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Carbonic anhydrase inhibitors: Inhibition of *Plasmodium falciparum* carbonic anhydrase with aromatic/heterocyclic sulfonamides—in vitro and in vivo studies

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ABSTRACT

A library of aromatic/heterocyclic sulfonamides possessing a large diversity of scaffolds has been assayed for inhibition of the carbonic anhydrase (CA, EC 4.2.1.1) from the malaria parasite *Plasmodium falciparum* (pfCA). Low micromolar and submicromolar in vitro inhibitors were detected, whereas several compounds showed ex vivo anti-*P. falciparum* activity, in cell cultures. One derivative, that is, 4-(3,4-dichlorophenylureido)thioureido-benzenesulfonamide was an effective in vitro pfCA inhibitor (K_I of 0.18 μM), inhibited the ex vivo growth of *P. falciparum* with an IC_{50} of 1 μM, and was also effective as an antimalarial agent in mice infected with *P. berghei*, an animal model of human malaria infection, with an ID_{50} of 10 mg/kg (chloroquine as standard showed an ID_{50} of 5 mg/kg). By inhibiting the first step of pyrimidine nucleotide biosyntheses, that is, the CA-mediated carbamoylphosphate biosynthesis, sulfonamide inhibitors of the protozoan CAs may have potential for the development of novel therapies of human malaria.

Malaria, a major parasitic disease of humans, is caused by protozoa of the genus *Plasmodium*, classified in the phylum Apicomplexa.[1] The disease afflicts 515 million and kills 1.5–2.7 million people each year, most of whom children in sub-Saharan Africa.[2–6] *P. falciparum* is responsible for most of these deaths.[2,6] In addition to the lack of effective vector control and vaccines, the limitations and toxicity of antimalarial drugs in current use, and the spread of drug-resistant malaria accompanied by a worldwide resurgence of the disease, highlights the need to develop quickly more effective and less toxic novel antimalarial drugs, possessing a different mechanism of action.[5,7–10] Drug screening procedures have rarely been applied to identify lead molecules for this disease, and there is a paucity of information on a number of metabolic pathways that can be exploited for malaria chemotherapy.[7–9] A better understanding of biochemical differences between the parasite and human metabolic processes may provide new targets for intervention in the fight against this disease.[10]

In 1998, Sein and Aikawa[11] proved the in situ carbonic anhydrase (CA, EC 4.2.1.1) activity in *P. falciparum*-infected red blood cells by using electron microscopy and CA-specific Hanssen's stain. Recently, we have demonstrated the existence of CA enzymatic activities in *P. falciparum* and in the related mouse parasite *P. berghei*, and the fact that this enzyme activity may be inhibited by sulfonamide CA inhibitors (CAIs).[12–14] Indeed, the metalloenzyme CA catalyzes the interconversion between carbon dioxide and bicarbonate, being essential in many physiologic processes both in eukaryotes and prokaryotes, with five distinct genetically unrelated gene families (α-, β-, γ-, δ-, and ζ-CA) encoding such enzymes all over the phylogenetic tree.[15–18] Malaria CAs belong to the α-class enzymes,[12–14] similarly to the human enzymes, of which 15 isoforms are presently known.[15] The *P. falciparum* CA has been designated as pfCA.[13,14]

The parasite is a purine auxotroph, that is, it is incapable of de novo purine biosynthesis owing to the missing enzymes in the biosynthetic pathway. It salvages the preformed purine bases/nucleosides (e.g., hypoxanthine, adenosine) from the human host and converts them to their mono-, di-, and triphosphates. The parasite can on the other hand synthesize pyrimidines de novo from HCO_3^-, adenosine 5′-triphosphate (ATP), glutamine (Gln), aspartate (Asp), and 5-phosphoribosyl-1-pyrophosphate (PRPP), as shown in Figure 1.[12–14] These unique properties on both purine and pyrimidine requirement of the parasite are key differences from the human host, in which both functional de novo

