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Plasmodium falciparum Carbonic Anhydrase is a Possible Target for Malaria Chemotherapy

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***Plasmodium falciparum* is responsible for the majority of life-threatening cases of human malaria. The global emergence of drug-resistant malarial parasites necessitates identification and characterization of novel drug targets. Carbonic anhydrase (CA) is present at high levels in human red cells and in *P. falciparum*. Existence of at least three isozymes of the α class was demonstrated in *P. falciparum* and a rodent malarial parasite *Plasmodium berghei*. The major isozyme CA1 was purified and partially characterized from *P. falciparum* (PfCA1). A search of the malarial genome database yielded an open reading frame similar to the α-CAs from various organisms, including human. The primary amino acid sequence of the PfCA1 has 60% identity with a rodent parasite *Plasmodium yoelii* enzyme (PyCA). The single open reading frames encoded 235 and 252 amino acid proteins for PfCA1 and PyCA, respectively. The highly conserved active site residues were also found among organisms having α-CAs. The *PfCA1* gene was cloned, sequenced and expressed in *Escherichia coli*. The purified recombinant PfCA1 enzyme was catalytically active. It was sensitive to acetazolamide and sulfanilamide inhibition. Kinetic properties of the recombinant PfCA1 revealed the authenticity to the wild type enzyme purified from *P. falciparum in vitro* culture. Furthermore, the PfCA1 inhibitors acetazolamide and sulfanilamide showed good antimalarial effect on the *in vitro* growth of *P. falciparum*. Our molecular tools developed for the recombinant enzyme expression will be useful for developing potential antimalarials directed at *P. falciparum* carbonic anhydrase.**

Keywords: Malaria; *Plasmodium falciparum*; *Plasmodium berghei*; Drug target; Carbonic anhydrase; Acetazolamide

INTRODUCTION

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium*. The disease afflicts approximately 5 hundred million and kills up to 2.5 million annually, mainly children in African countries.[1] Four species infect humans but *P. falciparum* is responsible for the majority of deaths. The limitation and toxicity of antimalarial drugs currently used, and the spread of drug-resistant malarial parasites accompanied by a world-wide resurgence of malaria, requires the development of new drugs for management of the disease. In intraerythrocytic stage of development, the parasites require purines and pyrimidines for DNA and RNA synthesis during their exponential growth and replication. The parasites, known as purine auxotroph, salvage the preformed purines from the human host, but they have to synthesize pyrimidines *de novo* from HCO_3^-, adenosine 5′-triphosphate, glutamine and aspartate.[2–4] These properties on both purine and pyrimidine biosynthesis represent key differences between the parasite and human host.

Carbonic anhydrase (CA; EC 4.2.1.1) is a zinc-containing enzyme catalyzing the reversible hydration of CO_2[$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$]. The first CA was purified from bovine red cells in 1933,[5] followed by the identification of several isozymes widely distributed in mammals, plants and bacteria.[6–8] Recent advances regarding the crystal structure and biochemistry of CAs from various organisms revealed that they evolved independently and have been categorized into 4 classes: α, β, γ and δ.[9] The first three classes have no significant sequence identity and structural differences, their active sites function with a single zinc ion essential for catalysis. Recently, we have demonstrated the existence of three active CA isozymes in the human malarial parasite

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P. falciparum.[10] The major isozyme, *P. falciparum* CA1 (PfCA1), has been purified and its characteristics have been partially studied due to the paucity of parasitic materials from *in vitro* culture with human red cells.

Based on our previous observation, this prompted us to identify the *PfCA1* gene in the malarial genome database, and then clone and heterologously express it in *Escherichia coli*. This is the first CA cloned and functionally expressed from a protozoan parasite. The recombinant enzyme is catalytically active for both *p*-nitrophenylacetate and α-naphthylacetate as substrates, and was strongly inhibited by acetazolamide (2-acetylamino-1,3,4-thiazole-5-sulfonamide, AAZ) and sulfanilamide (4-aminobenzenesulfonamide, SFA). Physical and kinetic properties indicate authenticity of the recombinant PfCA1 to the wild type enzyme purified from *P. falciparum*. The PfCA1 also differs from the human CA II. The PfCA1 inhibitors AAZ and SFA show good antimalarial effect on the *in vitro* growth of *P. falciparum*. In addition to *P. falciparum* CAs, we report the existence of four isozymes of CA and their sensitivity to AAZ inhibition in a rodent parasite *P. berghei*.

