



HAL
open science

Reconsidering anion inhibitors in the general context of drug design studies of modulators of activity of the classical enzyme carbonic anhydrase

Jean-Yves Winum, Alessio Nocentini, Andrea Angeli, Fabrizio Carta, Raivis Zalubovskis, Simone Carradori, Clemente Capasso, William Donald, Claudiu Supuran

► To cite this version:

Jean-Yves Winum, Alessio Nocentini, Andrea Angeli, Fabrizio Carta, Raivis Zalubovskis, et al.. Reconsidering anion inhibitors in the general context of drug design studies of modulators of activity of the classical enzyme carbonic anhydrase. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 2021, 36 (1), pp.561-580. 10.1080/14756366.2021.1882453 . hal-03526764

HAL Id: hal-03526764

<https://hal.umontpellier.fr/hal-03526764v1>

Submitted on 9 Jun 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License



Reconsidering anion inhibitors in the general context of drug design studies of modulators of activity of the classical enzyme carbonic anhydrase

Alessio Nocentini, Andrea Angeli, Fabrizio Carta, Jean-Yves Winum, Raivis Zalubovskis, Simone Carradori, Clemente Capasso, William A. Donald & Claudiu T. Supuran

To cite this article: Alessio Nocentini, Andrea Angeli, Fabrizio Carta, Jean-Yves Winum, Raivis Zalubovskis, Simone Carradori, Clemente Capasso, William A. Donald & Claudiu T. Supuran (2021) Reconsidering anion inhibitors in the general context of drug design studies of modulators of activity of the classical enzyme carbonic anhydrase, Journal of Enzyme Inhibition and Medicinal Chemistry, 36:1, 561-580, DOI: [10.1080/14756366.2021.1882453](https://doi.org/10.1080/14756366.2021.1882453)

To link to this article: <https://doi.org/10.1080/14756366.2021.1882453>



© 2021 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.



Published online: 21 Feb 2021.



Submit your article to this journal [↗](#)



Article views: 2293



View related articles [↗](#)



View Crossmark data [↗](#)



Citing articles: 41 View citing articles [↗](#)

REVIEW ARTICLE



Reconsidering anion inhibitors in the general context of drug design studies of modulators of activity of the classical enzyme carbonic anhydrase

Alessio Nocentini^a , Andrea Angeli^a , Fabrizio Carta^a, Jean-Yves Winum^b, Raivis Zalubovskis^{c,d}, Simone Carradori^e , Clemente Capasso^f , William A. Donald^g and Claudiu T. Supuran^a 

^aNeurofarba Department, Pharmaceutical and Nutraceutical Section, University of Florence, Florence, Italy; ^bIBMM, Univ. Montpellier, CNRS, ENSCM, Montpellier, France; ^cLatvian Institute of Organic Synthesis, Riga, Latvia; ^dInstitute of Technology of Organic Chemistry, Faculty of Materials Science and Applied Chemistry, Riga Technical University, Riga, Latvia; ^eDepartment of Pharmacy, "G. d'Annunzio" University of Chieti-Pescara, Chieti, Italy; ^fInstitute of Biosciences and Bioresources, National Research Council, Napoli, Italy; ^gSchool of Chemistry, University of New South Wales, Sydney, Australia

ABSTRACT

Inorganic anions inhibit the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) generally by coordinating to the active site metal ion. Cyanate was reported as a non-coordinating CA inhibitor but those erroneous results were subsequently corrected by another group. We review the anion CA inhibitors (CAIs) in the more general context of drug design studies and the discovery of a large number of inhibitor classes and inhibition mechanisms, including zinc binders (sulphonamides and isosteres, dithiocabamates and isosteres, thiols, selenols, benzoxaboroles, ninhydrins, etc.); inhibitors anchoring to the zinc-coordinated water molecule (phenols, polyamines, sulfocoumarins, thioxocoumarins, catechols); CAIs occluding the entrance to the active site (coumarins and derivatives, lacosamide), as well as compounds that bind outside the active site. All these new chemotypes integrated with a general procedure for obtaining isoform-selective compounds (the tail approach) has resulted, through the guidance of rigorous X-ray crystallography experiments, in the development of highly selective CAIs for all human CA isoforms with many pharmacological applications.