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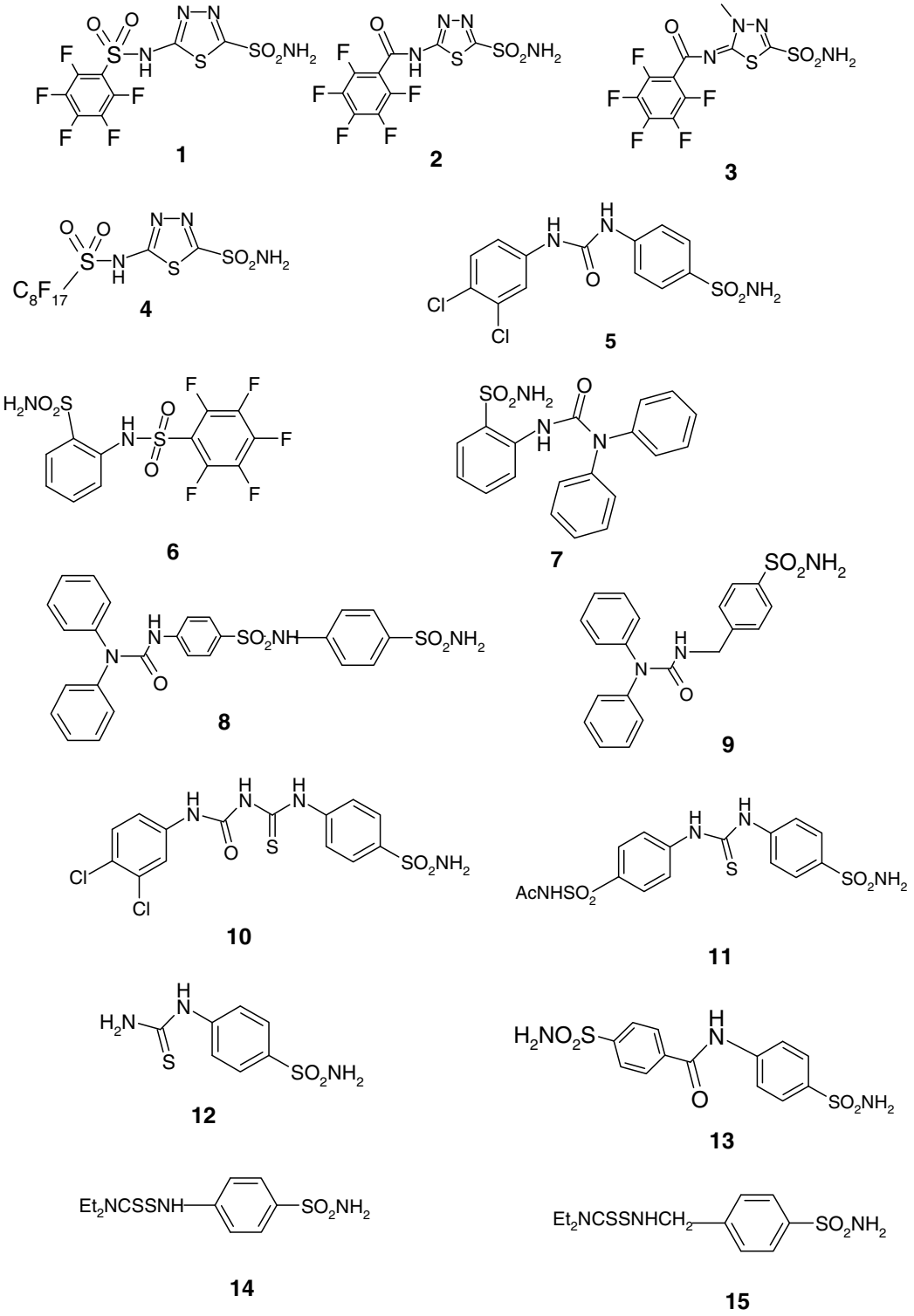
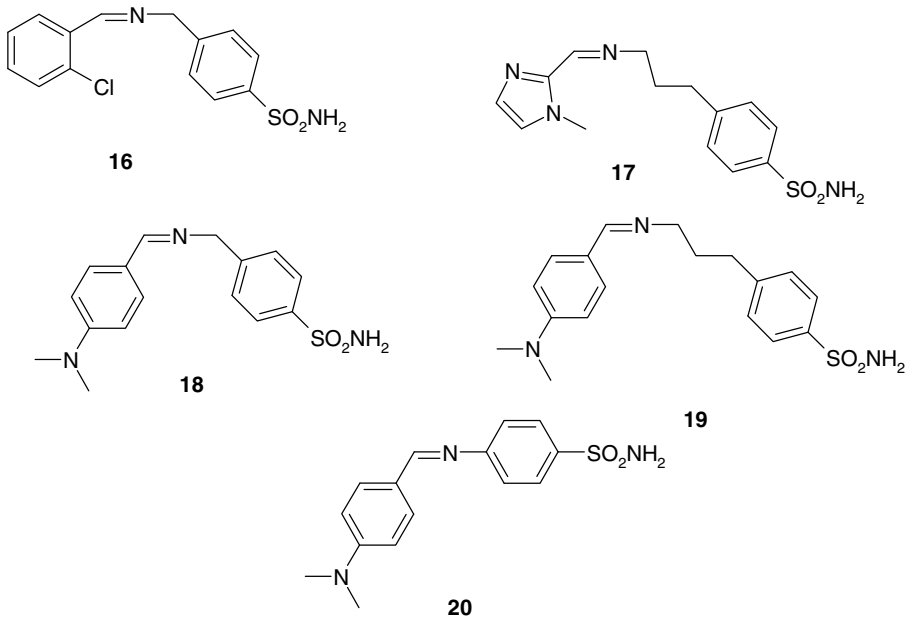


