The Alpha-Carbonic Anhydrase from the Malaria Parasite and its Inhibition

Jerapan Krungrai¹,² and Claudiu T. Supuran²

¹Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, 1873 Rama 4 Road, Pathumwan, Bangkok 10330, Thailand and ²Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino (Florence), Italy

Abstract: Plasmodium falciparum is the protozoan parasite responsible for the majority of life-threatening cases of human malaria, causing more than one million deaths a year. The global emergence of drug-resistant malarial parasites necessitates identification and characterization of novel drug targets. At present, α-carbonic anhydrase (CA) genes are identified in limited numbers of parasites in both protozoa and helminthes, however, the malarial genes are found in four species of Plasmodium. The CA gene of P. falciparum encodes an α-carbonic anhydrase enzyme possessing catalytic properties distinct of that of the human host CA I and II isozymes. P. falciparum native and recombinant enzymes have been prepared. A library of aromatic sulfonamides, most of which were Schiff’s bases derived from sulfanilamide/homosulfanilamide/4-aminoethyl-benzenesulfonamide and substituted-aromatic aldehydes, or ureido-substituted sulfonamides are very good inhibitors for P. falciparum enzyme with Ki values in the range of 80 nM-0.50 μM. The 4-(3,4-dichlorophenylureidoethyl)-benzenesulfonamide is the most effective antimalarial activity against growth of P. falciparum in vitro with an IC50 of 2 μM. The structure of the groups substituting the aromatic-ureido- or aromatic-azonethine fragment of the molecule and the length of the parent sulfonamide (i.e., from sulfanilamide to 4-aminoethylbenzenesulfonamide) from which the Schiff’s base obtained, are the critical parameters for the enzyme inhibitory activities of these aromatic sulfonamide derivatives, both against the malarial as well as human enzymes. This review provides further support that the CA may have essential roles in the parasite metabolism. Thus, the aromatic sulfonamide CA inhibitors may have potential for development of novel antimalarial drugs.

Key Words: Alpha-carbonic anhydrase, carbonic anhydrase inhibitor, aromatic sulfonamides, antimalarial agents, drug design, malaria parasite, protozoa, helminthes.

1. INTRODUCTION

Carbonic anhydrases (CAs, also known as carbonate anhydrases, EC 4.2.1.1) are Zn²⁺-metalloenzymes, which catalyze the reversible hydration of CO₂ in forming HCO₃⁻ [1].

\[
\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \quad \text{(Eq. 1)}
\]

The first CA was purified from bovine red cells in 1933 [2], followed by the identification of several isozymes ubiquitously distributed in mammals, plants, archaea and bacteria [3-5]. At present, the CAs in protozoa and helminthes parasites are found to be sparsely [6,7]. Recent advances regarding the crystal structure and biochemistry of CAs from various organisms reveal that they evolved independently and are divided into four classes [8-11]. These are the α-CAs (mainly in mammals, vertebrates, bacteria, algae, protozoa and cytoplasm of plant), the β-CAs (ubiquitously in bacteria, algae and plant chloroplast); the γ-CAs (predominantly in archaea and bacteria); and the δ-CAs (present in diatoms) [12,13]. In mammalian tissues, sixteen α-CAs isozymes have been identified where they function in diverse essential process [14-18].

The α, β and γ classes have no significant sequence identity and structural differences, their active sites function with a single Zn²⁺ atom essential for catalysis. The catalytic and inhibition mechanism of α-CAs is well established [1]. X-ray crystallographic data of the human CA H showed that Zn²⁺ is located at the bottom of a 15 A deep active site cleft, being coordinated by three histidine residues (His 94, His 96 and His 119) and a water molecule/hydroxide ion. The histidine cluster (His 4, His 10, His 15, His 17, His 64) is also critical importance in the catalytic cycle of the enzyme. The overall enzyme-catalyzed reaction of the CA is illustrated in Fig. (1). The active form of the enzyme is the basic one, with hydroxide bound to Zn²⁺ (Fig. 1A). This strong nucleophile attacks the CO₂ molecule bound in a hydrophobic pocket in its neighborhood (Fig. 1B), leading to formation of HCO₃⁻ coordinated to Zn²⁺ (Fig. 1C).