ARTICLE HISTORY

Received 19 December 2020
Revised 13 January 2021
Accepted 20 January 2021

KEYWORDS

carbonic anhydrase; inhibitor; sulphonamide; inhibition mechanisms; cyanate



1. Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) was discovered almost a century ago by Meldrum and Roughton¹ who described its efficient catalysis for the reversible hydration of blood CO₂ to bicarbonate and protons. Subsequently, such a process was shown to be relevant physiologically for many systems in organisms throughout the phylogenetic tree^{2,3}. Relevant discoveries on the catalytic and inhibition mechanisms of this enzyme were thereafter mainly done by a Swedish scientist, in the period from the 60s to the 80s, S. Lindskog, who proposed the catalytic mechanism that is still valid today⁴, i.e. the enzyme contains a nucleophilic zinc hydroxide species that can hydrate CO₂ in a two-step, "ping-pong" process, as follows: (i) the attack of CO₂ by the zinc hydroxide bound within the active site of the enzyme resulting in formation of bicarbonate, which is released into solution, and (ii) regeneration of the zinc hydroxide nucleophilic species by a proton transfer reaction from a zinc-coordinated water molecule to the reaction medium, which is the rate-determining step of the catalytic cycle⁴. The first investigation of anion CA inhibitors (CAIs) were also performed by Lindskog on both native and cobalt(II)-substituted enzymes⁵, demonstrating by means of electronic spectroscopic techniques (using the cobalt-enzyme) that such inhibitors coordinate to the catalytic metal ion in a tetrahedral or trigonal-bipyramidal geometry³⁻⁵. The first crystallographic studies of a CA isoform were also reported by researchers from Sweden

(Lindskog's and Nyman's groups)⁶, whereas the first complete structure, that of human (h) isoform hCA II was published in 1972⁷.

Sulphonamides were reported to be CAIs in 1940 by Mann and Keilin⁸ who also reported some side effects that were observed with the newly discovered (in 1940) antibacterial drug sulphanilamide (4-amino-benzenesulfonamide). Krebs on the other hand published the first comprehensive structure-activity relationship (SAR) study of sulphonamide inhibitors⁹ and together with Roughton, one of the co-discoverers of the enzyme, proposed CA as "a tool in studying the mechanism of reactions involving H₂CO₃, CO₂ or HCO₃⁻"¹⁰, which is role that these enzymes still have today after thousands of valuable studies that have been performed worldwide¹¹⁻¹⁵. This is probably due primarily to the fact that CAs are drug targets for a multitude of disorders, which are aspects that will be discussed later in this review.

Nowadays, eight CA genetic families are known in organisms from across the phylogenetic tree, more precisely α -, β -, γ -, δ -, ζ -, η -, θ - and ι -CAs¹⁶⁻²³. Many representatives from these classes have been fully characterised and X-ray crystallographic structures are now known for several (and in some cases many) members of the α -, β -, γ -, ζ - and θ - classes¹⁶⁻²³, which constitutes an excellent example of convergent evolution at the molecular level. However, the structures of δ -, η - and ι -CAs have not yet been reported to date.

CONTACT Claudiu T. Supuran  claudiu.supuran@unifi.it  Neurofarba Department, Pharmaceutical and Nutraceutical Section, University of Florence, Via Ugo Schiff 6, Sesto Fiorentino, Florence, 50019, Italy