and salvage pathways of the purine and pyrimidine synthesis exist.[19] The enzymes involved in these sequential steps in the pathway leading to DNA/RNA synthesis in *Plasmodium* are CA; CPS II, carbamoylphosphate synthetase II; ATC, aspartate transcarbamoylase, DHO, dihydroorotase; DHOD, dihydroorotate dehydrogenase; OPRT, orotate phosphoribosyltransferase; OMPDC, orotidine 5′-monophosphate decarboxylase; NMPK, nucleoside monophosphate kinase; RNR; ribonucleotide reductase; TS, thymidylate synthetase; CTPS, cytidine 5′-triphosphate synthetase.[14,19] The mitochondrial electron transport system (ETS) is also linked to the enzyme DHOD of the pyrimidine pathway, functioning as electron disposal (Fig. 1).[14,19]

Since the pyrimidine biosynthetic pathway shown above represents a key difference between the parasite and its human host, it constitutes an important feature for the possible targeting of pfCA for the design of novel antimalarials. Indeed, pfCA catalyzes the formation of HCO_3^- as a substrate for the first enzyme in the pyrimidine biosynthetic pathway, carbamoylphosphate synthetase II (CPS II). Thus, inhibitors of pfCA may affect the entire biosynthetic pathway of Figure 1, leading to antimalarials which 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.[5,7–9] Indeed, in a previous work[13] we investigated a small number of aromatic sulfonamides, most of which were Schiff's bases derived from sulfanilamide/homosulfanilamide/4-aminoethylbenzene-sulfonamide and substituted-aromatic aldehydes, or ureido-substituted sulfonamides, some of which proved to be effective inhibitors of the parasitic enzyme pfCA. Here we extend the previous research[13] for detecting potent sulfonamide CAIs targeting malaria CAs. In this paper we report the in vitro pfCA inhibition studies with a library of aromatic/heterocyclic sulfonamides possessing a large diversity of scaffolds. The most effective CAIs were also assayed ex vivo, in cell cultures, for the inhibition of the parasite growth, as well as in vivo, in an animal model of human malaria, that is, mice infected with *P. berghei*.