MATERIALS AND METHODS

Materials and Chemicals

Restriction enzymes, biochemicals and chemicals were purchased from Promega, Sigma, Fluka and Amersham Biosciences. These were of the highest quality commercially available and were used without further purification. Nickel(II)-nitrilotriacetic acid (Ni^{2+}-NTA)-agarose affinity gel was obtained from Qiagen. Molecular mass markers for SDS-PAGE and gel filtration chromatography were purchased from Amersham Biosciences and BioRad.

Cultivation of *P. berghei* and *P. falciparum*

P. berghei was cultivated in Swiss albino mice with 50–60% parasitemia before collecting the blood. The *P. berghei*-infected blood, mainly at the trophozoite stage, was passed through CF-11 cellulose columns to remove all white blood cells and platelets. *P. falciparum* (T_9 isolate) was cultivated by a modification of the candle jar method of Trager and Jensen,[11] using a 5% hematocrit of human red cell type O suspended in RPMI 1640 medium supplemented with 25 mM Hepes, 32 mM $NaHCO_3$ and 10% fresh human serum type O. The cultures, started at low parasitemia (~1–2%), were changed with the medium twice daily until the cultures had ~30% parasitemia and then harvested for enzyme and nucleic acid preparations. The parasites were isolated from the infected red cells by incubating in 0.15% saponin in the RPMI medium for 20 min at 37°C. The host cell-free parasites were obtained after centrifugation at 8,000 × g for 10 min and washed at least 4 times with phosphate buffered saline (5 mM phosphate buffer/145 mM NaCl/pH 8.0) (PBS) and 1 mM phenylmethylsulfonyl fluoride (PMSF), and then lysed according to the reported procedure.[4]

Purification of Enzyme on Fast Protein Liquid Chromatographic System

The parasite supernatant obtaining after centrifugation of the parasite lysate at 27,000 × g for 60 min was dialyzed with 20 mM Na_2HPO_4 (pH 6.0) containing 1 mM PMSF, and concentrated. It was then loaded onto a Pharmacia Mono S cation-exchange fast protein liquid chromatographic (FPLC) column, which had been equilibrated with the phosphate buffer. The column was washed with the same buffer and then eluted with a linear gradient of phosphate buffer from pH 6.0–8.0 at a flow rate of 1 ml per min. The eluates were collected into 30 fractions, each 1.0 ml-fraction was assayed for CA activity and protein concentration. All *P. berghei* CA activities were eluted at fractions 3–8, pooled and prepared for activity staining on nondenaturing-PAGE. For the *P. falciparum* CA, the major isozyme CA1 was further purified to near homogeneity using two more sequential columns on Mono Q anion-exchange and Superose 12 gel filtration FPLC as described previously.[10]

Enzyme Assay and Kinetic Studies

The esterase activity of CA was measured by using the modified method of Armstrong *et al.*[12] The enzyme activity was determined by following the change in absorbance at 348 nm of *p*-nitrophenylacetate to 4-nitrophenoxide ion (extinction coefficient = 18.1 $M^{-1}\,cm^{-1}$) over a period of 3 min at 37°C using a Shimadzu 1601 spectrophotometer equipped with a temperature-controlled unit. The enzymatic reaction, in a total volume of 1.0 ml, contained 10 mM Tris–HCl buffer, pH 8.0, 0.25 mM *p*-nitrophenylacetate and 10–100 μl enzyme preparations. This measurement was then repeated in the presence of the inhibitor AAZ at a concentration of 0.1 mM, to obtain the net CA activity. One unit of enzyme activity was expressed as 1 μmol of *p*-nitrophenylacetate hydrolyzed per min at 37° C. Kinetic constants, K_m and k_{cat}, were determined by fitting data to the Michaelis-Menten equation using non-linear regression with an Elsevier Biosoft Enzfitter program. Inhibitor constants (K_i) were determined from Dixon's plots as described.[13]

Identification and Characterization of *P. falciparum* Carbonic Anhydrase Homolog

Homology search of the parasite PfCA1 was performed with the BLAST program of the U.S.

National Center for Biotechnology Information server.[14] Using α-CA sequences from other organisms, significant homology for PfCA1 was found within a sequence on chromosome 11 in a malaria genome database. A single continuous *P. falciparum* open reading frame (ORF) encoding CA homolog was further characterized by the TBLASTN program.[14] In addition, an ORF encoding CA homolog was identified in *P. yoelii* genome database (PyCA). Pair-wise and multiple sequence alignments of PfCA1 and PyCA with other organisms were performed using the CLUSTALW program.[15]

Cloning and Sequencing of *P. falciparum* Carbonic Anhydrase

Genomic DNA was isolated from *P. falciparum* by DNAzol™ reagent (Invitrogen). PCR was used to amplify DNA encoding PfCA1. The forward primer was 5′TCT**GGATCC**ATGAAAGATTTAAAGGAGAGAGAA3′ and the reverse primer was 5′CCC**AAGCTT**TTATTTATTACCTGAGCCGACGTG3′, which introduce *Bam*H I and *Hind* III restriction sites respectively (shown in bold).

The PCR cycling parameters include denaturation at 95°C (1 min), annealing at 55°C (1 min) and extension at 68°C (3 min). After 30 cycles, a single band of the predicted size was visualized on an 0.8% agarose gel. The PCR products from gDNA were purified from the gel by using gel extraction kit (Qiagen). PCR products were ligated into a cloning vector pDrive (Qiagen). The PfCA1 sequence was determined in both directions by the dideoxy chain-termination method using an automated Applied Biosystems Procise sequencer. The construct plasmid was subcloned into expression vectors. Attempts were done with at least three expression vectors having different promoters, i.e., pQE30 (Qiagen), pTOPO (Invitrogen), pET15b (Novagen). The *PfCA1* was expressed only with the pET15b expression vector. This approach will produce the recombinant protein fused to N-terminal His_6-thrombin cleavage site and the expressed recombinant protein can be detected by monoclonal antibody directed against His_6-tag.

Recombinant Protein Expression and Purification of *P. falciparum* Carbonic Anhydrase from *E. coli*

The competent *E. coli* BL21 (DE3) cells were transformed with the pET15b having the fused His_6-PfCA1. The cells were grown in LB medium (37°C) to an $OD_{600\ nm}$ of 0.5, and induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), harvested by centrifugation (8,000 × g) after IPTG induction for 3 h at 37°C, washed three times with ice-cold PBS, and stored as cell paste by freezing in −80°C until used.

All protein purification steps were performed at 4°C or on ice. Frozen cell pellets were suspended in four cell paste volume of buffer A (50 mM NaH_2PO_4, pH 8.0/300 mM NaCl/10 mM imidazole). A protease inhibitor cocktail was added to the cell suspension. The mixture was sonicated with an ultrasonic homogenizer. The *E. coli* lysate was then centrifuged for 30 min at 18,000 × g.

The supernatant (8 ml) was loaded onto a 2 ml bed volume of Ni^{2+}-NTA-agarose affinity gel equilibrated with buffer A. The column was washed with 20 ml of buffer B (50 mM NaH_2PO_4, pH 8.0/300 mM NaCl/20 mM imidazole), and then eluted with 6 ml of buffer C (50 mM NaH_2PO_4, pH 8.0/300 mM NaCl/250 mM imidazole). The eluted protein from the Ni^{2+}-NTA-agarose column was dialyzed extensively against PBS, prior to thrombin protease treatment (10 unit/mg protein, overnight at 22°C) for His_6-tag removal. The recombinant protein, after concentration using centricon 10 devices, was assayed for CA activity staining on nondenaturing-PAGE gel. The purified enzyme was determined for kinetic properties and inhibitory effect by AAZ and SFA inhibitors using the esterase assay as described.[12]

In Vitro Antimalarial Test

Growth of *P. falciparum* during drug-screening tests was measured by using incorporation of [^{3}H] hypoxanthine into parasite DNA and RNA and asynchronized culture with starting parasitemia of 0.5% as described.[16] Aliquots of stock solution of drugs were placed in 96-well tissue culture plates, to final concentrations of 0.001–1000 μM in sterile water after the addition of *P. falciparum* infected red cell suspension (0.5%) in RPMI 1640 culture medium. The plates were incubated in candle jars at 37°C for 24 h. [^{3}H] hypoxanthine (0.5 μCi; 1 Ci/mol) in 25 μl of the culture medium was then added to each well. The incorporation of [^{3}H]hypoxanthine in each well was examined after 48 h of drug-treated culture and the radioactivity was measured by liquid scintillation counting. The drug-free control of *P. falciparum*-infected red cells incubated under the same condition had radioactivity of 18,000 ± 1,000 cpm. The control red cells without harboring parasites incubated as described had 400 ± 50 cpm. All compounds were run in triplicate at each concentration. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of the compound causing 50% inhibition of the [^{3}H] hypoxanthine incorporation, compared with the drug-free control of the parasite culture. In parallel studies, antimalarial activity against *P. falciparum* in *in vitro* growth was quantified by measuring % parasitemia in a 96-hour culture in the presence of the drugs at various concentrations.[16] All compounds

were tested in triplicate at each concentration used. The morphological changes of *P. falciparum* were also observed in the culture treated with 100 μM AAZ in one intraerythrocytic cycle (~44–48 h) starting with the synchronized ring stage.