© 2021 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

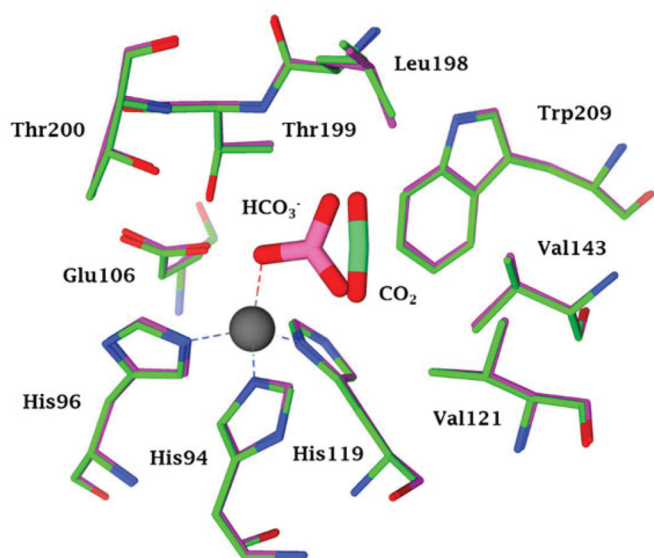


Figure 1. Active site view of the superimposed hCA II adducts with its main substrates: CO₂ (green, pdb 2VVA) and bicarbonate (pink, pdb 2VVB)²⁴. The zinc ion is shown as a grey sphere with its three coordinated protein ligands (green/red), His94, 96 and 119. Amino acid residues involved in the binding of the substrates/inhibitors, at the bottom of the active site, are also shown.

Importantly, the progress in X-ray crystallography over the last few decades has revealed the elusive binding modes of CO₂ and bicarbonate, which are the two main substrates of the enzyme^{24,25} (Figure 1). Indeed, as seen in Figure 1 for the α -class hCA II, CO₂ is bound in a hydrophobic pocket that is lined with residues Val121, Val143, Trp209 and Leu198 in close proximity (but not directly coordinated) to the zinc ion, whereas bicarbonate is coordinated to the metal ion in a monodentate fashion. Highly relevant studies from the groups of McKenna and Djinovic-Carugo²⁴ based on high pressure and cryo-crystallographic data demonstrated that the substrate binding modes are probably similar in all CA classes. Such a conclusion was further supported by crystallographic structures of β -CAs by McKenna's group²⁵, in which the binding mode of CO₂ to a β -CA expressed in the bacterium *Pseudomonas aeruginosa* (psCA3) was reported.

2. Anion inhibitors

As mentioned above, Lindskog³⁻⁵ reported some of the first anion CA inhibition studies, considering the well-known fact that many (in)organic anions have a high affinity for complexing transition metal ions in solution or within the active sites of metalloenzymes²⁶⁻³⁰. Such pioneering studies in this field, before the X-ray crystallographic techniques became widely available (i.e. around 1995), used various biophysical techniques, such as circular dichroism, electron paramagnetic spectroscopy (EPR), electronic spectroscopy (UV/Vis), and NMR techniques together with metal-substituted CAs (incorporating Co(II), Cu(II), Mn(II), Ni(II), Cd(II) and other transition metal ions which have the desired spectral/paramagnetic properties, of which Zn²⁺, a d¹⁰ metal ion, is devoid). This allowed the investigation of the interactions of CA with various anions such as cyanide, cyanate, thiocyanate, azide, halides, carboxylates, hydrogen sulphide, and more complex anions (ferrocyanide, ferricyanide, etc.)²⁷⁻³⁰. Such interesting and valuable studies were mainly reported by the groups of Coleman^{29,30} and Bertini-Scozzafava^{27,28}, which reinforced the earlier conclusions³⁻⁵

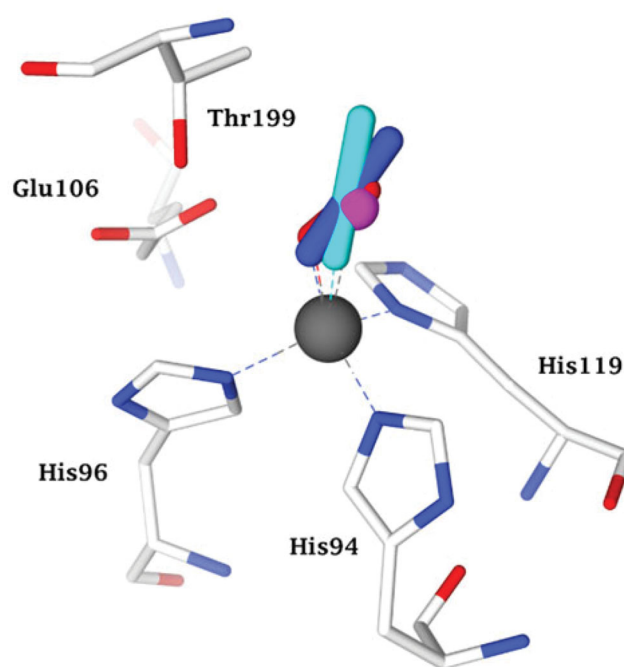


Figure 2. