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ABSTRACT
Anion inhibition study of the β-class carbonic anhydrase, AgaCA, from the malaria mosquito Anopheles gambiae is reported. A series of simple as well as complex inorganic anions, and small molecules known to interact with CAs were included in the study. Bromide, iodide, bisulphite, perchlorate, perrhenate, peroxysulphate, and peroxysulphate were ineffective AgaCA inhibitors, with Ks > 200 mM. Fluoride, chloride, cyanate, thiocyanate, cyanide, bicarbonate, carbonate, nitrite, nitrate, sulphate, stannate, selenate, tellurate, diprophosphate, divanadate, tetraborate, selenocyanide, and triithiocarbonate showed Ks in the range of 1.80–9.46 mM, whereas N,N-diethylthiodicarbamate was a submillimolar AgaCA inhibitor (Ks of 0.65 mM). The most effective AgaCA inhibitors were sulphamide, sulphanilic acid, phenylboronic acid and phenylarsionic acid, with inhibition constants in the range of 21–84 μM. The control of insect vectors responsible of the transmission of many protozoan diseases is rather difficult nowadays, and finding agents which can interfere with these processes, as the enzyme inhibitors investigated here, may arrest the spread of these diseases worldwide.

1. Introduction
Malaria is a tropical disease transmitted mainly by mosquitoes of the genus Anopheles. The disease itself is caused by parasites of the genus Plasmodium, of which five species affect human red blood cells: P. falciparum, P. ovale, P. vivax, P. knowlesi and P. malariae. Of these P. falciparum causes the most severe infection. Malaria causes non-specific symptoms of which most common are fever and headache, and in severe cases the disease may lead to death.

Carbonic anhydrases (CAs, EC 4.2.1.1) are enzymes that catalyse the reversible hydration reaction of carbon dioxide according to the following reaction: \( \text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{H}^+ \). CAs are typically zinc-containing metalloenzymes, but the \( \gamma \) form uses cadmium or zinc (in an inter-exchangeable manner), as an alternative metal cofactor. In addition, \( \gamma \)-CAs may contain iron(II) within the active site, at least in some anaerobic Archaea. The reaction catalysed by CAs is crucial in the regulation of acid/base balance in organisms. Additionally, this reaction participates in removing carbon dioxide out of tissues, takes part in biosynthetic reactions such as gluconeogenesis and ureagenesis, and is involved in many other physiological processes as well. Seven classes of carbonic anhydrases have been identified: the \( \alpha \), \( \beta \), \( \gamma \), \( \delta \), \( \zeta \), \( \eta \) and \( \theta \)-CAs. Of these, \( \beta \)-CAs appear to be the family with the widest distribution. They have been described from various groups of organisms including the Bacteria and Archaea domains as well as in all species of plants and some fungi among Eukarya. In addition, our previous studies suggested the widespread occurrence of at least one single-copy of a \( \beta \)-CA gene among animal species distinct from chordates.

\( \beta \)-CAs were first reported from two invertebrate species: the fruit fly Drosophila melanogaster and the worm Caenorhabditis elegans. Additionally, a \( \beta \)-CA was characterised from the unicellular parasite Leishmania donovani chagasi which is one of the causative agents for visceral leishmaniasis. Previously two \( \xi \)-CAs have been characterised from the mosquito Anopheles gambiae. Despite the recent finding that Leishmania parasites encode \( \beta \)-CAs, some protozoan parasites possess only \( \xi \)- or \( \eta \)-CAs. For example, P. falciparum seems to encode only the \( \eta \)-CAs. The exact expression pattern of \( \beta \)-CAs in protozoan organisms is currently unclear, even though a previous report suggested the presence of this enzyme family in a number of protozoans and metazoans. Recently, \( \xi \)-CA was characterised from unicellular protozoa responsible of Chagas’ disease, Trypanosoma cruzi. \( \beta \)-CAs are not present in vertebrates, which may lead to the design of \( \xi \)-CA-specific inhibitors that could be used against invertebrate pathogens and pathogen vectors with minimal side effects on vertebrate species. However, no such inhibitors have been reported so far. We performed sequence searches in the genomes of different pathogen vectors and found out that Anopheles gambiae encodes for one \( \beta \)-CA in addition to several \( \xi \)-CAs. The aim of this study was to characterise further the \( \beta \)-CA, AgaCA, from malaria mosquito Anopheles gambiae, which is one of the most important malaria vector organisms. Kinetic and anion inhibition studies with a large set of inhibitors were carried out to characterise its catalytic activity and inhibition profile, considering the fact...
that we have reported earlier only the sulphonamide inhibition profile of this enzyme. 

2. Materials and methods

2.1. Construction of β-CA fusion protein

Anopheles gambiae cDNA was obtained from Professor Michael Lehane (Liverpool School of Tropical Medicine, UK). The β-CA gene was retrieved from NCBI protein databases using Blast. The full-length β-CA gene was identified and amplified from cDNA by PCR using Phusion Hot Start High Fidelity DNA Polymerase (Finnzymes, Espoo, Finland). The detailed PCR method has been described previously by our group. The PCR product was separated from the gel and dissolved using I-IustraTM GEX PCR DNA and GEL Band Purification Kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Validity of the PCR product was verified by sequencing.

For recombinant protein production, the β-CA gene was constructed and cloned into the pFastBac1TM vector. As the forward primer used in the initial amplification of the β-CA gene was 5′-CGCGGATCCATGGAGCGTATATTGCGAGGC-3′ (F2), and the reverse primer was 5′-GGCTCTGGATTATGTTGATGTGTTGTTGGAACCCACGGGGAAGAGTATGCTCCGTACCTC-3′ (R2). The latter primer contains nucleotide repeats to create the C-terminal polyhistidine tag with six histidines. In addition, the forward primer contained the restriction site for BamHI and the reverse primer for histidine tag with six histidines. In addition, the forward primer contains nucleotide repeats to create the C-terminal polyhistidine tag with six histidines. In addition, the forward primer contained the restriction site for BamHI and the reverse primer for histidine tag with six histidines. In addition, the forward primer contained the restriction site for BamHI and the reverse primer for histidine tag with six histidines. In this case, the protein was transferred to 50 mM Tris-HCl, pH 7.5. To remove the His tag, the recombinant protein was treated with 150 μL of resin-coupled thrombin (Thrombin CleanCleave KIT, Sigma, Milan, Italy) per 1 mg of protein with gentle shaking at +20 °C overnight, according to the manufacturer’s instructions. Protein concentration was determined using the DC Protein AssayTM (Bio-Rad, Berlin, Germany) with three different dilutions.

2.2. Production of A. gambiae β-CA

The Sf9 insect cells were grown in Insect-Xpress protein-free cell culture medium (Lonza, Verviers, Belgium) in an orbital shaker at 27 °C (125 rpm) for 3 d after infection. Protein purification was performed after centrifugation (5000 × g, 20 °C, 8 min) from the supernatant. Purification was performed using the Protino® Ni-NTA Agarose (from Macherey-Nagel, Munich, Germany) under native binding conditions with wash and elution buffers made according to the manufacturer’s instructions. The purification procedure per 400 ml of insect cell medium was as follows: 3 L of native binding buffer (50 mM NaH2PO4, 500 mM NaCl, pH 8.0) and 8 ml of the nickel-chelating agarose were added to the medium, and the His-tagged protein was then allowed to bind to the resin on a magnetic stirrer at 25 °C for 3 h. The resin was washed with 40 + 20 ml of washing buffer (50 mM NaH2PO4, 500 mM NaCl, 20 mM imidazole, pH 8.0). The protein was then eluted with elution buffer (50 mM NaH2PO4, 500 mM NaCl, 250 mM imidazole, pH 8.0). After washing buffer (50 mM NaH2PO4, 500 mM NaCl, 20 mM imidazole, pH 8.0). After