HCO₃⁻ is then displaced by a water molecule and liberated into solution, leading to the acid form of the enzyme, with water coordinated to Zn²⁺ (Fig. 1D), which is catalytically inactive. The basic form A is then regenerated by a proton transfer reaction from the active site to its environments, e.g., active site His 64.

The type of α-CA kinetic mechanism reveals a “zinc hydroxide” catalysis that also extends to the β- and γ-CA classes [10,19]. This can be summarized,

\[
\text{E-Zn}^{2+}\text{-OH} + \text{CO}_2 \rightleftharpoons \text{E-Zn}^{2+}\text{-HCO}_3^- \quad \text{(Eq. 2)}
\]

\[
\text{E-Zn}^{2+}\text{-HCO}_3^- + \text{H}_2\text{O} \rightleftharpoons \text{E-Zn}^{2+}\text{-H}_2\text{O} + \text{HCO}_3^- \quad \text{(Eq. 3)}
\]

\[
\text{E-Zn}^{2+}\text{-H}_2\text{O} \rightleftharpoons \text{H}^+\text{-E-Zn}^{2+}\text{-OH}^- \quad \text{(Eq. 4)}
\]

\[
\text{H}^+\text{-E-Zn}^{2+}\text{-OH}^- \rightleftharpoons \text{E-Zn}^{2+}\text{-OH}^- + \text{H}^+ \quad \text{(Eq. 5)}
\]

where E is an CA enzyme, and reaction 5 (Eq. 5) is the rate-limiting step in catalysis, that is, the proton transfer that regenerates the zinc hydroxide species of the enzyme.

The X-ray crystallographic structures of many adducts of the CAs with aliphatic, aromatic or heterocyclic sulfonamides have been reported [1,9,20,21], proving that the sulfonamide inhibitor is directly bound to Zn²⁺ of the enzyme through the sulfonamide moiety. The interactions between the bound inhibitor and the enzyme active site are critical for the affinity of this class of inhibitors to the different isozymes and obviously, for design of novel drugs from the sulfonamide-based structure. Thus, the CA inhibition and activation is novel therapeutic applications for treatment of many human diseases, for instance, Alzheimer, cancer, diabetics, glaucoma, obesity and osteoporosis [12-14,18,19, 21,22].

This review will describe the state of the art of parasite CAs information in both protozoa and helminthes, including nematodes. This will focus mainly on the protozoan parasite Plasmodium falciparum, which is responsible for the majority of life-threatening cases of human malaria. Putative metabolic roles of the parasite CAs will be proposed. Some aromatic sulfonamides behave as inhibitors of P. falciparum CA (PfCA). It is apparent that 4-(3,4-dichlorophenylureidoethyl)-benzenesulfonamide is the most effective inhibitor for the in vitro growth of P. falciparum. Our review provides further support the CAs have essential roles in the parasite...
metabolism. Thus, sulfonamide CA inhibitors may have the potential for development of novel antimalarial drugs.

2. PROTOZOA AND HELMINTHES PARASITE CARBONIC ANHYDRASES

Carbonic anhydrase has previously been identified in all organisms so far examined: animals, plants, yeast, archaea and bacteria [3-5,23,24]. The α-CA isozymes of mammals, in particular the human and bovine, have been thoroughly investigated [13,18,22,25,26]. Using the classification and taxonomy of the parasite by Cox [27], the subkingdom protozoa (unicellular eukaryotic organisms) is divided into seven phyla and the subkingdom helminthes is classified into three phyla: Platyhelminthes, Acanthocephala and Nematoda. We have used the bioinformatics approach [28,29] to identify the parasite genes encoding putative CA activity and the results are summarized in Table I, compared their amino acid sequences identities to the mosquito (Aedes aegypti) and human isozymes CA I-III, and VI.