Other Methods

Human CA II was purified from normal red blood cells, which had been cultivated as well as *P. falciparum*-infected red cell using the same procedure as described for the malarial CA. Mouse red cells were prepared from the normal Swiss albino mice. The number of cells was determined by a hematocytometer. Parasitemia and parasite morphological characteristics were performed on methanol-fixed and Giemsa-stained blood film of the malarial culture and then counted by using a Nikon microscope, equipped with a camera unit.

Protein concentrations were determined by the method of Bradford[17] using bovine serum albumin as standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Bio-Rad minislab gel apparatus with a 5% acrylamide stacking gel and 10% acrylamide running gel in the discontinuous buffer system of Laemmli.[18] Nondenaturing-PAGE was performed by using the modified Laemmli's method in the absence of SDS and reducing agents of all reagents used, and 5% acrylamide running gel was applied. The enzyme activity was also detected on the nondenaturing-PAGE gel after electrophoresis in the presence of substrate α-naphthylacetate using the method described by Tashian.[19] The inhibitor AAZ (at 1 mM) was also included during enzyme activity staining as a control. The proteins on the SDS-PAGE gels were stained with Coomassie Blue R dye and visualized by a Kodak 1D image analysis software.

Western blot analysis was performed to confirm the authenticity of the His_6-tagged recombinant protein by using the method as described.[20] The detection system was QIA express™ kit (Qiagen) containing monoclonal antibody directed against His_6-tag with horseradish peroxidase-conjugate.

RESULTS AND DISCUSSION

The enzyme CA has previously been characterized from many species of animals, plants, yeast, bacteria, including human protozoa.[6–8,10] The crystal structures of the enzyme CA isolated from many sources have been identified.[9] The enzyme is the target for sulfonamide drugs, such as AAZ and methazolamide, for the treatment of human glaucoma.[8,21–22] In the present study, it is shown that in addition to the human parasite *P. falciparum*, the rodent parasite *P. berghei* propagated in the mouse system has its own CA activity and exhibits at least four isozymes, which are sensitive to the inhibitor AAZ. The *PfCA1* gene encoding *P. falciparum* CA1 was identified in the malarial genome database, and then cloned and functionally expressed in *E. coli*. The recombinant enzyme is catalytically active and sensitive to AAZ and SFA inhibition. It has authenticity to the wild type native enzyme purified from *P. falciparum*, and is also different from the human CA II. The PfCA1 inhibitors AAZ and SFA show good antimalarial effects on the *in vitro* growth of *P. falciparum* as observed by both [^{3}H] hypoxanthine incorporation and morphological abnormality, suggesting the therapeutic potential of the malarial parasite enzyme.

Existence of Carbonic Anhydrase Activity in Rodent Malarial Parasite *P. berghei*

To demonstrate the existence of CA activity in *P. berghei*, the isolated parasites freed from mouse red cells were used for CA assay by the esterase method, including the specific inhibitor AAZ, and compared to the CA activities in normal and *P. berghei*-infected red cells. The results are shown in Table I. There was a 4–5-fold increase in total activity of the enzyme in the infected red cells, compared to the uninfected and normal red cells. It was found that specific activity (mU/mg protein) of the enzyme in isolated parasites was ~9–10 times more than that in the normal red cells. When subjecting the parasite supernatant to the cation-exchange Mono S FPLC column, most CA activities were associated in the fractions 3–8 eluting at pH 6.2–6.6. These fractions were pooled, concentrated and then analyzed on nondenaturing-PAGE, followed by CA activity staining on the gels (Figure 1). There were four-activity bands, typing as isozymes CA1, CA2, CA3, and CA4, in the order of their mobility on the gel from cathode to anode, as described previously for the *P. falciparum* enzyme.[10] The isozymes CA2 and CA3 were major forms. In the presence of 1 mM AAZ, all four

TABLE I Carbonic anhydrase activity in normal and *P. berghei*-infected mouse red cells and in isolated parasites

	Activity[a]	
Cell	mU/10^9 cells	mU/mg protein
Normal mouse red cell	250 ± 20[b]	0.04 ± 0.005
P. berghei-infected red cell[c]	1,100 ± 130	0.63 ± 0.07
Isolated parasites	960 ± 90	0.35 ± 0.03

[a] mU of enzyme activity is expressed as nmol per min at 37°C. [b] The values are mean ± S.D., taken from three separate experiments of parasite and enzyme preparations. [c] The red cells were infected with ~60–70% parasitemia, harboring mainly the trophozoite stage.

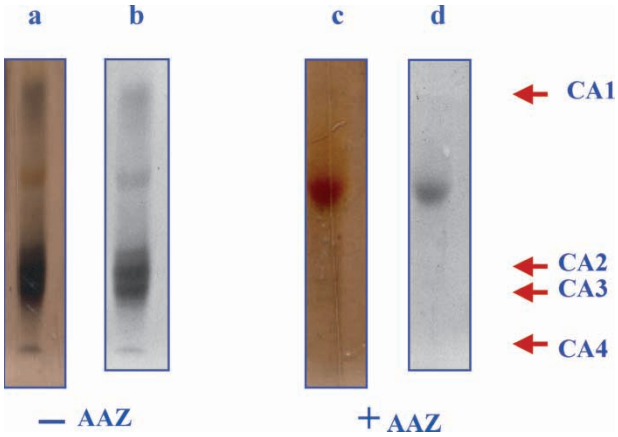

FIGURE 1 Enzyme activity staining of *P. berghei* carbonic anhydrase on nondenaturing-PAGE gels using α-naphthylacetate as substrate in the absence (a,b) and presence (c,d) of 1 mM acetazolamide (AAZ). The enzyme was concentrated from the pooled fractions 3–8 eluting at pH 6.2–6.6 of the cation-exchange Mono S FPLC column. Four-activity bands were observed to be sensitive to AAZ inhibition, and typing as CA1, CA2, CA3, and CA4 based on their mobility of the gels.

malarial CA activities were completely inhibited. Our results indicate the existence of at least four CA isozymes in *P. berghei.* This is consistent to our previous study on the occurrence of three CA isozymes in *P. falciparum*,[10] however, the majority of CA isozymes and their sensitivity to AAZ inhibition are markedly different between the human and the rodent malarial parasites. Purification of the major isozymes in *P. berghei* remains to be studied.

Cloning and Expression of *P. falciparum* Carbonic Anhydrase in *E. coli*

Recently, the nucleotide sequence of the *P. falciparum* genome, having 14 chromosomes with 23 Mb, has been completed.[23] It is now possible to identify the sequences that encode CA isozymes in this parasite. By using the bioinformatics approaches, TBLASTN searching of the malarial genome database was performed with the protein CA sequences obtained from other organisms. The search of the malarial genome database yielded an open reading frame (ORF) on chromosome 11 similar to the α-CAs from various organisms, including human. The primary amino acid sequence of the PfCA1 gene has ~60% identity with a rodent parasite enzyme, namely *P. yoelii* (PyCA). The single ORFs encoded 235 and 252 amino acid proteins for PfCA1 and PyCA, respectively. Low homology (~35–51%) of the PfCA1 and PyCA were found when compared to the insect *Drosophila melanogaster* and human CA I, II sequences. Nevertheless, the highly conserved active site residues responsible for binding of substrate and catalysis were also found among organisms having α-CAs (Figure 2). The consensus signature sequence remained variable.

The full-length *PfCA1* gene was cloned using PCR and genomic DNA extracted from *P. falciparum*, and the nucleotide of the cloned gene was sequenced to confirm it authenticity. The construct plasmid having *PfCA1* gene was then expressed in *E. coli*. The recombinant PfCA1 protein expression was obtained by a cloning and expression strategy using

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D.melanogaster      MSHHWGYTEENGPAHWAKEYPQASGHRQSPVDITPSSAKKGSELNVAPLKWKYVPEHTKS
H.sapiens           MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDP--SLKPLSVSYDQATSLR
P.falciparum        MKDLKERELKNISDVYLNLFDD-DNYAWNNYNKPWMKG------DFFYYYEYFIKKIVIN
P.yoelii            MHTLKERELKNLSDFYLNAFYDNDEYSWNNFNRPWFKG------DIFYYYENLINKIIIN
                    *        .* .  : : :   .    .  :    ..      ..

D.melanogaster      LVNPGYCWRVDVN-GADSELTGGPLGDQIFKLEQFHCHWGCTDSKGSEHTVDGV--SYSG
H.sapiens           ILNNGHAFNVEFDDSQDKAVLKGGPLDGTYRLIQFHFHWGSLDGQGSEHTVDKK--KYAA
P.falciparum        RQNNIFQIKAARDGIIPFGVLFTTEQPAMFYADQIHFHA------PSEHTFQGSGNRREI
P.yoelii            RQNNMFKIKASNNEIIPFGVLFTTDEPTIFYSHHINFHS------PSEHTFEGSGNRRHI
                      *   .  ..  :     :        :   ::: *        ****.:

D.melanogaster      ELHLVHWNTTKYKSFGEAAAAPDGLAVLGVFLKAGNHHAELDKVTSLLQFVLHKGDRVTL
H.sapiens           ELHLVHWN-TKYGDFGKAVQQPDGLAVLGIFLKVGSAKPGLQKVVDVLDSIKTKGKSADF
P.falciparum        EMQIFHSTNYFYDIQDDKSKYKKKYGLHIYNNLKKNSKETSKKDSSRYHSYLMSFLMNSL
P.yoelii            EMQIYHSTNEIYDYDENK-----WNGVFEKKNYKKKNNETN-----IQHSYILTFLMNSL
                    *::: * .   *    .        .:        . :          .    .     :

D.melanogaster      PQGCDPGQLLPDVHTYWTYEGSLTTPPCSESVIWIVFKTPIEVSDDQLNAMRNLNAYDVK
H.sapiens           TN-FDPRGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNGEG
P.falciparum        SNEQLQNKYNKKKR--------------------IKKMKNQYEVISITFTSAEINASTINA
P.yoelii            SNPHLGQQYTKNKKRNKRSKSLYNSIRLDENGKNTKRENQYQVISITFSSAEIDKSTINN
                    .:        .                              :   .* .  .   .

D.melanogaster      EECPCNEFNGKVINNFRPPLPLGKRELREIGGH-
H.sapiens           EPE------ELMVDNWRPAQPLKNRQIKASFK--
P.falciparum        FKKLPSEKFLRTIINVSSAVHVGSGNK-------
P.yoelii            FKKLPSEKFLKTILEASQNVPVGSGEKNIFIYFS
                              : :      : . :
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FIGURE 2 Amino acid sequences of *P. falciparum* and *P. yoelii* CAs, deduced from the open reading frame of *PfCA1* and *PyCA* genes. The predicted amino acid sequences of PfCA1 (235 residues) and PyCA (252 residues) are shown aligned with *D. melanogaster* (270 residues) and human CA II isozyme (260 residues). The amino acids responsible for binding and catalysis are identical among the four sequences and shown by boldface letters. The identical amino acids and conservative replacements are shown by star and dot symbols, respectively.

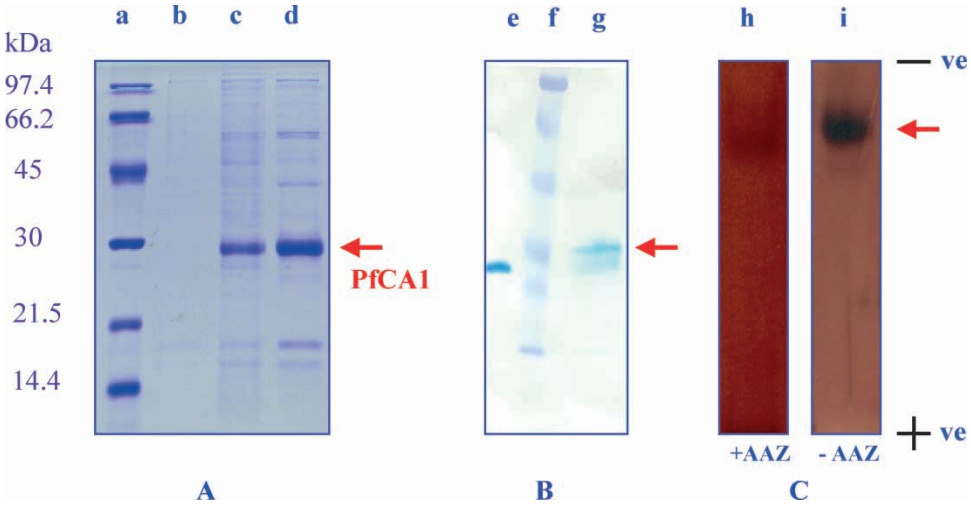

FIGURE 3 Analysis of recombinant *P. falciparum* carbonic anhydrase from IPTG-induced *E. coli* harboring PfCA1 construct pET15b plasmid. Panel A shows a SDS-PAGE analysis of purified PfCA1 from Ni^{2+}-NTA chromatography (>90% purity). The gel was stained with Coomassie Blue R; lane a, known proteins with molecular masses indicated in kDa; lane b, lanes c and d, 7.5 and 15 μg of the purified enzyme respectively; arrow indicates position of the recombinant enzyme. Panel B shows Western blot analysis of purified PfCA1 with monoclonal antibody directed against His_6 with HRP conjugate; lane e, 2 μg of control recombinant His_6-tagged dihydrofolate reductase (molecular mass of 26 kDa) obtained from Qiagen; lane f, pre-stained marker proteins; lane g, 2 μg of purified PfCA1. Panel C shows activity staining on denaturing-PAGE gels using α-naphthylacetate as substrate in the presence (lane h) or absence (lane i) of 1 mM acetazolamide (AAZ). The concentrated recombinant PfCA1 enzyme, after His_6-tag removal by thrombin, was used.

a His_6-tagged fusion protein at N-terminus, containing a thrombin cleavage site. It was shown that the *PfCA1* was functionally expressed in *E. coli* as soluble protein. The recombinant protein in the IPTG-induced *E. coli* supernatant was purified using Ni^{2+}-NTA agarose-affinity chromatographic column, and analyzed by SDS-PAGE (Figure 3A). The purity of the enzyme preparation was >90% after analysis by a Kodak 1D image software. It has a molecular mass of 29 ± 1 kDa, close to the molecular mass of the deduced amino acid sequence of PfCA1 (Figure 2) and the native CA1 purified from the malarial culture.[10] The authentic recombinant protein having N-terminal His_6-tag was confirmed using the Western blot analysis and monoclonal antibody directed against His_6-tag (Figure 3B). Furthermore, the recombinant PfCA1 was shown to express its activity after His_6-tag removal on the nondenaturing-PAGE gels, and was completely inhibited by 1 mM AAZ (Figure 3C). The purified recombinant PfCA1 was then studied kinetically for comparison with the wild type native *P. falciparum* CA1.

Kinetic Characterization of Recombinant *P. falciparum* Carbonic Anhydrase

Characterization of the purified recombinant PfCA1 was performed and compared with the purified human red cell CA II and the native CA1 purified from *P. falciparum* culture as described previously.[10] The kinetic parameters including K_m, k_{cat}, K_i of two specific inhibitors, AAZ and SFA, were found to be similar between the native and recombinant enzymes (Table II). AAZ and SFA were found to be typical competitive inhibitors for the malarial enzyme as determined by Dixon's plots (data not shown). The kinetic and inhibitory constants were different between the human and the parasite enzymes. These results suggest that the recombinant PfCA1 enzyme shows most properties similar to the native enzyme. The protein obtained here will be

TABLE II Comparison of kinetic parameters and inhibitory constants of human red cell CA II, native and recombinant *P. falciparum* carbonic anhydrases

Source	K_m[a] (mM)	k_{cat} (min^{-1})	K_i^{AAZ} (nM)	K_i^{SFA} (μM)
Human CA II	10.1 ± 0.8[b]	74.1 ± 5.7	99 ± 6	145 ± 2
Native PfCA1	3.7 ± 0.2	10.4 ± 1.2	247 ± 14	56 ± 4
Recombinant PfCA1	5.6 ± 0.3	8.2 ± 1.6	315 ± 26	84 ± 10

[a] This assay is based on esterase assay using *p*-nitrophenylacetate as substrate. [b] The values are mean ± S.D., taken from 3–4 separate experiments of the enzyme preparations.

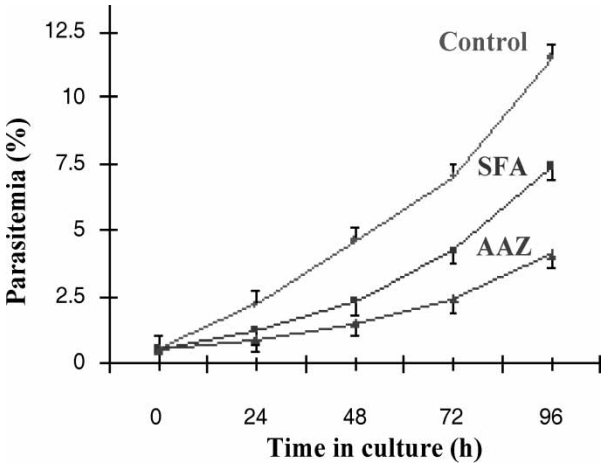

FIGURE 4 Antimalarial activity of the PfCA1 inhibitors, acetazolamide (AAZ) and sulfanilamide (SFA). *P. falciparum* growth was started with 0.5% parasitemia (mixed stages) at 2.5% red cell suspension and monitored every 24 h for up to 96 h at 37°C. The growth of *P. falciparum* in the absence of inhibitors is shown (●). 100 μM AAZ (▲) or 100 μM SFA (■) was present during the 96-h growth.

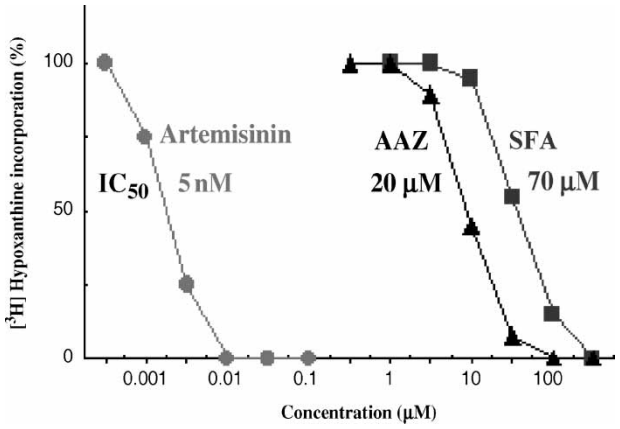

FIGURE 6 Inhibition of [^{3}H]hypoxanthine incorporation by *in vitro* culture of *P. falciparum* at various concentrations of drugs. Growth of *P. falciparum* was started with 0.25% parasitemia (mixed stages) at 0.5% red cell suspension. (●) antimalarial artemisinin, a Chinese traditional drug; (▲) acetazolamide, AAZ; (■) sulfanilamide (SFA).

used for drug-screening tests and crystal structure analysis for a purpose of mechanism-based drug design in the near future.

Antimalarial Properties of *P. falciparum* CA Inhibitors

We hypothesized that inhibition of *P. falciparum* growth in the erythrocytic stage requires inhibition of both human and *P.falciparum* CAs, since both human host cell and parasite contain relatively high CA activities.[10] The antimalarial properties of CA inhibitors were tested against *in vitro* growth of *P. falciparum* by lowering the % red cell suspension from 10% to 2.5%. Both AAZ and SFA drugs (100 μM) tested at 2.5% red cell suspension showed a strong antimalarial effect on *P. falciparum* growth with higher than 50% inhibition (Figure 4), whereas at 10% red cell suspension they showed little activity (data not shown). Interestingly, AAZ at 100 μM shows its antimalarial property by interfering with the intracellular development of *P. falciparum* in a stage-dependent manner (Figure 5). The morphological abnormality, as shown by clumping of nucleus and cytosol, of the AAZ-treated parasites in the human host red cells were markedly enhanced at the latter stages of development, i.e., trophozoite and schizont (Figure 5, D–F). The control culture shows healthy parasites during an intraerythrocytic development (Figure 5, A–C). By using [^{3}H] hypoxanthine incorporation for monitoring growth of *P. falciparum* in *in vitro* culture, which were started with mixed stages at 0.5% red cell suspension, the IC_{50} values in the mixed stages of parasite develop-

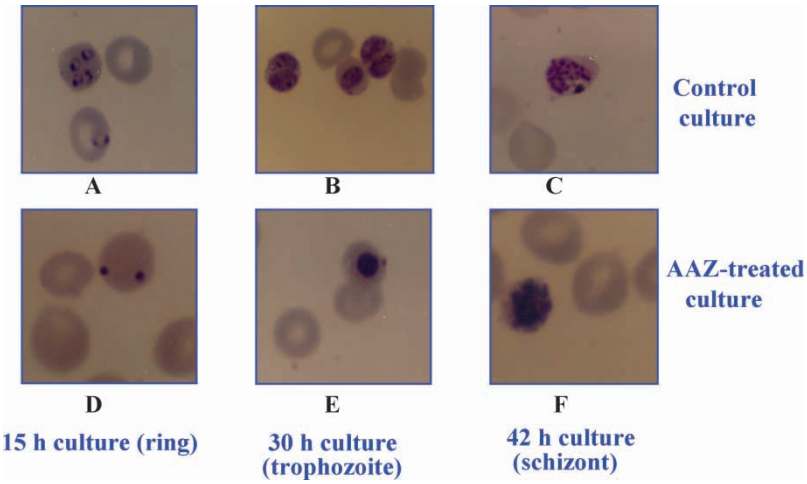

FIGURE 5 Effect of acetazolamide (AAZ) on *P. falciparum* morphology during an intraerythrocytic cycle (ring, trophozoite and schizont stages). The morphological changes were examined in the absence (panels A, B and C; control culture) or in the presence of 100 μM AAZ (panels D, E and F; AAZ-treated culture) at various times of *P. falciparum* culture starting with ring stage parasite.

ment in the human red cell for AAZ and SFA were determined to be 20 μM and 70 μM, respectively (Figure 6). This condition was also used for the control antimalarial drug artemisinin which had IC_{50} of 5 nM. The IC_{50} values for AAZ and SFA reported here are ~10-fold less than the values reported under different conditions for drug testing, i.e., 5% red cell suspension.[10]

Based on these results, which are consistent with the role of carbonic anhydrase in the malarial parasite[10] and the inhibition of *P. falciparum* enzyme by the sulfonamide-based drugs, it is appropriate to target this enzyme for the development of new antimalarial drugs.

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