2.3. CA activity measurements and inhibition studies

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.6) or 20 mM TRIS (pH 8.3) as buffers, and 20 mM NaClO4 (for maintaining constant the ionic strength). Perchlorate is not inhibiting the enzyme at concentrations up to 100 mM, data not shown, as for many other CAs investigated earlier by our group, following the initial rates of the CA-catalysed CO2 hydration reaction for a period of 10–100 s. The CO2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalysed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitors (10 mM) were prepared in distilled-deionised water and dilutions up to 0.01 mM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng–Prusoff equation, as reported earlier, and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in house as reported earlier. The concentration of AgaCA used in the experiments reported in the paper was 13.2 nM.

3. Results and discussion

As shown in the introduction, there exists only few studies on insect CAs. Apart our initial reports on the presence of a β-CA in Drosophila melanogaster and Anopheles gambiae, some sulphonamide and dithiocarbamate studies were reported for the inhibition of the first enzyme, but no other inhibition studies (except the sulphonamide ones) are available for AgaCA. It should be mentioned that recently a CA was also reported and its activity/inhibition investigated from another insect species, the honey bee Apis mellifera. In this paper, we report the first extensive anion inhibition study of the β-CA from Anopheles gambiae, AgaCA, with a large series of simple and complex anions.

In the previous work, we observed that AgaCA has a significant catalytic activity for the physiologic, CO2 hydration reaction to bicarbonate and protons, with the kinetic parameters shown in Table 1. AgaCA has a catalytic activity which is similar to that of the human cytosolic isoform hCA I, and is also inhibited quite effectively by the sulphonamide. The widely clinically used compound, acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulphonamide), showed an inhibition constant of 27.3 nM (Table 1).

Inorganic anions constitute an important class of CA inhibitors (CA(i)) both inorganic, complexing anions and more complex anions were investigated for their interaction with a large number of enzymes belonging to all CA families. Such studies may lead...
to the discovery of novel classes of pharmacologically relevant CAIs: indeed, the dithiocarbamates were discovered, considering the simple anion trithiocarbonate (CS$_2^{2-}$) as an inhibitor, and showed significant in vitro and in vivo activities in pathologies related to CA dysregulation, such as glaucoma.

In Table 2, the inhibition of AgaCA with a panel of such anions is shown. Inhibition data for the widespread cytosolic isoforms hCA I and II, as well as for the enzyme from D. melanogaster, are also shown, for comparison reasons. The following may be noted from the inhibition data of Table 2:

(i) Anions with low propensity for inhibiting AgaCA were bromide, iodide, bisulphite, perchlorate, perhenate, perrhenate, and peroxodisulphate, which showed K$_I >$ 200 mM. Whereas perchlorate is generally the anion with less affinity for metal ions in solution and metalloenzyme (in fact it does not inhibit significantly any CA investigated so far), the data for the heavy halogenides and bisulphite are rather surprising, considering the fact that iodide and bromide are rather effective hCA I and DmBCA inhibitors (Table 2). Bisulphite is a weak hCA I and II inhibitor but it is more effective as a DmBCA inhibitor.

(ii) Azide and hydrogensulphide, anions which show a high affinity for many metal ions, were rather weak AgaCA inhibitors, with K$_I$ of 12.4–25.1 mM. They were much more effective as DmBCA inhibitors and are micromolar hCA I inhibitors (Table 2). Thus, there are significant differences in the affinity of these inhibitors for various CAs, with the mosquito enzyme definitely less sensitive to these inhibitors compared to other insect or human CAs.

(iii) Most of the investigated anions showed inhibition constants in the range of 1.80–9.46 mM, being thus weak CAIs, but normally this is the range in which most simple/complex anions interact with most CAs. They include fluoride, chloride, cyanate, thiocyanate, cyanide, bicarbonate, carbonate, nitrite, nitrate, sulphate, stannate, selenate, tellurate, diphosphate, divanadate, tetraborate, selenocyanide, and trithiocarbonate. It should be observed that this series includes both anions with a high affinity for complexing metal ions (such as cyanate, thiocyanate, and cyanide) as well as anions with lower affinity for cations, such as nitrite, nitrate, and sulphate. It is interesting to note that for the halogenides, those incorporating light elements (F, Cl) were more effective than the halogenides incorporating heavy elements, which is opposite to the inhibitory effects observed with these anions against hCA I and II (Table 2). Bicarbonate was twice better as an AgaCA inhibitor compared with carbonate, whereas sulphate, which is a weak hCA I and II inhibitor, showed a <10 mM activity against AgaCA.

(iv) The most effective AgaCA inhibitors were sulphamide, sulphamic acid, phenylboronic acid, and phenylarsenic acid, with inhibition constants in the range of 21–84 mM. These compounds are known to act as efficient CAIs against many CAs and were used as leads to obtain potent inhibitors, some of which inhibit these enzymes in the low nanomolar range. Indeed, these simple molecules incorporate zinc-binding functions of the sulphonamide, sulphamide, sulphanilate, boronic acid, etc., which have been extensively employed to design highly effective CAIs.

4. Conclusions

We report here an anion inhibition study of the β-class CA, AgaCA, from the mosquito Anopheles gambiae, the vector responsible of malaria transmission. A series of simple as well as complex inorganic anions, together with small molecules known to interact with CAs were included in the study. Bromide, iodide, bisulphite, perchlorate, perhenate, perruthenate, and peroxodisulphate were ineffective AgaCA inhibitors, with K$_I >$ 200 mM. Fluoride, chloride, cyanate, thiocyanate, cyanide, bicarbonate, carbonate, nitrite, nitrate, sulphate, stannate, selenate, tellurate, diphosphate, divanadate, tetraborate, selenocyanide, and trithiocarbonate showed K$_I$ 

### Table 1. Kinetic parameters for the CO$_2$ hydration reaction catalysed by the human cytosolic isoforms hCA I and II (α-class CAs) and the β-CAs from Drosophila melanogaster (DmBCA) and Anopheles gambiae (AgaCA) measured at 20°C, pH 7.6 in 20 mM HEPES buffer (for hCA I and II) and 20°C, pH 8.3 in 20 mM TRIS buffer (for the β-CAs), in the presence 20 mM NaClO$_4$ (for maintaining constant ionic strength). Inhibition data with the clinically used sulphamide, acetylsalicylic (5-acetamido-1,3,4-thiadiazole-2-sulphonamide) are also provided.

| Enzyme     | Activity level | Class | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) | $K_i$ (acetylsalicylic acid) (nmM) |
|------------|----------------|-------|---------------------------------|-----------------------------------|
| hCA I      | Moderate       | α     | $2.0 \times 10^2$              | 5.0 $\times 10^4$                |
| hCA II     | Very high      | α     | $1.4 \times 10^2$              | 1.5 $\times 10^4$                |
| DmBCA      | High           | β     | $9.5 \times 10^2$              | 1.1 $\times 10^4$                |
| AgaCA      | Moderate       | β     | $7.2 \times 10^2$              | 5.6 $\times 10^4$                |

*From ref.20.
From ref.9.
This work.

### Table 2. Inhibition constants of anionic inhibitors against isozymes hCA I, II (α-class), and DmBCA (D. melanogaster) and AgaCA (A. gambiae) for the CO$_2$ hydration reaction, at 20°C.

| Inhibitor | hCA I$^{a}$ | hCA II$^{b}$ | DmBCA$^{b}$ | AgaCA$^{a}$ |
|-----------|-------------|--------------|-------------|-------------|
| F$^{-}$   | >300        | >300         | 0.80        | 9.42        |
| Cl$^{-}$  | 0.6         | 200          | 0.97        | 8.74        |
| Br$^{-}$  | 4           | 63           | 1.04        | >200        |
| I$^{-}$   | 0.3         | 26           | 1.18        | >200        |
| CN$^{-}$  | 0.0007      | 0.03         | 0.73        | 9.46        |
| SCN$^{-}$ | 0.2         | 1.6          | 1.28        | 6.41        |
| N$_2$O$_5$| 0.0005      | 0.02         | 0.67        | 8.34        |
| HCN$^{-}$ | 0.0012      | 1.5          | 1.12        | 12.40       |
| CO$_2$    | 12          | 85           | 26.90       | 4.34        |
| HCO$_3^-$ | 15          | 73           | 0.86        | 9.25        |
| NO$_3^-$  | 7           | 35           | 43.74       | 6.50        |
| NO$_2^-$  | 8.4         | 63           | 28.60       | 4.55        |
| HS$^{-}$  | 0.0006      | 0.04         | 1.01        | 25.10       |
| HSO$_3^-$ | 18          | 89           | 1.29        | >200        |
| SO$_2$    | 63          | >200         | 1.36        | 9.03        |
| ClO$_4^-$ | >200        | >200         | >200        | >200        |
| BrO$_3^-$ | 0.57        | 0.83         | nt          | 1.80        |
| SeO$_3^{2-}$ | 118     | 112          | nt          | 9.41        |
| TeO$_4^{2-}$ | 0.66   | 0.92         | nt          | 4.96        |
| PO$_4^{3-}$ | 25.8      | 48.5         | nt          | 8.52        |
| V$_2$O$_7^{2-}$ | 0.54 | 0.57         | nt          | 7.98        |
| Bi$_2$O$_7^{2-}$ | 0.64 | 0.95         | nt          | 7.95        |
| ReO$_4^{3-}$ | 0.11     | 0.75         | nt          | >200        |
| RuO$_4^{2-}$ | 0.10    | 0.69         | nt          | >200        |
| SiO$_2$   | 0.11        | 0.084        | nt          | >200        |
| SeCN$^{-}$ | 0.0085     | 0.086        | nt          | 8.68        |
| CS$_2^{2-}$ | 0.0087     | 0.0088       | nt          | 8.19        |
| Et$_2$NCS$^{-}$ | 0.79   | 3.1          | nt          | 6.65        |
| H$_2$NSO$_2$NH$_2$ | 0.31 | 1.13         | 0.15        | 0.054       |
| H$_2$NSO$_2^+$ | 0.021     | 0.39         | 2.45        | 0.021       |
| Ph-B(OH)$_2$ | 58.6     | 23.1         | 22.39       | 0.047       |
| Ph-ASO$_3^2$ | 31.7     | 49.2         | 32.60       | 0.084       |

*From ref.20.
From ref.9.
This work.
As sodium salt; nt: not tested.

*Errors in the range of 5–10% of the shown data, from three different assays, by a CO$_2$ hydration stopped-flow assay.
in the range of 1.80–9.46 mM, whereas N,N-diethyl-dithiocarbamate was a submillimolar AgaCA inhibitor, with a $K_i$ of 0.65 mM. The most effective AgaCA inhibitors were sulphamide, sulphamic acid, phenylboronic acid, and phenylarsenic acid, with inhibition constants in the range of 21–84 $\mu$M. The control of insect vectors responsible of the transmission of many protozoan diseases is rather difficult nowadays, and finding agents which can interfere with these processes, as the enzyme inhibitors investigated here, may arrest the spread of these diseases worldwide.

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Disclosure statement

The authors do not declare any conflict of interest.

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References

1. Whitty CJ, Chiodini PL, Laloo DG. Investigation and treatment of imported malaria in non-endemic countries. BMJ 2013;346:f2906.
2. a) Sly WS, Hu PY. Human carbonic anhydrases and carbonic anhydrase deficiencies. Annu Rev Biochem 1995;64:375–401. b) Supuran CT. Carbonic anhydrases: from biomedical applications of the inhibitors and activators to biotechnological use for CO$_2$ capture. J Enzyme Inhib Med Chem 2013;28:229–30. c) Supuran CT. How many carbonic anhydrase inhibition mechanisms exist? J Enzyme Inhib Med Chem 2016;31:345–60. d) Alterio V, Di Fiore A, D’Ambrosio K, et al. Multiple binding modes of inhibitors to carbonic anhydrases: how to design specific drugs targeting 15 different isoforms? Chem Rev 2012;112:4421–68.
3. a) Xu Y, Feng L, Jeffrey PD, et al. Structure and metal exchange in the cadmium carbonic anhydrase of marine diatoms. Nature 2008;452:56–61. b) Alterio V, Langella E, Viparelli F, et al. Structural and inhibition insights into carbonic anhydrase CDCA1 from the marine diatom Thalassiosira weissflogii. Biochimie 2012;94:1232–41.
4. a) Lane TW, Saito MA, George GN, et al. Biochemistry: a cadmium enzyme from a marine diatom. Nature 2005;435:42. b) Del Prete S, Vullo D, De Luca V, et al. Biochemical characterization of the $\delta$-carbonic anhydrase from the marine diatom Thalassiosira weissflogii, TweCA. J Enzyme Inhib Med Chem 2014;29:906–11.
5. a) Macauley SR, Zimmerman SA, Apolinario EE, et al. The archetype gamma-class carbonic anhydrase (Cam) contains iron when synthesized in vivo. Biochemistry 2009;48:817–9. b) Zimmerman SA, Ferry JG, Supuran CT. Inhibition of the archaean beta-class (Cab) and gamma-class (Cam) carbonic anhydrases. Curr Top Med Chem 2007;7:901–8.
6. a) Tripp BC, Bell CB, Cruz F, et al. A role for iron in an ancient carbonic anhydrase. J Biol Chem 2004;279:6683–7. b) Innocenti A, Zimmerman S, Ferry JG, et al. Carbonic anhydrase inhibitors. Inhibition of the zinc and cobalt gammaclass enzyme from the archaean Methanosarcina thermophila with anions. Bioorg Med Chem Lett 2004;14:3327–31.
7. a) Capasso C, Supuran CT. An overview of the alpha-, beta- and gamma-carbonic anhydrases from Bacteria: can bacterial carbonic anhydrases shed new light on evolution of bacteria? J Enzyme Inhib Med Chem 2015;30:325–32. b) Supuran CT, Capasso C. Carbonic anhydrase from Porphyromonas Gingivalis as a drug target. Pathogens 2017;6:E30. c) Capasso C, Supuran CT. Inhibition of bacterial carbonic anhydrases as a novel approach to escape drug resistance. Curr Top Med Chem 2017;17:1237–48. d) Supuran CT, Capasso C. New light on bacterial carbonic anhydrases phylogeny based on the analysis of signal peptide sequences. J Enzyme Inhib Med Chem 2017;32:832–40.
8. a) Supuran CT. Structure and function of carbonic anhydrases. Biochem J 2016;473:2023–32. b) Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nat Rev Drug Discov 2008;7:168–81. c) Vullo D, Kumar RSS, Scozzafava A, et al. Sulphonamide inhibition studies of the $\beta$-carbonic anhydrase from the bacterial pathogen Clostridium perfringens. J Enzyme Inhib Med Chem 2018;33:31–6. d) Supuran CT. Legionella pneumophila carbonic anhydrases: underexplored antibacterial drug targets. Pathogens 2016;5:E44.
9. Syrjanen L, Tolvanen M, Hilvo M, et al. Characterization of the first beta-class carbonic anhydrase from an arthropod (Drosophila melanogaster) and phylogenetic analysis of beta-class carbonic anhydrases in vertebrates. BMC Biochem 2010;11:28.
10. Fasseas MK, Tsikou D, Fletemakis E, Katinakis P. Molecular and biochemical analysis of the beta class carbonic anhydrases in Caenorhabditis elegans. Mol Biol Rep 2010;37:2941–50.
11. Syrjanen L, Vermelho AB, de Almeida Rodrigues I, et al. Cloning, characterization and inhibition studies of a beta carbonic anhydrase from Leishmania donovani chagasi, the protozoan parasite responsible of leishmaniasis. J Med Chem 2013;56:7372–81.
12. Seron TJ, Hill J, Linser PJ. A GPI-linked carbonic anhydrase expressed in the larval mosquito midgut. J Exp Biol 2004;207:4559–72.
13. Smith KE, Vanekeris LA, Linser PJ. Cloning and characterization of AgCA9, a novel alpha-carbonic anhydrase from Anopheles gambiae. J Exp Biol 2007;210:3919–30.
14. a) Krungkrai J, Supuran CT. The alpha-carboxanic anhydrase from the malaria parasite and its inhibition. Curr Pharm Des 2008;631–40. b) Del Prete S, Vullo D, Fisher GM, et al. Discovery of a new family of carbonic anhydrases in the malaria pathogen Plasmodium falciparum – the $\eta$-carbonic anhydrase. Bioorg Med Chem Lett 2014;24:4389–96. c) Zolfaghari Emameh R, Barker H, Hytönen VP, et al. Beta carbonic anhydrases: novel targets for pesticides and anti-parasitic agents in agriculture and livestock husbandry. Parasit Vectors 2014;7:403.
15. Pan P, Vermelho AB, Capaci Rodrigues G, et al. Cloning, characterization, and sulfonamide and thiol inhibition studies of an α-carbonic anhydrase from Trypanosoma cruzi, the causative agent of chagas disease. J Med Chem 2013;56:1761–71.

16. Syrjänen L, Kuuslahti M, Tolvanen M, et al. The β-carbonic anhydrase from the malaria mosquito Anopheles gambiae is highly inhibited by sulfonamides. Bioorg Med Chem 2015;23:2303–9.

17. Altschul SF, Gish W, Miller W, et al. Basic local alignment search tool. J Mol Biol 1990;215:403–10.

18. Hilvo M, Baranauskiene L, Salzano AM, et al. Biochemical characterization of CA IX, one of the most active carbonic anhydrase isozymes. J Biol Chem 2008;283:27799–809.

19. Khalifah RG. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. J Biol Chem 1971;246:2561–73.

20. a) De Simone G, Supuran CT. (In)organic anions as carbonic anhydrase inhibitors. J Inorg Biochem 2012;111:117–29. b) Del Prete S, Vullo D, Osman SM, et al. Anion inhibitors of the β-carbonic anhydrase from the pathogenic bacterium responsible of tularemia, Francisella tularensis. Bioorg Med Chem 2017;25:4800–4. c) Nocentini A, Vullo D, Del Prete S, et al. Inhibition of the β-carbonic anhydrase from the dandruff-producing fungus Malassezia globosa with monothiocarbamates. J Enzyme Inhib Med Chem 2017;32:1064–70.

21. a) Scozzafava A, Briganti F, Mincione G, et al. Carbonic anhydrase inhibitors: synthesis of water-soluble, aminocarboxylic acid, and dipeptidyl sulfonamides possessing long-lasting intraocular pressure-lowering properties via the topical route. J Med Chem 1999;42:3690–700. b) Puccetti L, Fasolis G, Vullo D, et al. Carbonic anhydrase inhibitors. Inhibition of cytosolic/tumor-associated carbonic anhydrase isozymes I, II, IX, and XII with Schiff's bases incorporating chromone and aromatic sulfonamide moieties, and their zinc complexes. Bioorg Med Chem Lett 2005;15:3096–101.

22. a) Scozzafava A, Menabuoni L, Mincione F, Supuran CT. Carbonic anhydrase inhibitors. A general approach for the preparation of water-soluble sulfonamides incorporating polyanino – polycarboxylate tails and of their metal complexes possessing long-lasting, topical intraocular pressure-lowering properties. J Med Chem 2002;45:1466–76. b) Pacchiano F, Aggarwal M, Avvaru BS, et al. Selective hydrophobic pocket binding observed within the carbonic anhydrase II active site accommodate different 4-substituted-ureido-benzensulfonamides and correlate to inhibitor potency. Chem Commun (Camb) 2010;46:8371–3. c) Supuran CT, Mincione F, Scozzafava A, et al. Carbonic anhydrase inhibitors—Part 52. Metal complexes of heterocyclic sulfonamides: a new class of strong topical intraocular pressure-lowering agents in rabbits. Eur J Med Chem 1998;33:247–54.

23. a) Scozzafava A, Menabuoni L, Mincione F, et al. Carbonic anhydrase inhibitors: perfluoroalkyl/aryl-substituted derivatives of aromatic/heterocyclic sulfonamides as topical intraocular pressure-lowering agents with prolonged duration of action. J Med Chem 2000;43:4542–51. b) Abbate F, Winum JY, Potter BV, et al. Carbonic anhydrase inhibitors: X-ray crystallographic structure of the adduct of human isozyme II with EMATE, a dual inhibitor of carbonic anhydrases and steroid sulfatase. Bioorg Med Chem Lett 2004;14:231–4.

24. a) Syrjänen L, Tolvanen ME, Hilvo M, et al. Characterization, bioinformatic analysis and dithiocarbamate inhibition studies of two new α-carbonic anhydrases, CAH1 and CAH2, from the fruit fly Drosophila melanogaster. Bioorg Med Chem 2013;21:1516–21. b) Syrjänen L, Parkkila S, Scozzafava A, Supuran CT. Sulfonamide inhibition studies of the β-carbonic anhydrase from Drosophila melanogaster. Bioorg Med Chem Lett 2014;24:2797–801. c) Zolfaghari Emameh R, Syrjänen L, Barker H, et al. Drosophila melanogaster: a model organism for controlling Dipteran vectors and pests. J Enzyme Inhib Med Chem 2015;30:505–13.

25. Soydan E, Güler A, Byk S, et al. Carbonic anhydrase from Apis mellifera: purification and inhibition by pesticides. J Enzyme Inhib Med Chem 2017;32:47–50.

26. a) Carta F, Aggarwal M, Maresca A, et al. Dithiocarbamates: a new class of carbonic anhydrase inhibitors. Crystallographic and kinetic investigations. Chem Commun (Camb) 2012;48:1868–70. b) Carta F, Aggarwal M, Maresca A, et al. Dithiocarbamates strongly inhibit carbonic anhydrases and show antigenic action in vivo. J Med Chem 2012;55:1721–30. c) Maresca A, Carta F, Vullo D, Supuran CT. Dithiocarbamates strongly inhibit the β-class carbonic anhydrases from Mycobacterium tuberculosis. J Enzyme Inhib Med Chem 2013;28:407–11.

27. a) Supuran CT. Bortezomib inhibits bacterial and fungal β-carbonic anhydrases. Bioorg Med Chem 2016;24:4406–9. b) Alterio V, Cadoni R, Esposito D, et al. Benzoxaborole as a new chemotype for carbonic anhydrase inhibition. Chem Commun (Camb) 2016;52:11983–6. c) Nocentini A, Cadoni R, Del Prete S, et al. Benzoxaboroles as efficient inhibitors of the β-carbonic anhydrases from pathogenic fungi: activity and modeling study. ACS Med Chem Lett 2017;8:1194–8.