Table I demonstrates the limited information of all parasite genomic databases for the putative genes for the CAs. Up to now, the CA genes are identified in only seven species of the protozoan and helminthes parasites. In protozoa, these are Plasmodium, Theileria, Trypanosoma, Leishmania and Euamoeba. In helminthes, they are Ascaris (parasitic nematode) and Coenorhabditis (free-living nematode). The predicted protein sequences of these parasites show low identity (<25%) to the mosquito and the human CAs I-III and VI isozymes [30].

3. MALARIAL PARASITE CARBONIC ANHYDRASES

Malaria is a disease caused by genus Plasmodium, classified in the phylum Apicomplexa of subkingdom protozoa. The disease afflicts 515 million and kill up to 2.5 million annually, mainly children in African countries [31-35]. Four species infecting humans, P. falciparum is responsible for the majority of deaths [31,35]. The limitation and toxicity of antimalarial drugs in currently use, and the spread of drug-resistant malarial parasites accompanied by a worldwide resurgence of malaria requires the development of new drugs for management of the disease [34,36-39]. During intraerythrocytic stage of development, the malarial parasites require purines and pyrimidines for DNA/RNA synthesis and other metabolisms during this exponential growth and replication. The parasites, known as purine auxotroph, salvage the preformed purine base/nucleoside (e.g., hypoxanthine, adenosine) from the mammalian host, but they have to synthesize pyrimidine de novo from HCO$_3^-$, adenine 5'-triphosphate (ATP), glutamine (Gln), aspartate (Asp) and 5-phosphoribosyl-1-pyrophosphate (PRPP) [40-52]. The unique properties on both purine and pyrimidine requirement of the parasite are key different from the human host, that is, the parasite and pyrimidine synthesis are operated by both de novo and salvage pathways [43,52-54].

In 1998, Sein and Aikawa [6] demonstrated the in situ carbonic anhydrase activity for the first time in the P. falciparum-infected red cells by using electron microscopy and carbonic anhydrase-specific Hanssen’s stain. Recently, we have demonstrated the existence of CA activity in the human malarial parasite P. falciparum [7] and in the rodent malarial parasite P. berghei [55]. Details will be briefly described below.

3.1. The Presence of α-Carbonic Anhydrase Isozymes in P. falciparum and P. berghei

P. falciparum-infected red cells contain CA activity about 2 times higher than those of normal and uninfected red cells. The three developmental forms of the asexual stages, ring (young), trophozoite (growing) and schizont (mature) show stage-dependent activity, i.e., the specific activity of the CA enzyme is increased with the stages of parasite development in the human host red cell (Table 2).

There are at least three CA isozymes activity in P. falciparum [7]. A major α-CA isozyme, completely inhibited by an inhibitor of α-CA (CA1, acetazolamide, AZA), of P. falciparum CA1 (PICA1) has been purified and extensively characterized.

In P. berghei, there is a 5-fold increase in total activity of the CA enzyme in the infected red cells, compared to the uninfected and normal red cells. At least four isozymes are demonstrated in P.
bergheri [55]. The α isozyme PbCA2 and PbCA3 are major forms. All four P. bergheri CA activities were completely inhibited by AZA. The purified and native PbCA1 enzyme has a Kᵢ value of AZA higher than those of the human CA II and bacterial ones. Whereas, the yeast CA, the plant CA and the mammalian CA III are peculiarly insensitive to AZA [56-63].

3.2. P. falciparum α-Carbonic Anhydrase Gene and Its Recombinant Enzyme

In 2002, the nucleotide sequencing project of the human parasite P. falciparum and the rodent parasite P. yoelii genomes, having 14 chromosomes with approximately 23 Mb, were completed [39] and deposited in the Plasmodium genome database [64]. It is now possible to identify the sequences that encode CA isozymes in the malarial parasites. By using the bioinformatics approach, TBLASTN searching of the malarial genome database has been performed with the protein CA sequences obtained from other organisms. The search of the malarial genome database yields the possible to identify the sequences that encode CA isozymes in the malarial parasites. By using the bioinformatics approach, TBLASTN searching of the malarial genome database has been performed with the protein CA sequences obtained from other organisms. The search of the malarial genome database yields the open reading frame similar to the α-CAs from various organisms, including human. At least four Plasmodium species have putative α-CA genes. These include P. falciparum, P. chabaudi, P. yoelii and P. bergheri (Fig. 2).

The primary amino acid sequence of the P. falciparum gene has 47%, 42% and 40% identity with P. berghei, P. chabaudi and P. yoelii, respectively. High identity (>80%) are observed among the three rodent malarial parasites, P. chabaudi, P. yoelii and P. bergheri. Low identity (<25%) of the malarial sequences are found among the four Plasmodium species and the rodent parasite P. berghei. These include P. falciparum, P. chabaudi, P. yoelii and P. bergheri (Table 1).

Table 1. Putative α-Carbonic Anhydrases Identified in Some Protozoa and Helminthes Parasites (1-10), Mosquito (11) and Human (12-15). The Numbers are Percentage of Amino Acids Identity between Pairs of Organisms

| Organism a | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 |
|------------|------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| P. falciparum | 100 42 40 47 7 3 7 20 2 8 12 8 3 8 10 |
| P. chabaudi | 42 100 65 84 13 4 8 17 6 5 12 10 9 6 7 |
| P. yoelii | 40 65 100 85 10 10 6 26 8 8 10 19 17 16 13 |
| P. bergheri | 47 84 85 100 9 5 8 24 5 2 1 7 5 6 12 |
| Ascaris | 7 13 10 9 100 15 6 24 17 4 32 27 24 31 24 |
| Caenorhabditis | 3 4 10 5 15 100 9 10 10 5 18 23 25 25 19 |
| Entamoeba | 7 8 6 8 6 9 100 6 4 20 12 3 2 3 5 |
| Theileria | 20 17 26 24 24 10 6 100 11 7 18 14 17 19 18 |
| Trypanosoma | 2 6 8 5 17 10 4 11 100 2 21 18 20 15 18 |
| Leishmania | 8 5 8 2 4 2 5 20 7 2 100 5 4 2 1 6 |
| Mosquito | 12 12 16 10 32 18 12 18 21 5 100 29 28 28 22 |
| Human CAI | 8 10 19 7 27 23 3 14 18 4 29 100 60 53 31 |
| Human CAII | 3 9 17 5 24 25 2 17 20 2 28 60 100 58 33 |
| Human CAVI | 8 6 16 6 31 25 3 19 15 1 28 53 58 100 32 |
| Human CAVII | 10 7 13 12 24 19 5 18 16 6 22 31 33 32 100 |

*Name of Organisms Accession Numbers Number of Amino Acids

1 = P. falciparum AAN35993 418
2 = P. chabaudi CAH77957 486
3 = P. yoelii XP_726574 728
4 = P. bergheri XP_676575 363
5 = Ascaris suum BU606588 202
6 = Caenorhabditis elegans AABS3034 319
7 = Entamoeba histolytica EAL51300 188
8 = Theileria annulata CAH77957 486
9 = Trypanosoma brucei XP_828900 422
10 = Leishmania major CAJ02106 236
11 = Aedes aegypti AAL72625 298
12 = H. sapiens (CAI) P00915 261
13 = H. sapiens (CAII) P00918 260
14 = H. sapiens (CAIII) P07451 260
15 = H. sapiens (CAVII) P23280 308

Unit of enzyme activity is expressed as nmol per min at 37°C.
Values are mean ± S.D., taken from eight to ten of sample preparations.
Red cells are infected with more than 90% parasitaemia of mixed stages of ring (46 ± 10%), trophozoite (44 ± 6 %) and schizont (10 ± 3 %).
Fig. (2). Multiple alignments of the amino acid sequences of *P. falciparum*, *P. chabaudi*, *P. berghei* and *P. yoelii* CAs, deduced from the continuously single open reading frame of the parasite genes. The identical amino acids and conservative replacements are shown by star and dot symbols, respectively.
expressed in *Escherichia coli* by using pET-15b vector [55]. The recombinant PfCA1 protein shows authenticity to the native enzyme purified from *in vitro* *P. falciparum* culture [7]. The kinetic parameters including $K_m$, $k_{cat}$, $K_i$ of the inhibitor AZA are also found to be similar between the native and recombinant enzymes. The recombinant protein obtained is used for drug-screening test for a mechanism-based drug design, especially sulfonamide CA inhibitors.

4. *P. falciparum* α-CARBONIC ANHYDRASE INHIBITION

The pyrimidine biosynthetic pathway represents a key difference between the parasite and its human host as discussed earlier, this constitutes an important feature for the possible targeting of PfCA for the design of novel antimalarials. Since the PfCA catalyzes the formation of $HCO_3^-$ as a substrate for the first enzyme of the pyrimidine pathway, carbamoylphosphate synthase II (see details in section 6). 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 [34,36-38].

It is established that α-CAs are strongly inhibited by aromatic/heterocyclic sulfonamides, which bind in deprotonated state to $Zn^{2+}$ within the enzyme active site [1,5,9,21,65-67]. Some compounds belonging to this class, such as AZA, methazolamide, dichlorophenamide or indisulam among others, are widely used pharmacological agents, mainly as diuretics, antiglaucoma, antiepileptics or anticancer agents [1,9,12-14,18]. Indisulam is in advanced clinical trials for the treatment of solid tumors [1,65].

A series of sulfonamides, derivatives of sulfamide, sulfanilamide, homosulfanilamide and 4-aminoethylbenzenesulfonamide, of compounds 1-18, has been investigated for PfCA inhibition *in vitro*. Sulfonamides 1-18 have been prepared as previously reported [68-74]. Thus, details of the sulfonamide synthesis are not described here. The structures of sulfonamides 1-18 are shown in Fig. (3).

![Fig. (3). Structures of aromatic/heterocyclic sulfonamide carbonic anhydrase inhibitors 1-18.](image-url)
The library of sulfonamides have been 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 antitumor agents [68-74]. As may be seen from the structures of these derivatives, we have investigated aromatic sulfonamides possessing different spacers between the benzenesulfonamide moiety and the derivatized amino moiety. There is one exception, compound 8 [69] which is a sulfamide Schiff’s base, possessing a completely different zinc-binding function, for which we have 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 [75]. 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 PICA inhibition, we have also compared derivatives possessing diverse moieties than the azomethine one, of the sulfonamido (compound 16) or ureido type (compounds 17, 18).

Inhibition data against two human red blood cell isozymes, human CA I and human CA II, as well as PICA with sulfonamides 1-18 as well as AZA as standard inhibitor, are summarized in Table 3 [76].

Table 3. α-Carboxy Anhydrase Inhibition Data Against Human Isozymes I and II and P. falciparum Enzyme PICA with Sulfonamides 1-18 and Acetzolamide AZA

