amino acid sequence was also analysed using the program SMART [22] for finding functional domains. Phylogenetic tree analysis was performed using CLUSTAL W and PHYLIP [23].

Southern blot analysis

P. falciparum chromosomal DNA (2 μg/lane) was digested using the restriction endonucleases EcoRI, SpeI and XbaI, subjected to 0.8% agarose gel electrophoresis and transferred on to Hybond-N+ nylon membrane (Amersham Biosciences). A 661-bp cDNA fragment of *PfPDE1* (nts 1147–1807) was ^{32}P-radiolabelled using the Random Primer DNA Labelling Kit (TaKaRa Bio). Hybridization was performed in PerfectHyb™ (Toyobo, Osaka, Japan) containing the ^{32}P-labelled probe at 60 °C for 4 h. The membrane was washed with 2 × SSC (1 × SSC: 0.15 M NaCl and 15 mM sodium citrate, pH 7.0) and 0.1% (w/v) SDS at 25 °C for 5 min, followed by two 10 min washes with 0.1 × SSC and 0.1% (w/v) SDS at 60 °C. X-ray film was exposed to the membrane at − 80 °C for 12 h.

Stage-specific expression of *PfPDE1* mRNA

To investigate stage-specific expression of *P. falciparum* cells at asexual stages, cultures were synchronized by sorbitol lysis and treatment with 66% (v/v) Percoll™ (Amersham Biosciences) [24,25]. In brief, infected erythrocytes with late-stage asexual parasites, containing trophozoites and schizonts, were separated using Percoll™. These concentrated late-stage parasites were suspended with uninfected erythrocytes and cultured for 5–7 h. A proportion of the schizonts proceeded to the merozoite stage and invaded the fresh erythrocytes. These infected erythrocytes were treated with 66% (v/v) Percoll™ to prevent minimal contamination with gametocytes and then with sorbitol to remove the remaining late-stage parasites (trophozoites and schizonts). The resultant ring forms were returned to culture and, 21, 32, 38, 43 and 50 h later, each sample was centrifuged at 830 *g* for 5 min at 4 °C. The parasites were obtained by treating with 0.075% (w/v) saponin in PBS. Total RNA was obtained using Isogen and first-strand cDNA was prepared using the Advantage™ RT-for-PCR Kit (Clontech). Expression levels of *PfPDE1* transcripts were examined by PCR amplification using the 5′-primer 5′-AGCATGCTTTTCATGCTAGACATGAACCAC-3′ and the 3′-primer 5′-TTATTCAAATTTGATGAGCTCAAGTTTGCTTAG-3′, which generates a DNA fragment of 795 bp (nt 1870–2655). An 840 bp DNA fragment of the *P. falciparum* cysteine protease

falcipain-3 ([26]; GenBank™ accession number AF258791) was amplified by PCR using the 5′-primer 5′-AATAGTTTATATAAAAGGGGTATG-3′ and the 3′-primer 5′-TAATGGTACATAAGCTTCTGTTCC-3′ (nt 686–1528 of the cDNA) as a control. PCR amplification was carried out through 38 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. PCR products were separated using 1.5 % agarose gel electrophoresis, and the DNA fragments were stained with ethidium bromide.

Expression of PfPDE1 protein in *Escherichia coli*

To generate an expression plasmid for a catalytic domain of PfPDE1, the EcoT22I–SalI DNA fragment (approx. 1 kb) of pGEM-PfPDE1c was subcloned into the PstI and XhoI sites of the mammalian expression vector pcDNA4/HisMax A (Invitrogen) and the resultant plasmid named pHis-PfPDE1Δ. Transfection of this plasmid into COS-7 cells for recombinant PfPDE1 production was done according to the method described previously [12]. To express the PfPDE1 enzyme in *E. coli*, the BamHI–PstI DNA fragment (approx. 1 kb) of pHis-PfPDE1Δ was subcloned into the BamHI and PstI sites of the bacterial expression vector pQE-32 (Qiagen) and the resultant plasmid named pQE-PfPDE1Δ. Site-directed mutagenesis of the pQE-PfPDE1Δ plasmid was used to generate PfPDE1 mutants with alanine substitutions at Asp^{762} and Gly^{788}; the QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used following the manufacturer's protocol. To introduce the desired mutations, the following primers were used: 5′-ATTTTAAAGGCATCAGCTATTGGACACTCAACA-3′ with 5′-TGTTGAGTGTCCAATAGCTGATGCCTTTAAAAT-3′ for PfPDE1D762A, and 5′-GAATTCTATTTACAAGCTTTACTAGAAAAATCG-3′ with 5′-CGATTTTTCTAGTAAAGCTTGTAAATAGAATTC-3′ for PfPDE1G788A. The mutations were confirmed by DNA sequencing analysis.

The expression plasmid pQE-PfPDE1Δ, pQE-PfPDE1ΔD762A, pQE-PfPDE1ΔG788A or the control vector pQE-32 was introduced into *E. coli* JM109 cells. The recombinant *E. coli* cells were grown overnight at 37 °C in LB (Luria–Bertani) medium containing ampicillin (100 μg/ml). The culture was diluted 1:20 with fresh LB medium containing 2 % glucose and ampicillin (100 μg/ml), incubated at 37 °C in a shaking incubator for 2 h and then further incubated at 27 °C for 4 h, after addition of IPTG (isopropyl β-D-thiogalactoside) at a final concentration of 1 mM. The cells were then washed once with ice-cold PBS and resuspended in ice-cold lysis buffer (40 mM Tris/HCl, pH 7.5, 15 mM benzamidine, 5 μg/ml pepstatin A and 5 μg/ml leupeptin). After freeze–thaw treatment, suspended cells were disrupted by a sonicator (TOMY Seiko, Japan) for 15 s (5 times with 1 min intervals), and the lysates were centrifuged at 16000 *g* for 15 min at 4 °C. The supernatant was added to a plastic tube containing nickel nitrilotriacetate resin (Qiagen), equilibrated with the lysis buffer, and incubated with rotation at 4 °C for 3 h. The nitrilotriacetate resin was poured into a plastic column (0.8 cm × 5 cm) and allowed to drain. The packed resin was washed with wash buffer (40 mM Tris/HCl, pH 7.5, 15 mM benzamidine, 200 mM NaCl, 5 mM imidazole, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin), and the proteins were then eluted using elution buffer (40 mM Tris/HCl, pH 7.5, 15 mM benzamidine, 200 mM NaCl, 200 mM imidazole, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin). The PfPDE1 fractions were dialysed against the lysis buffer and stored at −80 °C until use.

SDS/PAGE, immunoblotting, and MS analysis

Proteins were subjected to SDS/PAGE (12.5 % gels) and stained using a Silver Staining Kit, Protein (Amersham Biosciences), omitting a step of fixation with glutardialdehyde. Immunoblot analysis was performed using PVDF membranes (Millipore) and anti-pentahistidine monoclonal antibody (Qiagen) as previously described [12]. Bound primary antibodies were detected using horseradish-peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.) and visualized with ECL® Western Blotting Detection System (Amersham Biosciences). A gel slice containing a 41 kDa protein was treated with 10 mM DTT (dithiothreitol) at 56 °C for 45 min, and alkylation was performed in the dark with 50 mM iodoacetic acid at 25 °C for 30 min. The dried sample was immersed in 40 μl of 5μg/ml trypsin solution in 25 mM NH_4HCO_3, and kept at 37 °C for 15 h. Tryptic peptides were extracted with 45 μl of 33 % NH_4HCO_3/66 % acetonitrile, 45 μl of 5 % formic acid/66 % acetonitrile and 75 μl of 5 % formic acid/80 % acetonitrile. The digests were subjected to automated LC (liquid chromatography)-MS/MS analysis HPLC using the Magic 2002 (Michrom BioResources, Auburn, CA, U.S.A.) connected online to a LCQ Deca ion-trap tandem mass spectrometer (Thermoquest, San Jose, CA, U.S.A.). Each chromatogram was subsequently analysed with the Mascot search algorithm (http://www.matrixscience.com).

PDE and protein assays

The PDE assay was performed using the radiolabelled nucleotide method as described previously [12,27]. In brief, the assay buffer contained 50 mM Tris/HCl, pH 8.0, 5mM $MgCl_2$ or 1 mM $MnCl_2$, 4 mM 2-mercaptoethanol, 0.33 mg/ml BSA, 13 nM [^{3}H]cGMP or 5 nM [^{3}H]cAMP, and unlabelled cGMP or cAMP. Reactions were started by adding enzyme solution to 500 μl of assay buffer and the tubes were incubated at 37 °C for 30 min. After boiling for 2 min, the mixtures were added to 100 μl of 1 mg/ml rattlesnake (*Crotalus atrox*) venom and incubated at 37 °C for 30 min. Reactions were stopped by the addition of 500 μl of methanol and the resultant solutions were applied to Dowex (1 × 8 200–400; Dow Chemical Company, Midland, MI, U.S.A.) columns. Aqueous scintillation mixture was added to each eluate and the radioactivity was measured using a scintillation counter. The K_m and V_{max} values were calculated from Lineweaver–Burk plots [28]. Relative V_{max} values were determined according to the method of McPhee et al. [29]. Relative concentrations of PfPDE1 proteins produced in *E. coli* were calculated by immunoblotting as described above. The membranes were incubated with ECL® reagents at 25 °C for 1 min and then exposed to X-ray film for 2–10 s, under conditions in which each X-ray film exposure did not reach saturation. The resultant films were scanned by ARCUS II (Agfa-Gevaert), and quantified using the Quantity One program (PDI, Inc., Huntington Station, NY, U.S.A.). The absorbances were plotted versus the amount of hexahistidine-tagged protein to measure the relative concentrations of PfPDE1 proteins. The protein concentration was determined using DC Protein Assay Kit (Bio-Rad), using the BSA as a standard.

PDE assay of *P. falciparum* lysates

Erythrocytes infected with *P. falciparum* Honduras-1 were washed with AIM buffer (10 mM Pipes, pH 6.7, 120 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, and 5 mM glucose) and treated with 0.075 % (w/v) saponin in AIM buffer to obtain the parasite. The cells washed with AIM buffer were suspended in ice-cold lysis buffer and disrupted by freeze–thaw treatment and sonication as described above. To remove cell debris and haem polymer, the lysates were centrifuged at 10000 *g* for 15 min at 4 °C. Then the supernatants were centrifuged at 100000 *g* for 60 min at 4 °C and fractionated into pellets and soluble (cytosolic) fractions.

The pellets were dissolved with ice-cold lysis buffer containing 0.5 % Triton X-100, centrifuged at 10000 *g* for 15 min at 4 °C and the resultant supernatants were used as the membrane fractions. Prepared cytosolic and membrane fractions were used in a PDE assay.

