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Carbonic anhydrase inhibitors. Inhibition of *Plasmodium falciparum* carbonic anhydrase with aromatic sulfonamides: towards antimalarials with a novel mechanism of action?

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Abstract—The malarial parasite *Plasmodium falciparum* encodes for an α-carbonic anhydrase (CA) enzyme possessing catalytic properties distinct of that of the human host, which was only recently purified. A series of aromatic sulfonamides, most of which were Schiff's bases derived from sulfanilamide/homosulfanilamide/4-aminoethylbenzenesulfonamide and substituted-aromatic aldehydes, or ureido-substituted such sulfonamides, were investigated for in vitro inhibition of the malarial parasite enzyme (pfCA) and the growth of *P. falciparum*. Several inhibitors with affinity in the micromolar range (K_I's in the range of 0.080–1.230 μM) were detected, whereas the most potent such derivatives were the clinically used sulfonamide CA inhibitor acetazolamide, and 4-(3,4-dichlorophenyl-ureidoethyl)-benzenesulfonamide, which showed an inhibition constant of 80 nM against pfCA, being four times more effective an inhibitor as compared to acetazolamide (K_I of 315 nM). The lipophilic 4-(3,4-dichlorophenylureido-ethyl)-benzenesulfonamide was also an effective in vitro inhibitor for the growth of *P. falciparum* (IC_{50} of 2 μM), whereas acetazolamide achieved the same level of inhibition at 20 μM. This is the first study proving that antimalarials possessing a novel mechanism of action can be obtained, by inhibiting a critical enzyme for the life cycle of the parasite. Indeed, by inhibiting pfCA, the synthesis of pyrimidines mediated by carbamoylphosphate synthase is impaired in *P. falciparum* but not in the human host. Sulfonamide CA inhibitors have the potential for the development of novel antimalarial drugs.

1. Introduction

Malaria is a disease caused by protozoan parasites belonging to the genus *Plasmodium*. The disease afflicts approximately 500 million people worldwide, causing 2.5 million people death annually, mainly children in African countries.[1] Four protozoan species infect humans, with *P. falciparum* being responsible for the majority of deaths.[1] The limitation and toxicity of antimalarial drugs currently used, and the spread of drug-resistant malarial parasites accompanied by a worldwide resurgence of malaria, requires the development of new drugs for the management of this disease.

In the intraerythrocytic stage of development, the malaria parasites require purines and pyrimidines for DNA and RNA synthesis during their exponential growth and replication.[2–4] The parasites, being purine auxotrophs, salvage the preformed purines from the human host, but must synthesize pyrimidines de novo from HCO_3^-, adenosine 5′-triphosphate, glutamine and aspartate.[2–4] The need of bicarbonate for these biosynthetic steps also involves the metallo-enzyme carbonic anhydrase (CA, EC 4.2.1.1), known to rapidly interconvert carbon dioxide and bicarbonate at neutral pH.[5–7]

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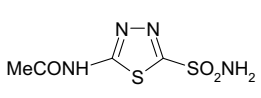
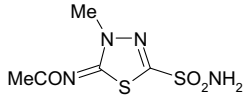
Indeed, the *P. falciparum* CA (abbreviated as pfCA) has only recently been isolated and characterized by Krungkrai co-workers,[8,9] who showed that this enzyme belongs to the α-class, similarly with the 14 isozymes presently known in higher vertebrates, including humans.[5–7,10] pfCA is involved in the first biosynthetic step leading to pyrimidines, that is, the synthesis of carbamoylphosphate from glutamine, in the presence of carbamoylphosphate synthase.[6c,8] It is well established that in this reaction bicarbonate and not carbon dioxide is the true substrate,[6c,8] and thus, the interconversion between these two species, catalyzed by pfCA, is critical for the entire metabolic pathway leading to pyrimidines. Details of pyrimidine/purine metabolism in the malaria parasites are extensively discussed elsewhere.[8,9] These pathways on both purine/pyrimidine biosynthesis represent key differences between the parasite and their human host, constituting an important feature for the possible targeting of pfCA for the design of novel antimalarials. Such compounds should possess a different mechanism of action as compared to the presently known drugs, most of which are rather toxic and lead to the emergence of drug resistance.[1–4]

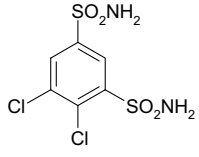
It is well known that α-CAs are strongly inhibited by aromatic/heterocyclic sulfonamides, which bind in deprotonated state to the Zn(II) ion within the enzyme active site.[5–7,10] Some compounds belonging to this class, such as acetazolamide **AZA**, methazolamide **MZA**, dichlorophenamide **DCP** or indisulam **IND** among others, are widely used pharmacological agents, mainly as diuretics, antiglaucoma, antiepileptics or anticancer agents (**IND** is in advanced clinical trials for the treatment of solid tumours).[5–7,10,11]



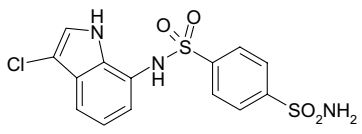
AZA



MZA



DCP



IND

It appeared thus of interest to try to target the α-CA from *P. falciparum* with different sulfonamide derivatives previously described,[5–7] in order to detect derivatives with good affinity for pfCA and possibly, selectivity towards this enzyme as compared to the human isozymes hCA I and hCA II, which are highly abundant in red blood cells.[12] A screening of different libraries of CA inhibitors previously reported by our group has been initiated in order to test the druggability of this new target, pfCA. Here we present a first study of pfCA inhibition with a series of aromatic sulfonamides, which led to the detection of an efficient in vitro inhibitor, highly promising for the development of conceptually novel compounds for the treatment of malaria.

2. Chemistry

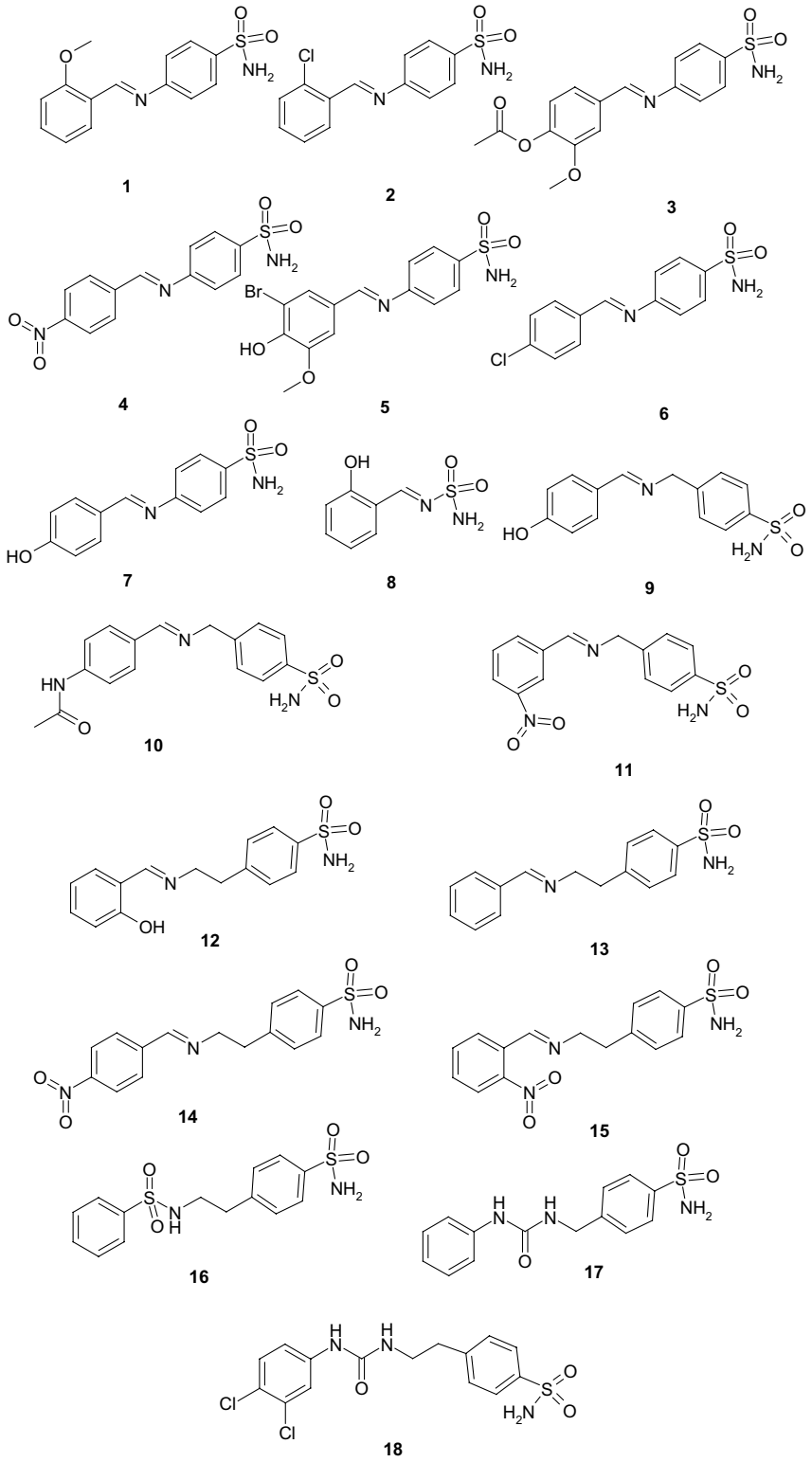
A series of sulfonamides, derivatives of sulfamide, sulfanilamide, homosulfanilamide and 4-aminoethylbenzenesulfonamide, of types **1**–**18**, has been investigated for pfCA inhibition in vitro.

The sulfonamides investigated here were previously reported by our group, mainly in the search of isozyme-selective CA inhibitors (CAIs), or for the design of novel topically acting antiglaucoma agents or CAIs with potential applications as antitumour agents.[13–19] As may be seen from the structures of these derivatives, our main objective was to investigate aromatic sulfonamides possessing different spacers between the benzenesulfonamide moiety and the derivatized amino moiety (with one exception, compound **8**,[15] which is a sulfamide Schiff's base, possessing thus a completely different zinc-binding function, for which we recently shown by means of X-ray crystallography that additional stabilization of the E–I adduct is achieved due to the presence of the heteroatom).[20] Most of the investigated derivatives are Schiff's bases obtained from sulfanilamide, homosulfanilamide or 4-aminoethylbenzenesulfonamide and different substituted aromatic aldehydes (compounds **1**–**7** and **9**–**15**). In order to see whether the derivatization of the amino moiety of the starting aminosulfonamides is a critical factor for pfCA inhibition, we also included derivatives possessing diverse moieties than the azomethine one, of the sulfonamido (compound **16**) or ureido type (compounds **17** and **18**). It should be stressed that these compounds are easily synthesized in one step from inexpensive materials, a factor quite important for the potential development of novel antimalarials. Indeed, this terrible disease principally affects poor countries, and the main drug houses are not interested in the development of drugs for poors. Thus, a simple and inexpensive compound effective as antimalarial may have better chances to become a drug candidate and to be investigated clinically even in resource poor areas.

3. In vitro CA inhibition

Inhibition data against two human red blood cell isozymes, hCA I and hCA II, as well as pfCA with sulfonamides **1**–**18** as well as acetazolamide (**AZA**) as standard inhibitor, are shown in Table 1.

The following SAR can be observed from the data of Table 1: (i) a first groups of derivatives, such as **3**, **4**, **7**, **8**, **10** and **11**, behave as low potency or ineffective CAIs of the protozoan enzyme, with inhibition constants against pfCA in the range of 3.2–7.4 μM for **3**, **4**, **7** and **11**, or even higher than 10 μM in the case of **8** and **10**. With exception of **8**, which is a sulfamide Schiff's base, the other ineffective pfCA inhibitors from this subgroup are all Schiff's bases derived from sulfanilamide/homosulfanilamide and aromatic aldehydes pos-



sessing various substituents at the aromatic moiety. Thus, a first SAR conclusion is that the nature of the group(s) substituting the aromatic ring of the aldehyde from which the Schiff's base was obtained is an important parameter for the pfCA inhibitory activity of these derivatives. It should also be stressed that these compounds are much more potent inhibitors of the major human isozyme investigated here, that is, hCA II (K_I's in the range of 0.04–0.19 μM), whereas they behave as more ineffective hCA I inhibitors (K_I's in the range of 3–14 μM); (ii) another groups of derivatives, such as **1**, **2**, **5**, **6**, **9**, **13**, **15** and **16**, act as medium potency pfCA inhibitors, with inhibition constants in the range of 0.54–1.23 μM. Except for **16**, which is a sulfonylated aminosulfonamide, all other derivatives in this subgroup are again Schiff's bases derived from sulfanilamide/homosulfanilamide/4-aminoethylbenzenesulfonamide, whereas the nature of the aldehyde from which they

Table 1. Carbonic anhydrase inhibition data against human isozymes I and II and *P. falciparum* enzyme pfCA by the esterase method,[21] and antimalarial test on *P. falciparum* growth in vitro with sulfonamides **1**–**18** and acetazolamide **AZA** as standard

Inhibitor	K_I (μM)[a]			IC_{50} (μM)[b]
	pfCA	hCA I	hCA II	
1	0.670	42	0.17	>50
2	0.535	13	0.29	>50
3	4.100	3	0.10	>50
4	6.980	13	0.05	>50
5	1.230	12	0.04	>50
6	0.735	25	0.28	>50
7	7.470	14	0.19	>50
8	>10	13	0.04	>50
9	0.620	8	0.07	>50
10	>10	10	0.11	>50
11	3.260	14	0.18	>50
12	0.465	11	0.13	>50
13	0.560	12	0.14	>50
14	0.500	10	0.02	>50
15	0.770	1	0.25	>50
16	0.824	0.69	0.28	>50
17	0.335	8	0.105	>50
18	0.080	0.12	0.13	2[c]
AZA	0.315	0.2	0.07	20[c]

[a] Inhibition constants against recombinant CA isozymes. Errors in the range of ±5–10% of the reported values, from three different assays.
[b] Molarity of inhibitor producing 50% inhibition of growth of parasitaemia in vitro.
[c] Mean from three different tests.

were obtained is the same as for compounds described above. Thus, the first SAR conclusion mentioned above is reinforced, being also possible to hypothesize that increasing the length of the parent sulfonamide (i.e., from sulfanilamide to 4-aminoethylbenzenesulfonamide) from which the Schiff's base was obtained, seem also to be beneficial for enhancing affinity for the protozoan enzyme, a situation generally also true for the other two α-CAs investigated here, that is, hCA I and hCA II. Moieties substituting the aldehyde part of the molecule leading to enhanced pfCA inhibitory properties were 2-methoxyphenyl-; 2- or 4-chlorophenyl-; 2- or 4-hydroxyphenyl- and 3-methoxy-4-hydroxy-5-bromophenyl among others (it should also be mentioned that the unsubstituted, benzaldehyde derived Schiff's base **13** also showed a good inhibitory activity). Also comparing derivatives **13** and **16**, which are quite similar except for the chemical functionality by which the tail is attached to the sulfonamide part (i.e., Schiff's base for **13** and secondary sulfonamide for **16**), it is clear that the first one was better for the pfCA inhibitory properties as compared to the second one.[5–7] The inhibition profile of these derivatives against the human isozymes hCA I and hCA II is rather similar on the other hand with that of the derivatives discussed above, being more efficient hCA II inhibitors as compared to their inhibition of the protozoan enzyme, and less effective hCA I inhibitors; (iii) several derivatives, such as **12**, **14**, **17**, **18** and the clinically used drug acetazolamide, **AZA**, showed much more effective pfCA inhibitory properties, with inhibition constants in the range of 80 nM to 0.50 μM. Two of these derivatives are 4-aminoethylbenzenesulfonamide derived Schiff's bases, two are ureido derivatives of homosulfanilamide/4-aminoethylbenzenesulfonamide, whereas acetazolamide is the only heterocyclic sulfonamide investigated here. It is interesting to note that the most effective pfCA inhibitor detected up to now is the urea **18**, which is almost four times more effective than the clinically used drug acetazolamide, being at the same time a rather efficient hCA I and hCA II inhibitor, with K_I's in the range of 120–130 nM. Acetazolamide on the other hand is a stronger hCA II inhibitor (K_I of 70 nM) and a less effective hCA I inhibitor (K_I of 200 nM) as compared to **18**. Thus, from this small library of investigated derivatives it is apparent that Schiff's bases and urea-based aromatic sulfonamides, mainly derived from homosulfanilamide/4-aminoethylbenzenesulfonamide, lead to potent pfCA inhibitors, and they also may appreciably inhibit the vertebrate red blood cell isozymes CA I and II. The nature of the groups substituting the aromatic-ureido- or aromatic-azomethine fragment of the molecule is also a critical parameter for the CA inhibitory activities of these aromatic sulfonamide derivatives, both against the protozoan as well as vertebrate enzymes.

4. In vitro antimalarial test

Cultivation of the malarial parasite in the presence of varying concentrations of sulfonamide CA inhibitor afforded us to determine the IC_{50} of these new sulfonamides (molarity of inhibitor producing a 50% decrease of the malarial parasite growth, see Experimental procedures for detail). All the investigated compounds were ineffective except for acetazolamide **AZA** and the ureido-sulfonamide derivative **18** (which were the most effective in vitro pfCA inhibitors—Table 1). Thus, acetazolamide has a medium potency efficiency for the inhibition of growth of *P. falciparum* in vitro, with an IC_{50} of 20 μM. The urea derivative **18** on the other hand was 10 times as effective an inhibitor, with an IC_{50} of 2 μM. The enhanced efficacy of this compound as compared to acetazolamide may also be explained by the fact that **18** is a much more liposoluble derivative as compared to acetazolamide, and thus its penetration through membranes is facilitated. On the contrary, acetazolamide is a very polar molecule, which has some difficulty in crossing biological membranes, and this may explain the 10 times lower activity (although the difference in inhibition constants between the two derivatives is only 4-fold). This is indeed a really encouraging data for the potential use of sulfonamide CA inhibitors for the treatment of malaria. Compounds with potent CA inhibitory properties should be further investigated. In addition, such compounds must possess a balanced lipo/hydrosolubility in order to achieve a good bioavailability. Work is in progress in our laboratories for detecting even more potent pfCA inhibitors.

5. Conclusion

A series of aromatic sulfonamides, most of which were Schiff's bases derived from sulfanilamide/homosulfanilamide/4-aminoethylbenzenesulfonamide and substituted-

aromatic aldehydes, or ureido-substituted such sulfonamides, were investigated for in vitro inhibition of the α-CA isozyme and the growth of the malarial parasite *P. falciparum*. Several micromolar inhibitors (K_I's in the range of 0.080–1.230 μM) were detected, but the most potent such derivatives were the clinically used sulfonamide CA inhibitor acetazolamide, and a previously reported compound, 4-(3,4-dichlorophenylureido-ethyl)-benzenesulfonamide, which showed an inhibition constant of 80 nM, being four times more effective an inhibitor as compared to acetazolamide. The lipophilic 4-(3,4-dichlorophenylureido-ethyl)-benzenesulfonamide was also an effective in vitro inhibitor for the growth of *P. falciparum* (IC_{50} of 2 μM), whereas acetazolamide achieved the same level of inhibition at 20 μM. This is the first study proving that antimalarials possessing a novel mechanism of action can be obtained, by inhibiting a critical enzyme for the life cycle of the parasite, which has not been considered up to now as a target for drug design.

6. Experimental part

6.1. Chemistry

Sulfonamides **1**–**18** were prepared as previously reported by this group.[13–19] Acetazolamide used as standard, as well as other reagents or solvents were of the highest purity available, from Sigma–Aldrich (Milan, Italy).

6.2. Biochemistry

Restriction enzymes, and biochemicals 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.

6.3. Cultivation of *P. falciparum*

P. falciparum (T_9 isolate) was cultivated by a modification of the candle jar method of Trager and Jensen,[22] using a 5% haematocrit 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 parasitaemia (~1–2%), were changed with the medium twice daily until the cultures had ~30% parasitaemia 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 PRMI medium for 20 min at 37 °C. The host cell-free parasites were obtained after centrifugation at 8000*g* for 10 min and washed at least four 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.[8,9]

6.4. Purification of pfCA 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 linear gradient of phosphate buffer from pH 6.0 to pH 8.0 at a flow rate of 1 mL/min. The eluates were collected into 30 fractions, each 1.0-mL fraction was determined for CA activity and protein concentration. For the *P. falciparum* CA, major isozyme CA1 was purified to near homogeneity using two more sequential columns on Mono Q anion exchange and Superose 12 gel filtration FPLC as described previously.[8]

6.5. Enzyme assay and kinetics

The esterase activity of pfCA was measured by using the modified method of Armstrong et al.[23] The enzyme activity was determined by following the change in absorbance at 348 nm of *p*-nitrophenylacetate to 4-nitrophenoxide ion, an extinction coefficient of 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 **AZA** 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 nonlinear regression of an Elsevier Biosoft Enzfitter program. Inhibitor constants (K_I) were determined from Dixon's plots as described previously.[8,9]

6.6. 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*HI and *Hin*dIII restriction sites, respectively (shown in bold).

The PCR cycling parameters include denaturation at 95 °C (1 min), annealling 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, that is, 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.

6.7. 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 (8000*g*) after IPTG induction for 3 h at 37 °C, washed three times with ice-cold PBS and stored as cell paste by freezing at −80 °C until use.

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 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 **AZA** using the esterase assay as described above, and was identical in every respect to the authentic enzyme isolated from the parasite.

6.8. 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 a synchronized culture with starting parasitaemia of 0.5% as described.[24] 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 ± 1000 cpm. The control red cells without harbouring 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 vitro growth was quantified by measuring % parasitaemia in a 96-h culture in the presence of the drugs at various concentrations.[24] All compounds were tested in triplicate at each concentration used. The morphological changes of *P. falciparum* was also observed in the culture treated with 100 μM **AZA** in one intraerythrocytic cycle (~44–48 h) starting with synchronized ring stage.

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