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Kᵢ (μM)</th>
<th>HumanCA I</th>
<th>HumanCA II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.670</td>
<td>42</td>
<td>0.17</td>
</tr>
<tr>
<td>2</td>
<td>0.535</td>
<td>13</td>
<td>0.29</td>
</tr>
<tr>
<td>3</td>
<td>4.100</td>
<td>3</td>
<td>0.10</td>
</tr>
<tr>
<td>4</td>
<td>6.980</td>
<td>13</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>1.230</td>
<td>12</td>
<td>0.04</td>
</tr>
<tr>
<td>6</td>
<td>0.735</td>
<td>25</td>
<td>0.28</td>
</tr>
<tr>
<td>7</td>
<td>7.470</td>
<td>14</td>
<td>0.19</td>
</tr>
<tr>
<td>8</td>
<td>&gt;10</td>
<td>13</td>
<td>0.04</td>
</tr>
<tr>
<td>9</td>
<td>0.620</td>
<td>8</td>
<td>0.07</td>
</tr>
<tr>
<td>10</td>
<td>&gt;10</td>
<td>10</td>
<td>0.11</td>
</tr>
<tr>
<td>11</td>
<td>3.260</td>
<td>14</td>
<td>0.18</td>
</tr>
<tr>
<td>12</td>
<td>0.465</td>
<td>11</td>
<td>0.13</td>
</tr>
<tr>
<td>13</td>
<td>0.560</td>
<td>12</td>
<td>0.14</td>
</tr>
<tr>
<td>14</td>
<td>0.500</td>
<td>10</td>
<td>0.02</td>
</tr>
<tr>
<td>15</td>
<td>0.770</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>16</td>
<td>0.824</td>
<td>0.69</td>
<td>0.28</td>
</tr>
<tr>
<td>17</td>
<td>0.335</td>
<td>8</td>
<td>0.105</td>
</tr>
<tr>
<td>18</td>
<td>0.080</td>
<td>0.12</td>
<td>0.13</td>
</tr>
<tr>
<td>AZA</td>
<td>0.315</td>
<td>0.2</td>
<td>0.07</td>
</tr>
</tbody>
</table>

The following SAR can be summarized from the data of Table 3:

1). The first group of derivatives, such as compounds 3, 4, 7, 8, 10 and 11, behave as low potency or ineffective CA inhibitors of the malarial enzyme, with inhibition constants (Ki) against PICA in the range of 3.2 – 7.4 μM for compounds 3, 4, 7 and 11, or even higher than 10 μM in the case of compounds 8 and 10. With exception of compound 8, which is a sulfamide Schiff’s base, the other ineffective PICA inhibitors from this subgroup are all Schiff’s bases derived from sulfanilamide/ homosulfanilamide and aromatic aldehydes possessing 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 PICA inhibitory activity of these derivatives. It should also be stressed that these compounds are much more potent inhibitors of the major human isozymes, i.e., human CA II (Ki-s in the range of 0.04-0.19 μM), whereas they behave as more ineffective human CA I inhibitors (Ki-s in the range of 3-14 μM).

2). The second group of derivatives, such as compounds 1, 2, 5, 6, 9, 13, 15 and 16, act as medium potency PICA inhibitors, with Ki-s in the range of 0.54 – 1.23 μM. Except for compound 16, which is a sulfonlated amino-sulfonamide, all other derivatives in this subgroup are the Schiff’s bases derived from sulfanilamide/ homosulfanilamide/4-aminoethylbenzenesulfonamide, whereas the nature of the aldehyde from which they 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 malarial enzyme, a situation generally also true for the other two α-CAIs, i.e., human CA I and human CA II. Moieties substituting the aldehyde part of the molecule leading to enhanced PICA inhibitory properties are 2-methoxyphenyl-; 2- or 4-chlorophenyl-; 2- or 4-hydroxypyphenyl- and 3-methoxy-4-hydroxy-5-bromophenyl among others. It should also be mentioned that the unsubstituted, benzaldehyde derived Schiff’s base 13 shows 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 compound 13 and secondary sulfonamide for compound 16), it is clear that the first one is better for the PICA inhibitory properties as compared to the second one [1,5,9,21]. The inhibition profile of these derivatives against the human isozymes CA I and CA II is rather similar on the other hand with that of the derivatives discussed above, being more efficient human CA II inhibitors as compared to their inhibition of the malarial enzyme, and less effective human CA I inhibitors.

3). The third group of several derivatives, such as compounds 12, 14, 17, 18 and the clinically used drug AZA, show much more effective PICA inhibitory properties, with Ki-s in the range of 80 nM–0.50 μM. Two of these derivatives are 4-aminoethylbenzenesulfonamide derived Schiff’s bases, two are ureido derivatives of homosulfanilamide/4-aminoethylbenzenesulfonamide, whereas AZA is the only heterocyclic sulfonamide. It is interesting to note that the most effective PICA inhibitor is the urea 18, which is almost 4 times more effective than the clinically used drug AZA, being at the same time a rather efficient human CA I and human CA II inhibitor, with Ki-s in the range of 120-130 nM. AZA on the other hand is a stronger human CA II inhibitor (Ki = 70 nM) and a less effective human CA I inhibitor (Ki = 200 nM) as compared to compound 18. Thus, from the small library of derivatives it is apparent that Schiff’s bases and urea-based aromatic sulfonamides, mainly derived from homosulfanilamide/4-aminoethylbenzenesulfonamide, lead to potent PICA inhibitors, and they also may appreciably inhibit the human 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 a critical parameter for the CA inhibitory activities of these aromatic sulfonamide derivatives, both against the malaria as well as human enzymes.
5. *P. falciparum* α-CARBONIC ANHYDRASE INHIBITORS
DISPLAY ANTIMALARIAL EFFECT

By using [3H]-hypoxanthine incorporation for monitoring growth of *P. falciparum* in *in vitro* culture [42,77], which are started with mixed stages at 0.5% red cell suspension, the 50% inhibitory concentration (IC$_{50}$) in the mixed stages of malarial parasite development in the human red cell for AZA is 20 μM. The effect of AZA has more pronounced in the ring/trophozoite forms than the schizont stage of *P. falciparum*. This is consistent with the stage-dependent activity of the enzyme that more maturing parasites contain higher activity. Pretreatment of the human red cell with AZA, which is totally abolished the host enzyme activity, show no pronounced effect on the parasite invasion. It is suggested that the CAI may directly affect the parasite carbonic anhydrase and lead to eventually death of the parasite in the host red cell.

All compounds of the above three groups of sulfonamides 1-18 are ineffective on the *P. falciparum* growth (IC$_{50}$ > 50 μM) except for the ureidosulfonamide derivative 18, which is the most effective *in vitro* PICA inhibitors. AZA has a medium potency efficiency for the inhibition of growth of *P. falciparum in vitro*. The compound 18 on the other hand is ten times as effective an inhibitor, with an IC$_{50}$ of 2 μM. The enhanced efficacy of this compound as compared to AZA may also be explained by the fact that compound 18 is a much more liposoluble derivative as compared to AZA, and thus its penetration through membranes is facilitated. On the contrary, AZA 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. The antimalarial effect of both compounds have been examined based on appearance of morphology during two parasite’s life cycles in intraerythrocytic stages, that is, they develop in human red cells as asexual stages (Fig. 4). Significant inhibition of the parasite growth, as shown by decreasing % parasitemia, is observed in the culture where the compound 18 is presented at 5 μM.

![Fig. (4). Antimalarial activity of *P. falciparum* carbonic anhydrase inhibitors, acetazolamide (AZA) and 4-(3,4-dichlorophenylureido-ethyl)-benzenesulfonamide, compound 18. *P. falciparum* culture was started with 0.5% parasitemia at 2.5% red cell suspension and monitored every 24 h for 96 h at 37 °C. *P. falciparum* was grown in the absence of inhibitor as control (●), 20 μM AZA (●●) or 5 μM (●●●) compound 18 was presented during the 96-h growth.](image)

The recent information on the CA inhibitor affecting the *P. falciparum* growth indicates the potential use of sulfonamide CA inhibitors for the treatment of malaria. This also provides that antimarial drugs 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. PUTATIVE METABOLIC ROLES OF MALARIAL CARBONIC ANHYDRASE

Human red cells infected with *P. falciparum* have more carbonic anhydrase enzymatic activity than do the uninfected red cells. The activity increases with the parasite maturation from ring to trophozoite and to schizont developmental stages in human red cells [7]. These activities increase parallel with other metabolic activities as the parasite develops and the biomass increases. *P. falciparum* CA is involved in the first biosynthetic step leading to pyrimidines, i.e., the synthesis of carbamoylphosphate from glutamine, in the presence of carbamoylphosphate synthase II (Fig. 5). It is well established, that in this reaction bicarbonate and not carbon dioxide is the true substrate, and thus, the interconversion between these two species, catalyzed by the malarial CA, is critical for the entire metabolic pathway leading to pyrimidine biosynthesis and they are responsible for DNA/RNA and lipid synthesis. The lines of evidence have suggested that the putative function of CA in providing HCO$_3^-$ for carbamoylphosphate synthetase II in the pyrimidine biosynthesis, phosphoenol-pyruvate carboxykinase, phosphoenolpyruvate carboxylase, acetyl CoA carboxylase for fatty acid synthesis [18,23,24,78,79]. In addition, the enzyme may play an additional role in HCO$_3^-$ transport across cell membrane [15,23] and maintaining intracellular pH in the malarial parasite [53,54,80]. Intracellular pH regulation is necessary for the balance of electrolytes and transportation of Na$^+$, K$^+$ and H$^+$ in the malarial parasite during its development in the host red cells. In addition, *P. falciparum* has been shown to be capable of CO$_2$-fixing activity [81]. It may be possible that the enzyme in the malarial parasite may be responsible for this function, at least it plays a role in CO$_2$/HCO$_3^-$ equilibration of all involved metabolic reactions.

7. CONCLUSION AND FUTURE PROSPECTS

Almost parasites in both protozoa and helminthes should have their own carbonic anhydrase activity, as demonstrated in all species of mammals, plants, archaea and bacteria so far examined [12-
14,18]. In malarial parasites, there are at least four species known to contain putative genes encoding the \( \alpha \)-CA class. One of \( P. falciparum \) genes has been cloned and functionally expressed in \( E. coli \). The recombinant enzyme is catalytically active and has the authentic properties to the wild type enzyme purified directly from \( P. falciparum \). A series of aromatic sulfonamides show good inhibitors on the malarial enzyme, whereas the most potent such derivatives are the clinically used sulfonamide CA inhibitor acetazolamide, and the lipophilic \( 4-\{(3,4\text{-diclorophenylureidoethyl)}\text{-benzenesulfon-

amide show effective antimalarial property for the growth of \( P. falciparum \). 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 malarial CA, the synthesis of pyrimidines mediated by carbamoylphosphate synthetase II is impaired in \( P. falciparum \) but not in the host, since the human is able to obtain pyrimidines by the salvage pathway. Sulfonamide CA inhibitors may have the potential for the development of novel antimalarial drugs. Compounds with potent CA inhibitory properties should be further investigated. In addition, such compounds must possess a balanced lipohydrophilicity in order to achieve a good bioavailability. Work is still in progress in our laboratories for detecting even more potent malarial CA inhibitors and their respective antimalarial activities on both \( \textit{in vitro} \) and \( \textit{in vivo} \) studies. Furthermore, the evolutionary relationship of the parasite enzymes to other organisms is still unclear. Finally, the functional roles of the enzyme in parasite metabolism need to be further investigated. Our ultimate goal is the elucidation of 3-D structure of the parasite enzyme for rational drug design leading further insights into its differences from the equivalent enzyme in human.

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REFERENCES

The Alpha-Carbonic Anhydrase from the Malaria Parasite