In vitro antimalarial activity assay

The following procedure is based on the antimalarial activity assay previously described [30]. Test compounds were dissolved in appropriate solvent and a serial dilution was prepared. In the 24-well plate, each well contained 1 ml of asynchronous *P. falciparum* Honduras-1 culture with a final Ht concentration of 3 % and 0.3 % parasitaemia, which contained 10 μl of test compound solution. Prepared culture was then incubated for 72 h using the gas conditions described above. To evaluate the antimalarial activity of the test compounds, more than 1000 erythrocytes, stained with Giemsa (Merck), were examined by microscopy. All of the test compounds were assayed in duplicate and, for zaprinast, the experiment was repeated three times. The ED_{50} value, which is a dose giving 50 % reduction in the increase of infected erythrocytes, was determined by comparison with drug-free controls cultured under the same conditions.

RESULTS

Cloning of chromosomal DNA and cDNA for a novel *P. falciparum* PDE

A search of draft genome sequence databases using the amino acid sequences of 11 mammalian PDEs (PDE 1–11) resulted in four putative *PfPDE* (*P. falciparum* PDE) genes. During the course of this study, whole genome sequencing of this organism was completed [1] and the protein-coding regions have been predicted by computer analysis (http://www.plasmodb.org/). On that web site, the above four putative *PfPDE* genes have been registered as PFL0475w, MAL13P1.118, MAL13P1.119 and PF14_0672. All these gene products contained a sequence consistent with the class I PDE signature sequence HDX_2HX_4N [31]. Based on the alignment of PDE catalytic domain sequences, a phylogenetic tree was inferred by the NJ method (Figure 1). The four putative *PfPDEs* did not belong to any PDE family previously described, but showed a low degree of evolutionary relatedness with PDE9A and the *Dictyostelium* PDE, RegA. Thus the *PfPDE*s constitute a new family of PDE. We focused on one of these *PfPDE*s, PFL0475w (here designated *PfPDE1*). The *PfPDE1* gene was found on chromosome 12 in the genome database. Based on this sequence, we designed PCR primers and performed PCR amplification using *P. falciparum* chromosomal DNA as a template to obtain chromosomal DNA for *PfPDE1*. PCR products of the appropriate size were detected, subcloned into the TA-cloning vector pGEM-T Easy and sequenced. The nucleotide sequences of the isolated clones carrying a chromosomal DNA for *PfPDE1* were in full agreement with the sequences found in the genome database (Figure 2A). Southern blot analysis of *P. falciparum* chromosomal DNA, digested with restriction endonucleases, EcoRI, SpeI and XbaI, indicated that *PfPDE1* is a single-copy gene (Figure 2B).

PfPDE1 cDNA was also obtained in the same way by PCR amplification using *P. falciparum* cDNA as a template. An open reading frame of 2655 bp was identified and predicted to encode an 884-amino-acid protein with a predicted molecular mass of 107 kDa. The cDNA sequence was A/T-rich [75.6 % (2112 bp/2655 bp)] and showed a characteristic codon usage similar to those of the *P. falciparum* genes reported to date. Two

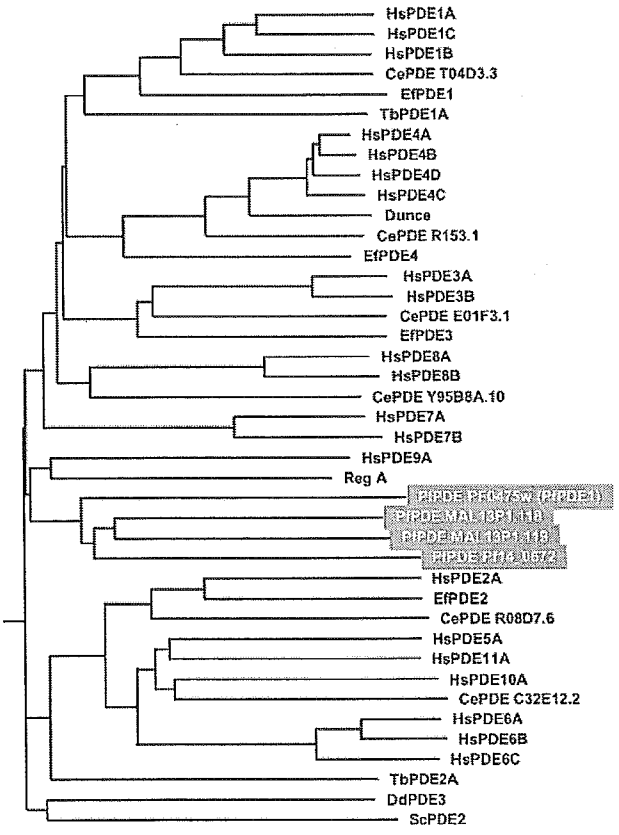

Figure 1 Phylogenetic tree of the PDE families inferred from their catalytic domain sequences

The phylogenetic tree was generated using the NJ algorithm of PHYLIP on the basis of a multiple alignment of the catalytic domain sequences of PDEs analysed with CLUSTALW. The following PDEs were included in the analysis: human PDE1A (HsPDE1A) (GenBank accession number, U40370), HsPDE1B (U56976), HsPDE1C (U40371), HsPDE2A (U67733), HsPDE3A (U36798), HsPDE3B (U38178), HsPDE4A (U68532), HsPDE4B (U85048), HsPDE4C (Z46632), HsPDE4D (L20969), HsPDE5A (D89094), HsPDE6A (M26061), HsPDE6B (X66142), HsPDE6C (X94354), HsPDE7A (U67932), HsPDE7B (AB038040), HsPDE8A (AF056490), HsPDE8B (AB085824), HsPDE9A (AF048837), HsPDE10A (AB020593), HsPDE11A (AB036704), *Ephydatia fluviatilis* PDE1 (EfPDE1) (AB017021), EfPDE2 (AB017022), EfPDE3 (AB017023), EfPDE4 (AB017024), *Caenorhabditis elegans* PDE (CePDE) T04D3.3 (Z81114), CePDE R153.1 (U28729), CePDE E01F3.1 (Z93376), CePDE Y95B8A.10 (AC024877), CePDE R08D7.6 (Z12017), CePDE C32E12.2 (U80032), *Trypanosoma brucei* PDE1A (TbPDE1A) (AF253418), TbPDE2A (AF263280), *Saccharomyces cerevisiae* (yeast) PDE2 (ScPDE2) (M14563), *Drosophila melanogaster* Dunce (AH006406), *Dictyostelium discoideum* RegA (AJ005398), *D. discoideum* PDE3 (DdPDE3) (AY162269).

motifs of a bivalent-cation-binding domain ($YHNX_2HG/AX_{23}E$ and $HDX_2HX_{24-26}E$) conserved in class I PDEs [32] are also found in PfPDE1 ($Y^{609}HtX_2HAX_{23}d^{639}$ and $H^{650}DX_2HX_{25}E^{680}$, conserved and irregular amino acids are shown in upper- and lower-case letters respectively). [32]. The catalytic domain sequence of PfPDE1 (amino acid residues 607–776) was compared with those of PDEs from human, yeast, *Dictyostelium* and trypanosome. The catalytic domain sequence of PfPDE1 exhibited the highest identity (36 %) to PDE9A among these PDE sequences. Comparison of the catalytic domain sequence of PfPDE1 with those of the three other predicted PfPDEs revealed the highest identity (40 %) and similarity (60 %) to MAL13P1.118. The amino acid sequence of PfPDE1 outside the catalytic domain was searched using SMART and BLAST. The SMART program revealed that N-terminal region of PfPDE1 has six putative transmembrane regions (Figure 3), suggesting that PfPDE1 is

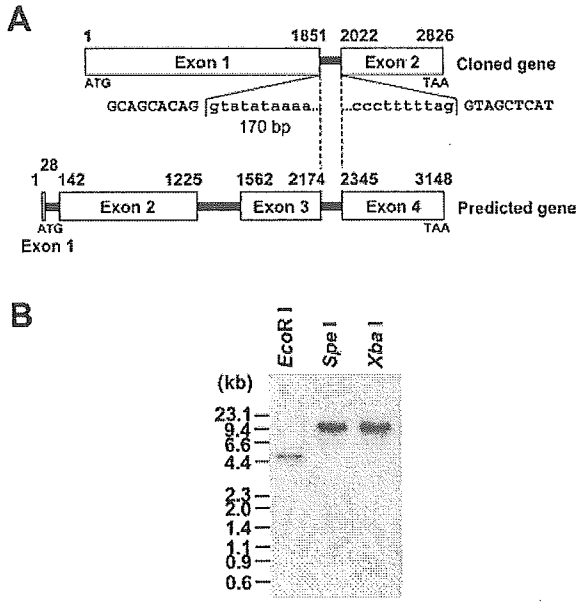

Figure 2 Structure of the *PfPDE1* gene

(**A**) The *PfPDE1* gene structure is illustrated. Open boxes with numbers represent exons. The computationally predicted organization is indicated above. Exon–intron organization, determined by comparison of the cDNA and genomic sequences, is shown below. Exon sequences are indicated by *upper*-case letters and intron sequences by lower-case letters. Exon–intron boundaries in common to both organizations are indicated by a broken line. A chromosomal region, which is computationally predicted as a second intron but actually is a coding region, is shown between thin lines. The initiation ATG codon of the cloned cDNA was indicated by a thick line. (**B**) Southern-blot analysis. *P. falciparum* chromosomal DNA, digested with the indicated (above the gel) restriction endonucleases, was fractionated and transferred on to a nylon membrane. The blot was hybridized with a ^{32}P-labelled *PfPDE1* cDNA fragment.

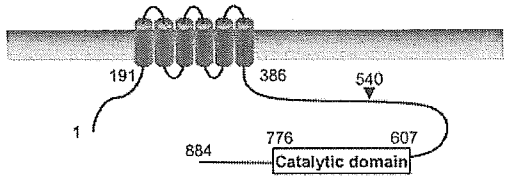

Figure 3 Schematic structure of PfPDE1

The cylinders represent hydrophobic segments that are thought to form transmembrane α-helices. An arrowhead indicates the start site of PfPDE1Δ.

a membrane protein. The BLAST search revealed no significant similarity to other reported sequences.

The *PfPDE1* cDNA sequence was compared with a computationally predicted coding sequence for PFL0475w and the PfPDE1 chromosomal DNA sequence. As shown in Figure 2(A), the *PfPDE1* gene was revealed to consist of two exons and a 170 bp intron, although the presence of three introns (nt 29–141, 1226–1561, and 2175–2344 in the predicted organization) has been predicted in the coding region of the genome sequence (accession no. NC004316). The nucleotide sequence, observed at the 5′ donor and 3′ acceptor splice sites, demonstrated that the sites are consistent with the canonical GT-AG rule (5′ donor; GTATATAAAAAA... and 3′ acceptor; ...ATCCCTTTTTAG) [33]. The second intron in the predicted organization (nt 1226–1561; see accession no. AE014845) was revealed to be a coding region of PfPDE1, composed of 112 amino acid residues

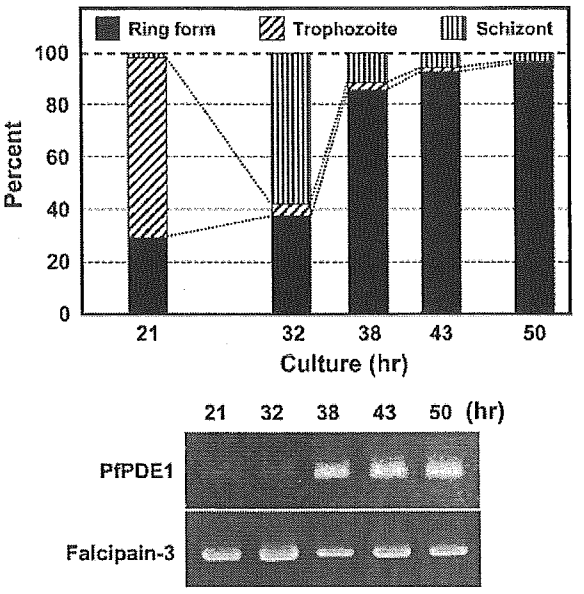

Figure 4 Stage-specific expression of *PfPDE1* transcripts

In order to obtain a synchronous culture, ring form-rich parasites, prepared as described in the Experimental section, were cultured. After 21, 32, 38, 43 and 50 h, each sample was collected and the stage-specific cDNAs were prepared. The percentage of ring form, trophozoite, and schizont was calculated by counting the numbers of each cell form (upper panel). Expression levels of *PfPDE1* transcripts (nt 1870–2655) and falcipain-3 (GenBank® accession number AF258791, nt 686–1528) were examined by RT-PCR. After separation on a 1.5% agarose gel, PCR products were detected with ethidium bromide staining (lower panel).

(Figure 2A). Finally, PfPDE1 contains six potential transmembrane regions (PFL0475w has been predicted as a membrane protein with three potential transmembrane regions).

Stage-specificity of *PfPDE1* expression in *P. falciparum*

Plasmodium has a complex life cycle that differs at morphological and biochemical levels. To investigate changes in *PfPDE1* expression during blood stage development, RT (reverse transcriptase)-PCR analysis was performed using total RNA isolated from synchronously cultured parasites as shown in Figure 4. Cell populations were determined by counting the numbers of the three cell forms. To eliminate PCR products amplified from contaminating chromosomal DNA, sense and antisense PCR primers were designed from coding sequences of exons 1 and 2 respectively. PCR products amplified from chromosomal DNA were not detected. The levels of *PfPDE1* transcripts were in accordance with the proportion of ring form. Amounts of mRNA loaded in each lane were confirmed by RT-PCR analysis of falcipain-3, which is expressed in all stages of asexual blood stages [26]. Thus stage-specific transcriptional regulation of *PfPDE1* was observed.

Production of recombinant PfPDE1 in *E. coli*

Production of the recombinant PfPDE1 protein as a hexahistidine-tagged N-terminally truncated form (PfPDE1Δ) was first attempted with COS cells, which we have previously employed successfully as described in [12]. However, no significant plasmid-directed PDE activity was detected in transfected COS cells, probably owing to an A/T-rich coding sequence of *PfPDE1*. Therefore an *E. coli* expression plasmid encoding a hexahistidine-tagged PfPDE1Δ (pQE-PfPDE1Δ) was generated and introduced into *E. coli*. After induction with IPTG, the cell extracts prepared

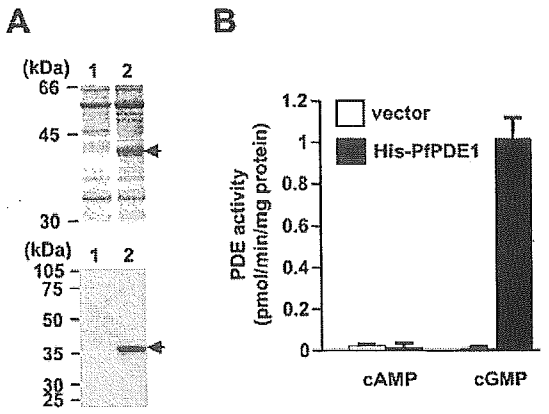

Figure 5 Production of the recombinant PfPDE1 protein

(**A**) Expression of the catalytic domain of PfPDE1 was examined by SDS/PAGE and immunoblotting. The control vector pQE-32 (lane 1) and pQE-PfPDE1Δ (lane 2) were used to transform the *E. coli* JM109 cells. The hexahistidine-tagged PfPDE1Δ was partially purified using a nickel affinity column. Partially purified PfPDE1 protein was separated by SDS/PAGE (12.5% gels), and visualized with silver staining (upper panel) and with immunoblotting using anti-pentahistidine antibody (lower panel). (**B**) Cyclic nucleotide hydrolytic activities of partially purified proteins from the cells carrying a control vector (open bars) and pQE-PfPDE1Δ (filled bars) were measured using 5 nM cAMP or 13 nM cGMP as a substrate. Bivalent cation used was 5 mM $MgCl_2$.

were subjected to nickel affinity column purification, as described in the Experimental section. SDS/PAGE analysis visualized by silver staining and immunoblot analysis using anti-pentahistidine antibody demonstrated the production of a 41 kDa protein in the lysates of the *E. coli* cells carrying pQE-PfPDE1Δ (Figure 5A). The value was in reasonable agreement with the molecular mass predicted for the hexahistidine-tagged PfPDE1Δ. The masses of tryptic peptides of the 41 kDa protein, determined by LC-MS/MS, corresponded to those of predicted tryptic fragments derived from the PfPDE1Δ sequence, such as 'ANTFISIGYK', 'LLYPLGVLEANFDKEK', 'AILSTDMK', 'LELIKFE', etc. (results not shown). The eluates were employed in the PDE assay using either 5 nM cAMP or 13 nM cGMP as a substrate. Partially purified proteins from PfPDE1Δ-transformed cells exhibited approx.135-fold higher levels of cGMP PDE activity than those from cells transformed with the control vector (Figure 5B). No significant cAMP hydrolytic activity of the PfPDE1Δ enzyme was observed under these conditions. cGMP hydrolysis was neither activated nor inhibited by cAMP at concentrations of up to 100 μM (results not shown). These results indicated that PfPDE1 is a cGMP-specific PDE.

Characterization of PfPDE1 activity

PDEs require bivalent cations for their activities [32,34]. For example, cGMP hydrolytic activity of human PDE9A is supported by Ca^{2+}, Mg^{2+} and Mn^{2+}, and this enzyme exhibits the highest activity with Mn^{2+} [34]. The catalytic domain sequence of PfPDE1 shows the highest similarity with that of PDE9A among mammalian PDEs, and both PfPDE1 and PDE9A are cGMP-specific PDEs, suggesting that PfPDE1 activity also may be supported by Mn^{2+}. The effect of several bivalent cations (Ca^{2+}, Mg^{2+} and Mn^{2+}) on PfPDE1 enzyme activity was examined, and as expected, Mn^{2+} was revealed to be a more potent activator of PfPDE1 than Mg^{2+} and Ca^{2+} at concentrations of 0.01–10 mM. Maximum activity was observed at 1 mM $MnCl_2$ (Figure 6). Ca^{2+} was less potent at concentrations of 0.01–1 mM.

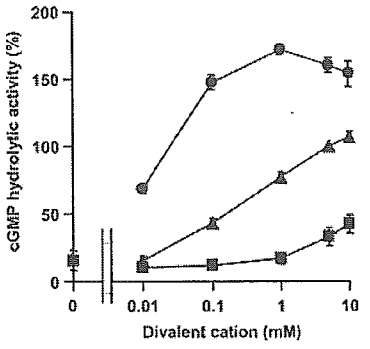

Figure 6 Effect of bivalent cation on PfPDE1

PDE activity of the catalytic domain of PfPDE1 expressed in *E. coli* was measured with 13 nM cGMP in the presence of different concentrations (0–10 mM) of $MgCl_2$ (▲), $MnCl_2$ (●), and $CaCl_2$ (■). The results are presented relative to the activity with 5 mM $MgCl_2$.

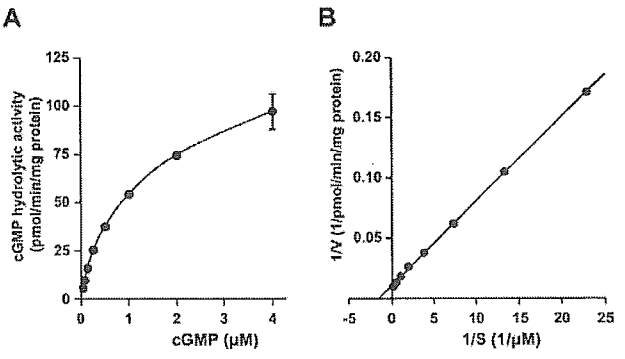

Figure 7 Kinetic analysis of partially purified PfPDE1

(**A**) Michaelis–Menten kinetics of PfPDE1. Partially purified PfPDE1 protein was prepared as described in the Experimental section. PfPDE1 was assayed at various cGMP concentrations (0.03125–4 μM). (**B**) Lineweaver–Burk plots of the same sets of data are shown. The data are expressed as the means ± S.E.M. for three independent experiments. K_m and V_{max} values are expressed as the means ± S.E.M. for triplicate assays. The bivalent cation used was 1 mM $MnCl_2$.

The K_m and V_{max} values of PfPDE1 in the presence of Mn^{2+} were calculated from Lineweaver–Burk plots of activities using cGMP (0.03125–4 μM) as a substrate. As shown in Figure 7, Lineweaver–Burk plots showed apparent linearity over the substrate concentration range examined. The K_m value of PfPDE1 for cGMP was 0.65 ± 0.033 μM, indicating that PfPDE1 is categorized into high-affinity cGMP PDEs. The V_{max} value was 91 ± 4.1 pmol/min per mg of protein with the partially purified recombinant protein. Consistent with the HDX_2HX_4N motif in the PfPDE1 catalytic domain, high affinity for cGMP also indicated that PfPDE1 is a class I, not a class II, PDE.

Inhibitory profile of PfPDE1

The effects of various PDE inhibitors on PfPDE1 were examined (Table 1). IC_{50} values for the non-specific PDE inhibitors IBMX, papaverine, theophylline and pentoxifylline were more than 100 μM. Furthermore, vinpocetine, EHNA, milrinone and rolipram, which are inhibitors for mammalian PDE1, PDE2, PDE3 and PDE4 respectively, were also inactive up to 100 μM. Compounds that inhibit PDE5 showed inhibitory effects on PfPDE1. Dipyridamole, E4021 and sildenafil demonstrated a moderate inhibitory effect ($IC_{50} = 22 \pm 0.58$, 46 ± 1.8, and 56 ± 11 μM, respectively). Among the PDE inhibitors tested, zaprinast

Table 1 Inhibitory effect of the various PDE inhibitors and chloroquine on PfPDE1

Partially purified PfPDE1 proteins produced in *E. coli* were used for the assay. The concentrations of cGMP used were 0.6 μM. IC_{50} values were calculated by linear regression. Data are expressed as the means $\pm$ S.E.M. for three independent determinations. All assays were performed in duplicate. The bivalent cation used was 1 mM $MnCl_2$.

Inhibitor	IC_{50} for PfPDE1 (μM)
Chloroquine	> 100
IBMX	> 100
Papaverine	> 100
Theophylline	> 100
Pentoxifylline	> 100
Vinpocetine	> 100
EHNA	> 100
Milrinone	> 100
Rolipram	> 100
Sildenafil	56 $\pm$ 11
E4021	46 $\pm$ 1.8
Dipyridamole	22 $\pm$ 0.58
Zaprinast	3.8 $\pm$ 0.23

Table 2 Comparison of IC_{50} for zaprinast of PfPDE1 mutants

Wild-type PfPDE1 enzyme and its mutants, PfPDE1D762A and PfPDE1G788A, were produced in *E. coli* and partially purified by using a nickel affinity column. The K_m values of PfPDE1 were calculated from Lineweaver–Burk plots of activities using cGMP. IC_{50} values for zaprinast were measured with 0.6 μM cGMP by linear regression. Data are expressed as the means $\pm$ S.E.M. for three independent determinations All assays were performed in duplicate. ND, not detected; NT, not tested.

Protein	K_m (μM)	IC_{50} for zaprinast (μM)
PfPDE1 (wild-type)	0.65 $\pm$ 0.033	3.8 $\pm$ 0.23
PfPDE1D762A	ND	NT
PfPDE1G788A	0.77 $\pm$ 0.054	5.6 $\pm$ 0.67

was the most effective antagonist for PfPDE1 with an IC_{50} value of 3.8 $\pm$ 0.23 μM. This value is approximately 10 times higher than that obtained for human PDE5 (IC_{50} = 0.5 μM; [35]). A potent antimalarial, chloroquine, showed 35 % inhibition at 100 μM.

Previous reports revealed that Asp^{754} and Gly^{780} of bovine PDE5A are critical for interaction with zaprinast [36,37]. The Asp residue is conserved among PDEs and the involvement of this residue in binding to the substrate cGMP has been reported [38]. Since PfPDE1 also contained Asp^{762} and Gly^{788} in the conserved positions, we examined a role of these residues for cGMP hydrolytic activity and zaprinast-sensitivity of PfPDE1. Two PfPDE1 mutants, PfPDE1D762A and PfPDE1G788A, with alanine substitutions at Asp^{762} and Gly^{788} respectively (see the Experimental section) were used. PfPDE1D762A showed no cGMP hydrolytic activity (Table 2), although the protein was produced at almost the same level as PfPDE1Δ in immunoblot analysis (results not shown), indicating that Asp^{762} is indispensable for the PDE activity. On the other hand, another PfPDE1 mutant, PfPDE1G788A, retained the cGMP-PDE activity with a K_m value of 0.77 $\pm$ 0.054 μM, which was about the same as that of the wild-type (0.65 $\pm$ 0.033 μM). The IC_{50} value of the mutant for zaprinast (PfPDE1G788A) was 5.6 $\pm$ 0.67 μM, and introduction of this amino acid substitution did not alter the zaprinast-sensitivity drastically.

cGMP hydrolytic activity in *P. falciparum*

Endogenous cGMP PDE activity in *P. falciparum* was examined. The cytosolic and membrane fractions were prepared from mixed

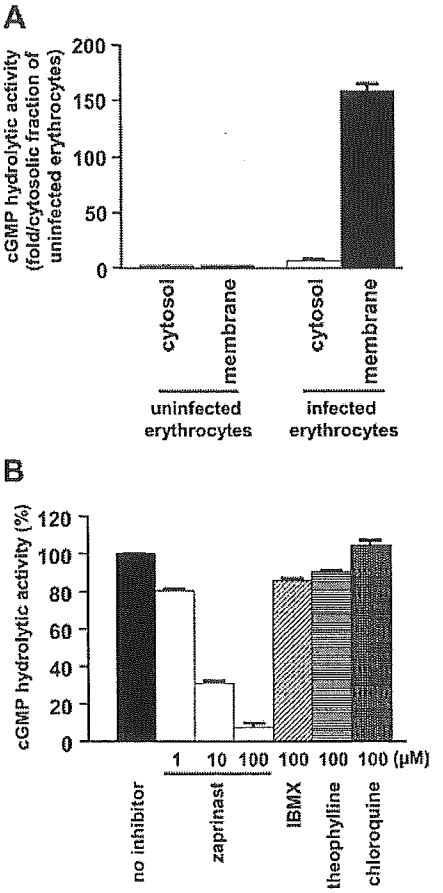

Figure 8 cGMP hydrolytic activity in *P. falciparum*

(**A**) Cytosolic and membrane fractions of *P. falciparum* were prepared as described in the Experimental section. PDE activity was measured using 13 nM cGMP as a substrate. The data are expressed as the means $\pm$ S.E.M. for three independent experiments. (**B**) Membrane fractions of *P. falciparum* were used for the PDE assay using 13 nM cGMP. Data are expressed as the means $\pm$ S.E.M. for three independent experiments. All assays were performed in duplicate. The bivalent cation used was 1 mM $MnCl_2$.

asexual blood-stage malaria parasites and cGMP PDE activities and their subcellular localization were tested. Consistent with the predicted protein feature of PfPDE1 as a membrane protein, most cGMP hydrolytic activity was detected in membrane fractions prepared from infected erythrocytes (Figure 8A). Uninfected erythrocytes showed no significant cGMP hydrolytic activities in either cytosolic or membrane fractions. Interestingly, zaprinast inhibited *P. falciparum* cGMP PDE activity of membrane fractions with an IC_{50} value of 4.1 $\pm$ 0.32 μM, which was in good agreement with the value obtained with recombinant PfPDE1 (Figure 8B). Furthermore, E4021 inhibited 85 % of cGMP hydrolytic activity at 100 μM. On the other hand, IBMX and theophylline were also weak inhibitors at a concentration of 100 μM. At this concentration, chloroquine did not inhibit the cGMP-hydrolytic activity at all.

In vitro antimalarial activity of zaprinast

Finally, we examined the effect of PDE inhibitors in the cell proliferation of asexual blood parasites using an *in vitro* antimalarial activity assay. A solvent for PDE inhibitors, DMSO, did not affect the increase in the *P. falciparum* cell number (results not shown). Intriguingly, treatment with zaprinast, a potent inhibitor

Table 3 ***In vitro*** **antimalarial activity of zaprinast**

Asynchronous *P. falciparum* Honduras-1 were cultured with final Ht 3 % and 0.3 % parasitaemia in the absence or presence of PDE inhibitors. After 72 h, more than 1000 erythrocytes stained with Giemsa were examined under microscopy. The ED_{50} value, which is a dose giving 50 % reduction of the increase of infected erythrocytes, was determined by comparison to drug-free controls cultured under the same conditions. Data are expressed as the means ± S.E.M. for three independent determinations.

Inhibitors	ED_{50} (μM)
IBMX	> 100
Theophylline	> 100
Zaprinast	35 ± 4.2

of both recombinant PfPDE1 enzyme and endogenous cGMP PDE activity in *P. falciparum*, resulted in growth inhibition of the parasites with $ED_{50} = 35 \pm 4.2$ μM (Table 3). In contrast, neither IBMX nor theophylline inhibited parasite growth at the concentration of 100 μM. These data reaffirm the importance of the involvement of zaprinast-sensitive PDE activity, including PfPDE1, in the asexual stage development.

DISCUSSION

Currently, PDEs comprise a superfamily of enzymes that are divided into three major classes (class I, II and III) on the basis of sequence similarity. Class I PDEs contain a signature sequence, HDX_2HX_4N, show high affinity for cAMP and cGMP and have been identified in organisms ranging from lower eukaryotes to mammals. *P. falciparum* was predicted to contain four class I PDE genes in its genome, based on sequence analysis. One of the *P. falciparum* PDEs, PfPDE1, showed high affinity for cGMP and was sensitive to some of the generally used PDE inhibitors, typical enzymatic characteristics of class I PDEs. Moreover, the PDE activity was lost by mutagenesis of the conserved Asp^{762}, which is predicted to be involved in the formation of a metal-binding pocket essential for class I PDEs [39]. Thus intracellular cyclic nucleotide levels in this lower organism would be multiply controlled by the four class I PDEs including PfPDE1.

The presence of six putative transmembrane domains in the N-terminal to central region of PfPDE1 suggested that PfPDE1 is a membrane protein. Interestingly, we found that the inhibitor sensitivity of cGMP hydrolytic activity in membrane fractions of *P. falciparum* at mixed asexual blood stages is similar to that of the recombinant PfPDE1 enzyme, suggesting the presence of PfPDE1 in these fractions. However, as well as PfPDE1, the other three putative PfPDEs are also predicted to contain transmembrane regions in their N-termini and it has been reported that theophylline-sensitive cGMP PDE activity, not a characteristic of PfPDE1, may exist in gametocytes of *P. falciparum* [6]. Thus, although inhibitor sensitivity of endogenous PDE activity is analogous to that of PfPDE1, it is unclear whether PfPDE1 is a major cGMP-PDE in asexual blood stages of *P. falciparum* or not. Therefore analysis of the three other putative PfPDEs is needed to clarify the regulation of cyclic nucleotide signalling through hydrolysis by the PDEs in *P. falciparum*.

Previous reports have discussed the cyclic nucleotide signalling in *P. falciparum*. cAMP is involved in the asexual stage development and erythrocyte invasion in *P. falciparum* and *Plasmodium berghei* [40,41]. In contrast, cGMP has been shown to play an important role in gametogenesis of both parasites [4,5]. Guanylate cyclase (PfGC) activity has been demonstrated in gametocytes. A gametocyte-activating factor, xanthurenic acid, is known to increase PfGC activity [6], suggesting that exflagellation, a requirement for fertilization, may be mediated by cGMP signalling. However, Deng and Baker [8] have reported predominant expression of *P. falciparum* cGMP-dependent protein kinase (PfPKG) in the ring stage. In mammals, cGMP has three major receptor proteins, namely cGMP-dependent protein kinase, cyclic nucleotide-gated ion channel and cGMP-regulated PDE. Since PfPDE1 and the other three putative PfPDEs do not have a cGMP-binding domain, and a cyclic nucleotide-gated ion channel has not yet been identified in *Plasmodium*, PfPKG is the only effector of cGMP. Stage-specific expression of PfPDE1 transcripts was consistent with PfPKG, but not PfGC, and the lysates prepared from the parasite in asexual blood stages actually contained cGMP hydrolytic activity. The presence of cGMP-PDE and PKG activities suggests a role for cGMP signalling in the development of the parasite at the asexual blood stages.

The *in vitro* antimalarial activity assay demonstrated that treatment of *P. falciparum* with zaprinast results in significant growth inhibition of the parasites in the asexual blood stages. Interestingly, a cell-permeable cGMP analogue, 8-bromo-cGMP, inhibited growth of the parasites in the asexual blood stages with ED_{50} values less than 10 μM (results not shown), indicating the significance of cGMP signalling in the survival of this organism. Zaprinast is a potent mammalian PDE5 inhibitor, and therefore it is important to know whether synthesis of a specific inhibitor for PfPDE1 is promising or not. Mutagenesis of Gly^{788} in PfPDE1, which is implicated in the zaprinast binding of bovine PDE5 [36,37], did not alter the inhibitory effects of this compound on PfPDE1. This indicates that the amino acid residues concerned with the binding of zaprinast differ between mammalian PDE5 and PfPDE1; that is, the discovery of a PfPDE1 specific inhibitor is achievable.

In addition to zaprinast ($IC_{50} = 3.8$ μM), PfPDE1 was also inhibited moderately by dipyridamole ($IC_{50} = 22$ μM). The antimalarial activity of dipyridamole has been reported previously [42,43]. A 50 % inhibition of *P. falciparum* has been observed at 30 nM dipyridamole after 24 h treatment [43]. However, the reported antimalarial activity of dipyridamole [42] is strong compared with that of zaprinast observed here. Dipyridamole is known to have various effects such as nucleoside transport inhibition [44], anti-tumour-drug-effluxing inhibition [45], blockage of chloride transport [46], band-3-mediated anion-exchange inhibition [47] and antioxidant properties [48]. It has been assumed that the antimalarial effects of dipyridamole are associated with the inhibition of some transport system [42]. On the other hand, dipyridamole is also established as a potent PDE inhibitor [49]; nevertheless, the involvement of its inhibitory effects on PDEs in the organism was not discussed in [42]. Dipyridamole-sensitive PDE may be included in the three other *Plasmodium* PDEs and dipyridamole might exhibit more potent antimalarial effects through inhibition of the enzyme than zaprinast.

In summary, we have described the first *P. falciparum* PDE, PfPDE1, and the effect of a PfPDE1 inhibitor, zaprinast, on antimalarial activity. PfPDEs in the asexual blood stages are not a target molecule of chloroquine, and therefore, considering the need for the development of a novel treatment of chloroquine-resistant malaria, PfPDEs must be a unique target for chemotherapy. Further investigation of the PfPDEs of *P. falciparum* will elucidate the involvement of cyclic nucleotide signalling in the physiological responses of this parasite and will provide us with a new treatment of the most lethal malaria.

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REFERENCES

1 Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S. et al. (2002) Genome sequence of the human malaria parasite Plasmodium falciparum. Nature (London) **419**, 498–511

2 Le Bras, J. and Durand, R. (2003) The mechanisms of resistance to antimalarial drugs in Plasmodium falciparum. Fundam. Clin. Pharmacol. **17**, 147–153

3 Miller, L. H., Baruch, D. I., Marsh, K. and Doumbo, O. K. (2002) The pathogenic basis of malaria. Nature (London) **415**, 673–679

4 Kawamoto, F., Alejo-Blanco, R., Fleck, S. L., Kawamoto, Y. and Sinden, R. E. (1990) Possible roles of Ca^{2+} and cGMP as mediators of the exflagellation of Plasmodium berghei and Plasmodium falciparum. Mol. Biochem. Parasitol. **42**, 101–108

5 Kawamoto, F., Fujioka, H., Murakami, R., Syafruddin Hagiwara, M., Ishikawa, T. and Hidaka, H. (1993) The roles of Ca^{2+}/calmodulin- and cGMP-dependent pathways in gametogenesis of a rodent malaria parasite, Plasmodium berghei. Eur. J. Cell. Biol. **60**, 101–107

6 Muhia, D. K., Swales, C. A., Deng, W., Kelly, J. M. and Baker, D. A. (2001) The gametocyte-activating factor xanthurenic acid stimulates an increase in membrane-associated guanylyl cyclase activity in the human malaria parasite Plasmodium falciparum. Mol. Microbiol. **42**, 553–560

7 Carucci, D. J., Witney, A. A., Muhia, D. K., Warhurst, D. C., Schaap, P., Meima, M., Li, J. L., Taylor, M. C., Kelly, J. M. and Baker, D. A. (2000) Guanylyl cyclase activity associated with putative bifunctional integral membrane proteins in Plasmodium falciparum. J. Biol. Chem. **275**, 22147–22156

8 Deng, W. and Baker, D. A. (2002) A novel cyclic GMP-dependent protein kinase is expressed in the ring stage of the Plasmodium falciparum life cycle. Mol. Microbiol. **44**, 1141–1151

9 Soderling, S. H. and Beavo, J. A. (2000) Regulation of cAMP and cGMP signalling: new phosphodiesterases and new functions. Curr. Opin. Cell Biol. **12**, 174–179

10 Francis, S. H., Turko, I. V. and Corbin, J. D. (2001) Cyclic nucleotide phosphodiesterases: relating structure and function. Prog. Nucleic Acid Res. Mol. Biol. **65**, 1–52

11 Hetman, J. M., Robas, N., Baxendale, R., Fidock, M., Phillips, S. C., Soderling, S. H. and Beavo, J. A. (2000) Cloning and characterization of two splice variants of human phosphodiesterase 11A. Proc. Natl. Acad. Sci. U.S.A. **97**, 12891–12895

12 Yuasa, K., Kotera, J., Fujishige, K., Michibata, H., Sasaki, T. and Omori, K. (2000) Isolation and characterization of two novel phosphodiesterase PDE11A variants showing unique structure and tissue-specific expression. J. Biol. Chem. **275**, 31469–31479

13 Shabsigh, R. (2004) Therapy of ED: PDE-5 Inhibitors. Endocrine **23**, 135–141

14 Liu, Y., Shakur, Y., Yoshitake, M. and Kambayashi, Ji J. (2001) Cilostazol (pletal): a dual inhibitor of cyclic nucleotide phosphodiesterase type 3 and adenosine uptake. Cardiovasc. Drug Rev. **9**, 369–386

15 Spina, D. (2004) The potential of PDE4 inhibitors in respiratory disease. Curr. Drug Targets Inflamm. Allergy. **3**, 231–236

16 Seebeck, T., Gong, K., Kunz, S., Schaub, R., Shalaby, T. and Zoraghi, R. (2001) cAMP signalling in Trypanosoma brucei. Int. J. Parasitol. **31**, 491–498

17 Zoraghi, R., Kunz, S., Gong, K. and Seebeck, T. (2001) Characterization of TbPDE2A, a novel cyclic nucleotide-specific phosphodiesterase from the protozoan parasite Trypanosoma brucei. J. Biol. Chem. **276**, 11559–11566

18 Rascón, A., Soderling, S. H., Schaefer, J. B. and Beavo, J. A. (2002) Cloning and characterization of a cAMP-specific phosphodiesterase (TbPDE2B) from Trypanosoma brucei. Proc. Natl. Acad. Sci. U.S.A. **99**, 4714–4719

19 Zoraghi, R. and Seebeck, T. (2002) The cAMP-specific phosphodiesterase TbPDE2C is an essential enzyme in bloodstream form *Trypanosoma brucei*. Proc. Natl. Acad. Sci. U.S.A. **99**, 4343–4348

20 Trager, W. and Jensen, J. B. (1976) Human malaria parasites in continuous culture. Science (Washington D.C.) **193**, 673–675

21 Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) Basic local alignment search tool. J. Mol. Biol. **215**, 403–410

22 Schultz, J., Milpetz, F., Bork, P. and Ponting, C. P. (1998) SMART, a simple modular architecture research tool: identification of signalling domains. Proc. Natl. Acad. Sci. U.S.A. **95**, 5857–5864

23 Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**, 4673–4680

24 Lambros, C. and Vanderberg, J. P. (1979) Synchronization of Plasmodium falciparum erythrocytic stages in culture. J. Parasitol. **65**, 418–420

25 Rivadeneira, E. M., Wasserman, M. and Espinal, C. T. (1983) Separation and concentration of schizonts of Plasmodium falciparum by Percoll gradients. J. Protozool. **30**, 367–370

26 Sijwali, P. S., Shenai, B. R., Gut, J., Singh, A. and Rosenthal, P. J. (2001) Expression and characterization of the Plasmodium falciparum haemoglobinase falcipain-3. Biochem. J. **360**, 481–489

27 Thompson, W. J., Terasaki, W. L., Epstein, P. M. and Strada, S. J. (1979) Assay of cyclic nucleotide phosphodiesterase and resolution of multiple molecular forms of the enzyme. Adv. Cyclic Nucleotide Res. **10**, 69–92

28 Lineweaver, H. and Burk, D. (1934) The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56**, 658–666

29 McPhee, I., Pooley, L., Lobban, M., Bolger, G. and Houslay, M. D. (1995) Identification, characterization and regional distribution in brain of RPDE-6 (RNPDE4A5), a novel splice variant of the PDE4A cyclic AMP phosphodiesterase family. Biochem. J. **310**, 965–974

30 Kim, H.-S., Shibata, Y., Wataya, Y., Tsuchiya, K., Masuyama, A. and Nojima, M. (1999) Synthesis and antimatarial activity of cyclic peroxides, 1,2,4,5,7-pentoxocanes and 1,2,4,5-tetroxanes. J. Med. Chem. **42**, 2604–2609

31 Beavo, J. A. and Reifsnyder, D. H. (1990) Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. Trends Pharmacol. Sci. **11**, 150–155

32 Francis, S. H., Colbran, J. L., McAllister-Lucas, L. M. and Corbin, J. D. (1994) Zinc interactions and conserved motifs of the cGMP-binding cGMP-specific phosphodiesterase suggest that it is a zinc hydrolase. J. Biol. Chem. **269**, 22477–22480

33 Senapathy, P., Shapiro, M. B. and Harris, N. L. (1990) Splice junctions, branch point sites, and exons: sequence statistics, identification, and applications to genome project. Methods Enzymol. **183**, 252–278

34 Fisher, D. A., Smith, J. F., Pillar, J. S., St Denis, S. H. and Cheng, J. B. (1998) Isolation and characterization of PDE9A, a novel human cGMP-specific phosphodiesterase. J. Biol. Chem. **273**, 15559–15564

35 Loughney, K., Hill, T. R., Florio, V. A., Uher, L., Rosman, G. J., Wolda, S. L., Jones, B. A., Howard, M. L., McAllister-Lucas, L. M., Sonnenburg, W. K. et al. (1998) Isolation and characterization of cDNAs encoding PDE5A, a human cGMP-binding, cGMP-specific 3′,5′-cyclic nucleotide phosphodiesterase. Gene **216**, 139–147

36 Turko, I. V., Francis, S. H. and Corbin, J. D. (1998) Potential roles of conserved amino acids in the catalytic domain of the cGMP-binding cGMP-specific phosphodiesterase. J. Biol. Chem. **273**, 6460–6466

37 Turko, I. V., Ballard, S. A., Francis, S. H. and Corbin, J. D. (1999) Inhibition of cyclic GMP-binding cyclic GMP-specific phosphodiesterase (Type 5) by sildenafil and related compounds. Mol. Pharmacol. **56**, 124–130

38 Zhang, W., Ke, H., Tretiakova, A. P., Jameson, B. and Colman, R. W. (2001) Identification of overlapping but distinct cAMP and cGMP interaction sites with cyclic nucleotide phosphodiesterase 3A by site-directed mutagenesis and molecular modeling based on crystalline PDE4B. Protein Sci. **10**, 1481–1489

39 Card, G. L., England, B. P., Suzuki, Y., Fong, D., Powell, B., Lee, B., Luu, C., Tabrizizad, M., Gillette, S., Ibrahim, et al. (2004) Structural basis for the activity of drugs that inhibit phosphodiesterases. Structure **12**, 2233–2247

40 Rangachari, K., Dluzewski, A., Wilson, R. J. and Gratzer, W. B. (1986) Control of malarial invasion by phosphorylation of the host cell membrane cytoskeleton. Nature (London) **324**, 364–365

41 McColm, A. A., Hommel, M. and Trigg, P. I. (1980) Inhibition of malaria parasite invasion into erythrocytes pretreated with membrane-active drugs. Mol. Biochem. Parasitol. **1**, 119–127

42 Akaki, M., Nakano, Y., Ito, Y., Nagayasu, E. and Aikawa, M. (2002) Effects of dipyridamole on Plasmodium falciparum-infected erythrocytes. Parasitol. Res. **88**, 1044–1050

43 Gero, A. M., Scott, H. V., O'Sullivan, W. J. and Christopherson, R. I. (1989) Antimalarial action of nitrobenzylthioinosine in combination with purine nucleoside antimetabolites. Mol. Biochem. Parasitol. **34**, 87–97

44 Scholtissek, C. (1968) Studies on the uptake of nucleic acid precursors into cells in tissue culture. Biochim. Biophys. Acta **158**, 435–447

45 Chen, H. X., Bamberger, U., Heckel, A., Guo, X. and Cheng, Y. C. (1993) BIBW 22, a dipyridamole analogue, acts as a bifunctional modulator on tumor cells by influencing both P-glycoprotein and nucleoside transport. Cancer Res. **53**, 1974–1977

46 Garcia, A. M. and Lodish, H. F. (1989) Lysine 539 of human band 3 is not essential for ion transport or inhibition by stilbene disulfonates. J. Biol. Chem. **264**, 19607–19613

47 Falke, J. J. and Chan, S. I. (1986) Molecular mechanisms of band 3 inhibitors. 2. Channel blockers. Biochemistry **25**, 7895–7898

48 Pick, E. and Mizel, D. (1981) Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. J. Immunol. Methods **46**, 211–226

49 Thompson, W. J. (1991) Cyclic nucleotide phosphodiesterases: pharmacology, biochemistry and function. Pharmacol. Ther. **51**, 13–33

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Chemotherapeutic efficacy of ascofuranone in *Trypanosoma vivax*-infected mice without glycerol

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Abstract

Ascofuranone, an antibiotic isolated from *Ascochyta visiae*, showed trypanocidal activity in *Trypanosoma vivax*-infected mice. A single dose of 50 mg/kg ascofuranone effectively cured the mice without the help of glycerol. Repeated administrations of this drug further enhanced its chemotherapeutic effect. After two, three, and four consecutive days treatment, the doses needed to cure the infection decreased to 25, 12, and 6 mg/kg, so that the total doses administered were 50, 36 and 24 mg/kg, respectively. Ascofuranone (50 mg/kg) also had a prophylactic effect against *T. vivax* infection within the first two days after administration. This prophylactic activity diminished to 80% by day 3 and completely disappeared four days after administration. Of particular interest in this study was that ascofuranone had trypanocidal activity in *T. vivax*-infected mice in the absence of glycerol, whereas co-administration of glycerol or repeated administrations of this drug are needed for *Trypanosoma brucei brucei* infection. Our present results strongly suggest that ascofuranone is also an effective tool in chemotherapy against African trypanosomiasis in domestic animals.

Keywords: *Trypanosoma vivax*; Chemotherapy; Ascofuranone; Mouse; Intramuscular

1. Introduction

Trypanosoma vivax is a causative agent of trypanosomiasis in livestock, and, even in the absence of tsetse fly, various blood-sucking insects can transmit mechanically [1]. This parasitic disease is spreading in the large area of sub-Saharan Africa and the 13 South American countries [2]. In Africa, the most commonly employed drugs for treating trypanosomiasis in cattle, sheep, and goats are currently homidium, isometamidium, and diminazene [3]. These drugs were discovered more than forty years ago and their use has been challenged by the appearance of drug-resistant trypanosomes [4–8]. New drugs that are nontoxic to the host and more effective and specific against African trypanosome infection in domestic animals are eagerly awaited [3,6,9,10].

The antibiotic ascofuranone (Fig. 1), which was isolated from the phytophathogenic fungus *Ascochyta visiae*, was discovered as an anti-tumor drug that is dependent on the activation of host macrophages [11–18]. In a previous report [19], we showed that ascofuranone strongly inhibits both mitochondrial O_2 consumption in a mitochondrial preparation and the in vitro growth of the bloodstream form of *T. b. brucei*. Moreover, we have shown that ascofuranone specifically inhibits the alternative oxidase (AOX or TAO) of the respiratory chain of the long slender (LS) forms of *T. b. brucei* [19–25], whereas glycerol inhibits the remaining anaerobic ATP production by mass action [19,20,23,27–31]. In the combination treatment with ascofuranone and glycerol,

Abbreviations: AOX, alternative oxidase; TAO, trypanosome alternative oxidase; LS, long slender; SHAM, salicylhydroxamic acid; TbAOX, *T. b. brucei* AOX; rTbAOX, recombinant TbAOX; TvAOX, *T. vivax* AOX; rTvAOX, recombinant TvAOX; PBS, phosphate buffered saline; GK, glycerol kinase; G-3-P, L-glycerol-3-phosphate.

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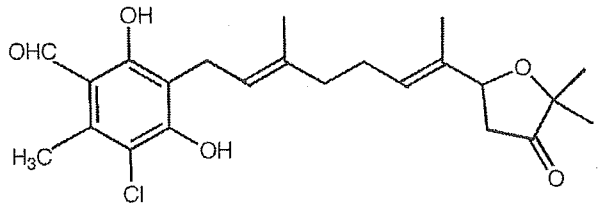

Fig. 1. Structure of ascofuranone.

trypanosomes were dramatically cleared from the infected mice. In fact, all bloodstream forms disappeared within 30 min of intraperitoneal treatment and within 180 min after oral administration [20]. Further, repeated administrations of ascofuranone also showed an anti-trypanosomal effect in the absence of glycerol against infection of *T. b. brucei* in mice [25]. These findings show that the mitochondrial respiratory chain of the African trypanosome is an attractive target for chemotherapy.

In an early study, Vickerman and Evans [32] showed that respiration of *T. vivax* was completely inhibited by 0.1 to 0.2 mM salicylhydroxamic acid (SHAM), an inhibitor of AOX. A combination of SHAM and glycerol was confirmed to have trypanocidal activity in *T. vivax*-infected mice [33,34], rats [29], and goats [35]. In a recent investigation, we cloned the *T. vivax* alternative oxidase (TvAOX) gene and characterized the recombinant enzyme [26]. We found that ascofuranone, the most potent known inhibitor of *T. b. brucei* AOX (TbAOX) [19], was also a competitive inhibitor of recombinant TvAOX (rTvAOX) with K_i value significantly lower than that of recombinant TbAOX (rTbAOX). Because of these findings and the current studies, we explored the chemotherapeutic efficacy of ascofuranone against *T. vivax* infection in mice.

2. Materials and methods

2.1. Chemicals

Ascofuranone [36] isolated from the fungus *Ascochyta visiae* was suspended in phosphate buffered saline (pH 7.5) containing 0.05% (v/v) Tween-20 (Wako Pure Chemical Co., Osaka, Japan) using a Teflon-glass homogenizer.

2.2. Trypanosomes

Trypanosoma vivax ILD at 1.2 [37] is a clone derived from of *T. vivax* Y486, isolated from an infected Zebu cow in Zaria, Nigeria and which is a naturally rodent-infective stock [38]. This clone was kindly provided by Dr. P.R. Gardiner, International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya [39]. The clone maintained in Balb/c mice (Japan SLC Co., Hamamatsu, Shizuoka, Japan) weighing 18 to 20 g by serial transfer at 6 day intervals was used throughout the experiment.

2.3. Animals

Balb/c mice (male, 6-week-old; Japan SLC Co.) were used for the current studies because they are susceptible to *T. vivax* infection [40].

2.4. Experimental treatments

Mice were inoculated intraperitoneally with 10^4 of bloodstream form of *T. vivax* from a previously infected mouse. After five days, ascofuranone was administered intramuscularly to trypanosome-infected mice every 24 h for one to four consecutive days. Four groups of five mice received daily intramuscular administration of ascofuranone (50, 25, 12, or 6 mg/kg) for one to four consecutive days. Control mice received only PBS containing 0.05% (v/v) Tween-20. After treatment, parasitemia was checked every day by observing tail blood smears. Mice that were negative for trypanosomes for 60 days after the last treatment were considered cured [41]. The animal research committee of Nagoya City University, Graduate School of Medical Sciences, approved this study.

3. Results

Experiments were started 5 days after infection of *T. vivax*. At the start of treatment, parasitemia level in infected mice was over 10^5 trypanosomes/ml. Without treatment, all mice died within 10 days after infection. In the present study, we adopted intramuscular administration of ascofuranone because intravenous and intramuscular injection is common in the treatment of infected domestic animals in the field. The chemotherapeutic efficacy of ascofuranone against *T. vivax* infection is summarized in Table 1. A single treatment with 50 mg/kg ascofuranone effectively cured all mice without the aid of glycerol. At a dose of 25 mg/kg ascofuranone, two consecutive days administration were needed for recovery from the infection. In mice treated once with 25 mg/kg ascofuranone, four out of five were cured, but bloodstream forms reappeared in the remaining mouse after 11 days, and it died 17 days after the treatment. In mice treated with 12 mg/kg ascofuranone, three consecutive days administration was required to permanently cure them from the infection (total dose: 36 mg/kg). In mice treated once with 12 mg/kg, one out of five mice was cured, but trypanosomes reappeared in four mice 6 to 8 days, and they died 12 to 14 days after the beginning of treatment. In mice treated twice with this dose of ascofuranone, two out of five were cured, but trypanosomes reappeared in three 8 to 11 days, and they died 14 to 17 days after the initiation of the treatment. When infected mice were treated with 6 mg/kg ascofuranone, four consecutive days administration was needed for the clearance of the infection (total dose: 24 mg/kg). When 6 mg/kg ascofuranone was administered for one or two consec-

Table 1
Effects of ascofuranone on *Trypanosoma vivax* infection in mice

Ascofuranone[a] (mg/kg)	Days of consecutive administration	Days of parasitemia reappearance and death occurred after beginning of treatment		Cure rate[c] (%)
		Parasitemia	Death	
PBS	5	N[b]	5	0
6	1	N[b]	6–7	0
	2	1–2	7–8	0
	3	7–9	13–15	40
	4	–	Cured	100
12	1	6–8	12–14	20
	2	8–11	14–17	40
	3	–	Cured	100
25	1	11	17	80
	2	–	Cured	100
50	1	–	Cured	100

[a] Ascofuranone was administered intramuscularly.
[b] Parasites did not disappear.
[c] Each group consists of five mice.

utive days, all infected mice died, and there was only a prolongation of the survival time. In mice treated for three consecutive days with 6 mg/kg ascofuranone, two out of five mice were cured, whereas the remaining mice relapsed 7 to 9 days, and they died 13 to 15 days after the beginning of treatment. Trypanosomes in the mice treated with 12 to 50 mg/kg ascofuranone disappeared from the blood within 12 h after the first administration. However, in infected mice receiving only a single administration of 6 mg/kg ascofuranone, trypanosomes did not disappear from the blood, and there was only a prolongation of the survival time. On the other hand, in infected mice administered 6 mg/kg ascofuranone for two to four consecutive days, there was a disappearance of trypanosomes from the blood within 24 to 48 h. Finally, the 50% lethal dose (LD_{50}) of ascofuranone administered intramuscularly for 4 consecutive days was estimated to be 800 mg/kg/day.

We next examined the prophylactic activity of ascofuranone. Groups of five mice each were injected intraperitoneally with 10^4 trypanosomes one to five days after administration of 50 mg/kg ascofuranone. As shown in Table 2, complete protection against *T. vivax* infection continued for two days, but this activity decreased to 80% by day three, and there was no effect observed 4 days after administration.

4. Discussion

Our present studies clearly demonstrate that ascofuranone acts as a trypanocidal drug in *T. vivax*-infected mice. In the preliminary experiments, we also confirmed trypanocidal effect of ascofuranone combination with glycerol in *T. vivax* infected mice (data not shown). However, we only determined the anti-trypanosomal activity of ascofuranone in a single or consecutive treatment without glycerol, because simple mode of administration is a very important factor in rural conditions. Fortunately, there was no requirement for glycerol as there is in mice infected with *T. b. brucei* [19,20], which is the causative agent of African sleeping sickness. All *T. vivax*-infected mice receiving a single 50 mg/kg dose of ascofuranone were effectively cured.

The idea that African trypanosomiasis might be cured with a combination of ascofuranone and glycerol is based on the effects of these chemicals on the unique energy-metabolizing pathway of the mammalian-infecting long slender (LS) bloodstream forms of this parasite. Energy production in the LS (host-infecting) forms of African trypanosomes is dependent on glycolysis within the glycosome, a peroxisome-like organelle specific to the parasite [30,42–44]. The mitochondrial cyanide-insensitive G-3-P oxidase system is used to re-oxidize NADH produced in the glycolytic pathway. This system oxidizes G-3-P produced in the glycosome using an electron transport system in the inner mitochondrial membrane that consists of G-3-P dehydrogenase, ubiquinone, and alternative oxidase (AOX) [19–29,42,45–58]. Under anaerobic conditions or in the presence of a respiratory inhibitor such as SHAM, G-3-P accumulates inside the glycosome and is converted to glycerol by the reverse reaction of glycerol kinase (GK) [30,31,42–44,59]. In this anaerobic glycolytic pathway, glucose is degraded to same amounts of pyruvate and glycerol, with a net synthesis of 1 mol of ATP per molecule of glucose degraded, even under anaerobic conditions or in the presence of inhibitors [30,31,42–44,59]. Therefore, inhibition of cyanide-insensitive respiration alone is not sufficient to kill the trypanosome, and the parasite can survive as long as glycerol does not accumulate in the medium. As we described previously [19], ascofuranone inhibited the electron-transport system of the LS forms of *T. b. brucei*, especially a mitochondrial enzyme TbAOX.

In the presence of ascofuranone, G-3-P cannot be re-oxidized, and G-3-P accumulates inside the glycosome and is converted to glycerol by the reverse reaction of glycerol kinase (GK). This alternative glycolytic pathway is inhibited by glycerol via mass action [19,20,23,27–31]. This combination treatment of ascofuranone and glycerol showed dramatic clearance of *T. b. brucei* infection in mice within 30 min after intraperitoneal administration [20]. Kralova et al. [59] have investigated the differences in the properties of GKs within the subgenus *Trypanozoon* (*T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. evansi*, and *T. equiperdum*) and other Trypanosomatidae. The activity of GK for glycerol and G-3-P could be attributed to the Ala at position 137. An alanine occurs in GK of the subgenus *Trypanozoon*, and it has the ability to produce large amounts of glycerol, whereas GK of *T. vivax* and *T. congolense* contains a serine and has little or no capacity to produce glycerol. The present results clearly demonstrate the property of *T. vivax* GK that G-3-P accumulated in the glycosome is not converted rapidly following administration of ascofuranone and it might cause the death of trypanosomes in the absence of glycerol.

In the recent study we cloned the TvAOX gene and expressed the recombinant enzyme in *E. coli* [26]. Ascofuranone, which is the most potent inhibitor of rTbAOX, inhibited rTvAOX at low concentration. Recombinant TvAOX was more sensitive to ascofuranone than rTbAOX. This higher sensitivity of rTvAOX was consistent with the efficacy of ascofuranone in *T. vivax*-infected mice: administration of 100 mg/kg ascofuranone for four consecutive days is needed to cure mice infected with *T. b. brucei* [25], whereas the *T. vivax*-infected mice were cured with four treatments of only 6 mg/kg ascofuranone.

Table 2
Prophylactic effect of ascofuranone against infection of *Trypanosoma vivax* in mice[a]

Days after administration[b]	No. of mice		Protection[c] rate (%)
	Protected	Dead	
1	5	0	100
2	5	0	100
3	4	1	80
4	0	5	0
5	0	5	0

[a] *T. vivax* (10^4) was inoculated intraperitoneally one to five days after administration of 50 mg/kg ascofuranone.
[b] Ascofuranone was administrated intramuscularly.
[c] Each group consists of five mice.

Our experiment showed that ascofuranone had a 100% to 80% prophylactic activity for three days after administration. Further immunological, biochemical, and cell biological investigations are necessary to understand this interesting phenomenon.

Our results strongly suggest that ascofuranone is a promising candidate as a chemotherapeutic agent to treat African trypanosomiasis in domestic animals. We therefore have an ongoing project in Africa to evaluate the efficacy of ascofuranone in *T. vivax*-infected livestock.

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References

[1] Gardiner PR. Recent studies of the biology of *Trypanosoma vivax*. Adv Parasitol 1989;28:229–317.
[2] Jones TW, Davila AM. *Trypanosoma vivax*—out of Africa. Trends Parasitol 2001;17:99–101.
[3] Wang CC. Molecular mechanisms and therapeutic approaches to the treatment of African trypanosomiasis. Annu Rev Pharmacol Toxicol 1995;35:93–127.
[4] Schonefeld A, Rottcher D, Moloo SK. The sensitivity to trypanocidal drugs of *Trypanosoma vivax* isolated in Kenya and Somalia. Trop Med Parasitol 1987;38:177–80.
[5] Codjia V, Mulatu W, Majiwa PA, Leak SG, Rowlands GJ, Authie E, et al. Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 3. Occurrence of populations of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium. Acta Trop 1993;53:151–63.
[6] Geerts S, Holmes PH, Eisler MC, Diall O. African bovine trypanosomiasis: the problem of drug resistance. Trends Parasitol 2001;17:25–8.
[7] Ainanshe OA, Jennings FW, Holmes PH. Isolation of drug-resistant strains of *Trypanosoma congolense* from the lower Shabelle region of Southern Somalia. Trop Anim Health Prod 1992;24:65–73.
[8] Sinyangwe L, Delespaux V, Brandt J, Geerts S, Mubanga J, Machila N, et al. Trypanocidal drug resistance in eastern province of Zambia. Vet Parasitol 2004;119:125–35.
[9] Kuzoe FAS. Current situation of African trypanosomiasis. Acta Trop 1993;54:153–62.
[10] Keiser J, Stich A, Burri C. New drugs for the treatment of human African trypanosomiasis: research and development. Trends Parasitol 2001;17:42–9.
[11] Magae J, Hosokawa T, Ando K, Nagai K, Tamura G. Antitumor protective property of an isoprenoid antibiotic, ascofuranone. J Antibiot 1982;35:1547–52.
[12] Magae J, Nagai K, Ando K, Yamasaki M, Tamura G. Effects of an antitumor agent, ascofuranone, on the macromolecular syntheses of intact cells. J Antibiot 1983;36:892–9.
[13] Magae J, Hotta M, Nagai K, Suzuki S, Ando K, Yamasaki M, et al. Activation of natural cytotoxic activity and concomitant reduction of triglyceride content of murine spleen, treated with an antitumor antibiotic, ascofuranone. J Antibiot 1986;39:676–81.
[14] Magae J, Suzuki S, Nagai K, Yamasaki M, Ando K, Tamura G. In vitro effects of an antitumor antibiotic, ascofuranone, on the murine immune system. Cancer Res 1986;46:1073–8.
[15] Magae J, Nagai K, Suzuki S, Yamasaki M, Ando K, Tamura G. Macrophage-specific effect on lipid metabolism by an antibiotic, ascofuranone. J Antibiot 1987;40:202–8.
[16] Magae J, Hosokawa T, Matsuda Y, Hotta M, Hayasaki J, Nagai K, et al. Suppression of hypertriglyceridemia of Ehrlich carcinoma-bearing mice by an antibiotic, ascofuranone. Cancer Res 1987;47:96–9.
[17] Magae J, Hayasaki J, Matsuda Y, Hotta M, Hosokawa T, Suzuki S, et al. Antitumor and antimetastatic activity of an antibiotic, ascofuranone, and activation of phagocytes. J Antibiot 1988;41:959–65.
[18] Magae J, Nagai K, Ando K, Tamura G. Differentiation of mouse and human myeloid leukemia cells induced by an antitumor antibiotic, ascofuranone. Agric Biol Chem 1988;52:3143–7.
[19] Minagawa N, Yabu Y, Kita K, Nagai K, Ohta N, Meguro K, et al. An antibiotic, ascofuranone, specifically inhibits respiration and in vitro growth of long slender bloodstream forms of *Trypanosoma brucei brucei*. Mol Biochem Parasitol 1996;81:127–36.
[20] Yabu Y, Minagawa N, Kita K, Nagai K, Honma M, Sakajo S, et al. Oral and intraperitoneal treatment of *Trypanosoma brucei brucei* with a combination of ascofuranone and glycerol in mice. Parasitol Int 1998;47:131–7.
[21] Fukai Y, Amino H, Hirawake H, Yabu Y, Ohta N, Minagawa N, et al. Functional expression of the ascofuranone-sensitive *Trypanosoma brucei brucei* alternative oxidase in the cytoplasmic membrane of *Escherichia coli*. Comp Biochem Physiol C 1999;124:141–8.
[22] Fukai Y, Nihei C, Yabu Y, Suzuki T, Ohta N, Minagawa N, et al. Strain-specific difference in amino acid sequences of trypanosome alternative oxidase. Parasitol Int 2002;51:195–9.
[23] Nihei C, Fukai Y, Kita K. Trypanosome alternative oxidase as a target of chemotherapy. Biochim Biophys Acta 2002;1587:234–9.
[24] Nihei C, Fukai Y, Kawai K, Osanai A, Yabu Y, Suzuki T, et al. Purification of active recombinant trypanosome alternative oxidase. FEBS Lett 2003;538:35–40.
[25] Yabu Y, Yoshida A, Suzuki T, Nihei C, Kawai K, Minagawa N, et al. The efficacy of ascofuranone in a consecutive treatment on *Trypanosoma brucei brucei* in mice. Parasitol Int 2003;52:155–64.
[26] Suzuki T, Nihei C, Yabu Y, Hashimoto T, Suzuki M, Yoshida A, et al. Molecular cloning and characterization of *Trypanosoma vivax* alternative oxidase (AOX) gene, a target of the trypanocide ascofuranone. Parasitol Int 2004;53:235–45.
[27] Clarkson Jr AB, Grady RW, Grossman SA, McCallum RJ, Brohn FH. *Trypanosoma brucei brucei*: a systematic screening for alternatives to the salicylhydroxamic acid–glycerol combination. Mol Biochem Parasitol 1981;3:271–91.
[28] van der Meer C, Versluijs-Broers JA, Opperdoes FR. *Trypanosoma brucei*: trypanocidal effect of salicylhydroxamic acid plus glycerol in infected rats. Exp Parasitol 1979;48:126–34.
[29] vad der Meer C, Versluijs-Broers JA. *Trypanosoma brucei* and *T. vivax*: salicylhydroxamic acid and glycerol treatment of acute and chronically infected rats. Exp Parasitol 1986;62:98–113.
[30] Clayton CE, Michels P. Metabolic compartmentation in African trypanosomes. Parasitol Today 1996;12:465–71.
[31] Michels PAM, Hannaert V, Bringaud F. Metabolic aspects of glycosomes in Trypanosomatidae—new data and views. Parastol Today 2000;16:482–9.
[32] Vickerman K, Evans DA. Studies on the ultrastructure and respiratory physiology of *Trypanosoma vivax* trypomastigote stages. Trans R Soc Trop Med Hyg 1974;68:145.
[33] Evans DA, Holland MF. Effective treatment of *Trypanosoma vivax* infections with salicylhydroxamic acid (SHAM). Trans R Soc Trop Med Hyg 1978;72:203–4.
[34] Evans DA, Brightman CA. Pleomorphism and the problem of recrudescent parasitemia following treatment with salicylhydroxamic acid (SHAM) in African trypanosomiasis. Trans R Soc Trop Med Hyg 1980;74:601–4.

[35] van der Meer C, Versluijs-Broers JA, van Duin CT, van den Ingh TS, Nieuwenhuijs J, Zwart D. Pitfalls of salicylhydroxamic acid plus glycerol treatment of *T. vivax* infected goats. Tropenmed Parasitol 1980;31: 275–82.
[36] Sasaki H, Hosokawa T, Sawada M, Ando K. Isolation and structure of ascofuranone and ascofuranol, antibiotics with hypolipidemic activity. J Antibiot 1973;26:676–80.
[37] Barry JD, Gathuo H. Antigenic variation in *Trypanosoma vivax*: isolation of a serodome. Parasitol 1984;89:49–58.
[38] Leeflang P, Buys J, Blotkamp C. Studies on *Trypanosoma vivax*: infectivity and serial maintenance of natural bovine isolates in mice. Int J Parasitol 1976;6:413–7.
[39] Vos GJ, Gardiner PR. Antigenic relatedness of stocks and clones of *Trypanosoma vivax* from East and West Africa. Parasitol 1900; 100:101–6.
[40] de Gee AL, Shah SD, Doyle JJ. *Trypanosoma vivax*: courses of infection with three stabilates in inbred mouse strains. Exp Parasitol 1982;54:33–9.
[41] Zhang ZQ, Giroud C, Baltz T. In vivo and in vitro sensitivity of *Trypanosoma evansi* and *T. equiperdum* to diminazene, suramin, Melcy, quinapyramine and isometamidium. Acta Trop 1991;50:101–10.
[42] Tielens AGM, Van Hellemond JJ. Difference in energy metabolism between Trypanosomatidae. Parasitol Today 1998;14:265–71.
[43] Visser N, Opperdoes FR. Glycolysis of *Trypanosoma brucei*. Eur J Biochem 1980;103:623–32.
[44] Hannaert V, Michels PAM. Structure, function, and biogenesis of glycosomes in Kinetoplastida. J Bioenerg Biomembr 1994;26:205–12.
[45] Clarkson Jr AB, Brohn FH. Trypanosomiasis: an approach to chemotherapy by the inhibition of carbohydrate catabolism. Science 1976;194:204–6.
[46] Opperdoes FR, Borst P, Bakker S, Leene W. Localization of glycerol-3-phosphate oxidase in the mitochondrion and particulate NAD^+-linked glycerol-3-phosphate dehydrogenase in the microbodies of the bloodstream form to *Trypanosoma brucei*. Eur J Biochem 1977; 76:29–39.
[47] Grady RW, Bienen EJ, Clarkson Jr AB. Esters of 3,4-dihydroxybenzoic acid, highly effective inhibitors of the sn-glycerol-3-phosphate oxidase of *Trypanosoma brucei brucei*. Mol Biochem Parasitol 1986; 21:55–63.
[48] Grady RW, Bienen EJ, Clarkson Jr AB. *p*-Alkyloxybenzhydroxamic acids, effective inhibitors of the trypanosome glycerol-3-phosphate oxidase. Mol Biochem Parasitol 1986;19:231–40.
[49] Clarkson Jr AB, Bienen EJ, Pollakis G, Grady RW. Trypanocidal CoQ analogues: their effect on other mitochondrial system. Comp Biochem Physiol B 1989;94:245–51.
[50] Clarkson Jr AB, Bienen EJ, Pollakis G, Saric M, McIntosh L, Grady R.W. Trypanosome alternative oxidase. Chemotherapy for trypanosomiasis. Proceedings of a workshop held at ILRAD, Nairobi, Kenya; 1989. p. 43–8.
[51] Clarkson Jr AB, Bienen EJ, Pollakis G, Grady RW. Respiration of bloodstream forms of the parasite *Trypanosoma brucei brucei* is dependent on a plant-like alternative oxidase. J Biol Chem 1989; 264:17770–6.
[52] Bienen EJ, Saric M, Pollakis G, Grady RW, Clarkson Jr AB. Mitochondrial development in *Trypanosoma brucei brucei* transitional bloodstream forms. Mol Biochem Parasitol 1991;45:185–92.
[53] Kornblatt JA, Nthale J, McOdimba F. Purification of *sn*-glycerol-3-phosphate dehydrogenase from *Trypanosoma brucei brucei*. Biochem Cell Biol 1992;70:136–41.
[54] Grady R.W, Bienen EJ, Dieck HA, Saric M, Clarkson Jr AB. N-*n*-alkyl-3, 4-dihydroxybenzamides as inhibitors of the trypanosome alternative oxidase: activity in vitro and in vivo. Antimicrob Agents Chemother 1993;37:1082–5.
[55] Priest JW, Hajduk SL. Developmental regulation of mitochondrial biogenesis in *Trypanosoma brucei*. J Bioenerg Biomembr 1994;26:179–91.
[56] Chaudhuri M, Ajayi W, Temple S, Hill GC. Identification and partial purification of a stage-specific 33 kDa mitochondrial protein as the alternative oxidase of the *Trypanosoma brucei brucei* bloodstream trypomastigotes. J Eukaryot Microbiol 1995;42:467–72.
[57] Chaudhuri M, Hill GC. Cloning, sequencing, and functional activity of the *Trypanosoma brucei brucei* alternative oxidase. Mol Biochem Parasitol 1996;83:125–9.
[58] Opperdoes FR. Progress in human African trypanosomiasis, sleeping sickness. Paris: Springer; 1999. p. 53–80.
[59] Kralova I, Rigden DJ, Opperdoes FR, Michels PA. Glycerol kinase of *Trypanosoma brucei*. Cloning, molecular characterization and mutagenesis. Eur J Biochem 2000;267:2323–33.