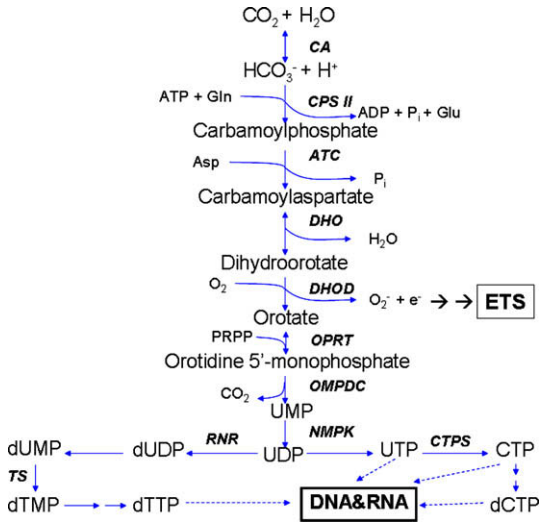


Figure 1. Involvement of α-CAs from *Plasmodium* parasites in the pyrimidine biosynthetic pathways (CA, carbonic anhydrase; CPS II, carbamoylphosphate synthetase II; ATC, aspartate transcarbamoylase; DHO, dihydroorotase; DHOD, dihydroorotate dehydrogenase; OPRT, orotate phosphoribosyltransferase; OMPDC, orotidine 5′-monophosphate decarboxylase; NMPK, nucleoside monophosphate kinase; RNR; ribonucleotide reductase; TS, thymidylate synthetase; CTPS, cytidine 5′-triphosphate synthetase).

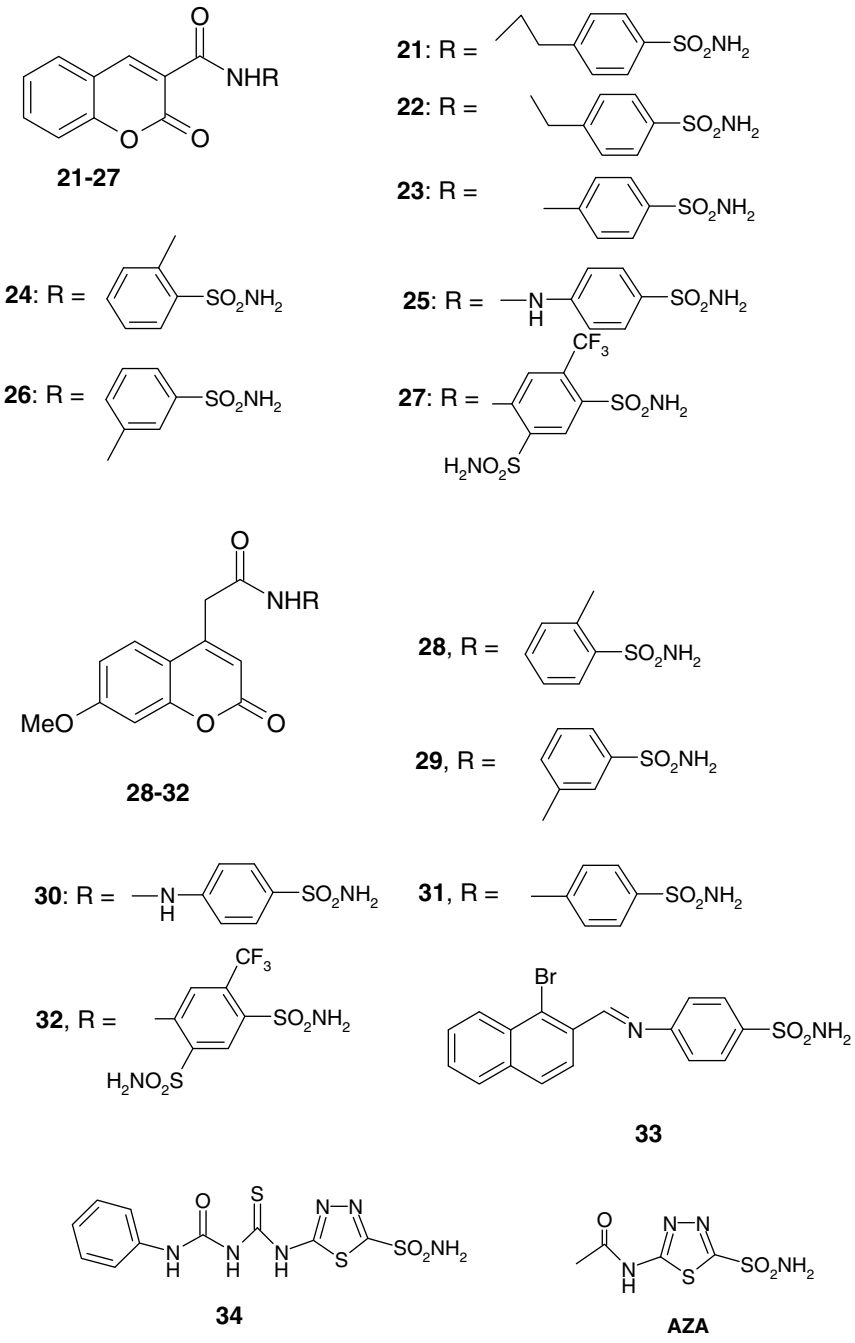
Sulfonamides **1–34** investigated here for the inhibition of recombinant, purified pfCA have been reported earlier by this group.[20–27] They include both aromatic (benzenesulfonamides as warheads to bind the Zn(II) ion within the CA active site)[15] as well as heterocyclic such compounds, that is, 1,3,4-thiadiazole- and 1,3,4-thiadiazoline-2-sulfonamides. Various tails are attached to these aromatic/heterocyclic sulfonamide scaffolds, such as the perfluoroaryl/alkyl-sulfonamido-; aryl/diaryl-ureido/thioureido-; arylcarboxamido-; diethyl-dithiocarbamoylamino-; aromatic Schiff's base, coumarinyl-3-carboxamido- as well as 7-methoxy-coumarin-4-yl-acetamido-, in order to include a large structural variation as well as physico-chemical properties to the test compounds. Indeed, these sulfonamides have been assayed earlier for the inhibition of the human isoforms CA I, II, IV, and IX, showing a great variation of potency and different affinities for the various human isoforms.[20–27]

In vitro inhibition data of sulfonamides **1–34** against recombinant purified pfCA, ex vivo data for the growth inhibition of *P. falciparum* in cell cultures as well as in vivo antimalarial data in mice infected with *P. berghei*, are shown in Table 1. Acetazolamide **AZA** has been included as standard sulfonamide CAI, as it has been investigated earlier for the in vitro and ex vivo inhibition of pfCA/*P. falciparum*.[13] Three antimalarial, clinically used drugs, that is, quinine, qinghausu, and chloroquine were also included in these assays as standards.[28]

Data of Table 1 show the following structure activity relationship (SAR) for the inhibition of pfCA with sulfonamides **1–34**:

(i) Several compounds among the tested sulfonamides, such as **8, 9, 17–21, 24**, and **26**, showed ineffective pfCA inhibitory activity, with inhibition constants in the range of 13.169 to >25 μM. They include various scaffolds, such as the *N*,*N*-diphenyl-ureas **8** and **9**, the Schiff's bases **17–20** or the coumarine-3-carboxamides **21, 24**, and **26**. SAR is difficult to interpret in these cases also considering the fact that some structurally related derivatives to these compounds showed much better pfCA inhibitory activity (see Discussion later in the text).

(ii) Another subgroup of derivatives, including **3, 5, 11, 15, 22, 23, 25, 27, 29, 33**, and **34** showed a better pfCA inhibitory activity than the compounds mentioned above, with K_Is in the range of 4.162–9.034 μM. Again the structures of these



medium potency inhibitors are very diverse, incorporating basically all classes of the investigated sulfonamides, such as the perfluoroaryl- (**3**), urea (**5** and **34**), thiourea (**11** and **34**), *N*,*N*-diethyldithiocarmaboylamino (**15**) coumarines (**22, 23, 25, 27**, and **29**) as well as Schiff's base (**33**) derivatives. The sulfonamide heads incorporated in these CAIs include both benezenesulfonamide as well as 1,3,4-thiadiazole/thiadiazolines warheads.

(iii) The remaining derivatives, including **1, 2, 4, 6, 7, 10, 12–14, 16, 28, 30–32**, and **AZA** were better pfCA inhibitors, showing low micromolar or submicromolar affinities for the enzyme, with K_Is in the range of 0.180–3.506 μM (Table 1). One may observe the large variation of inhibitory activity of these compounds, when small structural variations are incorporated. For example, incorporation of an extra-methyl group in **2**, as in the thiadiazoline **3**, led to a 2-fold decrease of the inhibitory activity of **3** compared to **2**. Replacement of the perfluorophenyl-sulfonyl moiety of **1** with the corresponding perfluoro-octylsulfonyl moiety present in **4**, led to a 13.45 times gain in inhibitory activity of the later compound compared to the former one. In fact **4**, together with the ureido-thiourea **10**, are among the most potent pfCA inhibitors ever detected up until now. Indeed, compound **10** possess the same 3,4-dichlorophenyl-urea moiety also found in **5**, but **10** has an additional thiourea fragment in its molecule, and it is 31 times more effective a pfCA inhibitor as compared to **5**. Probably the more elongated shape of **10** compared to the compact **5**, leads to better interactions with the enzyme active site and to this very good in vitro inhibition of pfCA. However, compound **34** possesses a similar elongated shape as **10**, and also the phenylureido-thiourea fragment, but **34** is a 37.4 times less effective

Table 1
In vitro inhibition data of recombinant purified pfCA (K_Is μM), ex vivo inhibition of growth of *P. falciparum* in cell cultures (IC_{50}, μM) and in vivo antimalarial activity in *P. berghei*-infected mice (ID_{50}, mg/kg) of sulfonamides **1–34**, acetazolamide **AZA** and antimalarial drugs quinine, qinghausu, and chloroquine, as standards

Compound	K_I (μM)	IC_{50} (μM)	ID_{50} (mg/kg)
1	2.583	>50	—
2	3.441	>50	—
3	6.867	>50	—
4	0.192	>50	—
5	5.580	>50	—
6	3.055	>50	—
7	1.992	>50	—
8	16.517	>50	—
9	16.038	>50	—
10	0.180	1.002	10.00*
11	4.622	>50	—
12	2.102	>50	—
13	3.053	>50	—
14	2.997	>50	—
15	8.392	>50	—
16	0.250	3.886	No effect*
17	>25	>50	—
18	>25	>50	—
19	>25	>50	—
20	>25	>50	—
21	13.169	>50	—
22	5.048	>50	—
23	5.816	>50	—
24	18.507	>50	—
25	4.057	>50	—
26	>25	>50	—
27	9.034	>50	—
28	1.436	>50	—
29	4.578	>50	—
30	3.506	>50	—
31	0.970	>50	—
32	2.519	>50	—
33	4.162	>50	—
34	6.730	>50	—
AZA	0.315**	20.0**	No effect*
Quinine	No inhibition	—	—
Qinghausu	No inhibition	—	—
Chloroquine	No inhibition	—	5.00*

In vivo data with the clinically used antimalarial drug chloroquine are provided for comparison. Compounds **10**, **16**, and **AZA** showed no cytotoxicity in human KB and BC cells (data not shown).

* Tested at 25, 10, 5, and 2.5 mg/kg body weight Ref. 30.

** From Ref. 13.

inhibitor as compared to **10**. Probably the two chlorine atoms present in **10** are crucial for the effective binding to the enzyme active site. It is also interesting to note the very effective pfCA inhibition with the Schiff's base **16**, incorporating again a chlorophenyl moiety in its molecule, which is at least 100 times more effective as an enzyme inhibitor as compared to the structurally related derivatives **17–20**, all quite ineffective pfCA inhibitors. Large variations of activity were also observed for the coumarines **21–32**, with only one compound possessing submicromolar pfCA inhibitory activity (**31**), the vast majority of these sulfonamides being medium potency inhibitors. The standard CAI acetazolamide, **AZA**, behaves as a strong pfCA inhibitor too, with a K_I of 0.315 μM, as shown earlier by us.[13] It is difficult to rationalize these data in the absence of an X-ray crystal structure of this enzyme, but we can state that small structural variations in the scaffold of tested sulfonamides lead to very different inhibition profiles, which is noteworthy, meaning that it is possible to detect much more effective pfCA inhibitors by an intense screening effort of various libraries of structurally diverse compounds. Data of Table 1 also demonstrate that antimalarials such as quinine, chloroquine or qinghausu, which do not possess moieties present in CAIs (sulfonamides and their bioisosteres), are not at all pfCA inhibitors up to millimolar concentrations (data not shown). Thus, the sulfonamides investigated here are specifically interacting with the pfCA active site, similarly to all sulfonamides targeting α-CAs investigated earlier.[15]

The most effective in vitro pfCA inhibitors, that is, **10, 16**, and **AZA** also showed interesting growth inhibition of *P. falciparum* parasite in ex vivo experiments (Table 1).[30] Indeed, **AZA** was a rather weak ex vivo inhibitor, with an IC_{50} of 20 μM, but two of the newly investigated derivatives, that is, **10** and **16**, showed appreciable activity, with IC_{50}s in the low micromolar range, of 1.002–3.886 μM. All other derivatives, irrespective of their K_I values against the purified pfCA enzyme, were ineffective inhibitors for the ex vivo growth of the parasite (Table 1), with IC_{50}s > 50 μM.

Acetazolamide (as standard), chloroquine (as clinically used antimalarial drug) and the two ex vivo active compounds, **10** and **16**, were also tested in vivo, in an animal model of human malaria, that is, mice infected with *P. berghei*, for their antimalarial activity (Table 1). It may be observed that **AZA** and compound **16** were ineffective antimalarials in this animal model (at doses of 25, 10, 5, and 2.5 mg/kg body weight, respectively) whereas chloroquine and compound **10** showed appreciable antimalarial activity, with ID_{50}s (amount of compound protecting 50% of the test animals against malaria infection) in the range of 5–10 mg/kg. Thus, the newly investigated sulfonamide **10** has a comparable activity with chloroquine, being slightly les effective (in vivo) as compared to this widely used drug, but its probable mechanism of action is very different compared to that of chloroquine, involving inhibition of the malaria parasites CA.

In conclusion, a library of aromatic/heterocyclic sulfonamides possessing a large diversity of scaffolds has been assayed for inhibition of CA from the malaria parasite *P. falciparum*. Low micromolar and submicromolar in vitro inhibitors were detected, whereas several compounds showed ex vivo anti-*P. falciparum* activity, in cell cultures. One derivative, 4-(3,4-dichlorophenylureido)thioureido-benzenesulfonamide (**10**) was an effective in vitro pfCA inhibitor (K_I of 0.18 μM), inhibited the ex vivo growth of *P. falciparum* with an IC_{50} of 1 μM, and was also effective as an antimalarial agent in mice infected with *P. berghei*, an animal model of human malaria infection, with an ID_{50} of 10 mg/kg (chloroquine as standard, showed an ID_{50} of 5 mg/kg). By inhibiting the first step of pyrimidine nucleotide biosyntheses, that is, the CA-mediated carbamoylphosphate biosynthesis, sulfonamide inhibitors of the protozoan CAs may have potential for the development of novel therapies of human malaria and thus shortcut the drug resistance to the clinically used antimalarials.

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28. The esterase activity of pfCA was measured by using the modified method of Armstrong et al.[29] The enzyme activity was determined by following the change in absorbance at 348 nm of *p*-nitrophenylacetate which ia hydrolyzed to the 4-nitrophenoxide ion (an extinction coefficient of 18.1 M^{-1} cm^{-1} for this last species was used) 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 solution. The measurement was also done by titration with the potent CAI **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 non-linear regression of an Elsevier Biosoft Enzfitter program. Inhibitor constants (K_I) were determined from Dixon's plots as described previously.[13]
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30. 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 a starting parasitemia of 0.5% as described.[31] 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 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 vitro growth was quantified by measuring % parasitemia in a 96-h culture in the presence of the drugs at various concentrations.[31] 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.
31. Krungkrai, J.; Krungkrai, S. R.; Phakanont, K. *Biochem. Pharmacol.* **1992**, *43*, 1295.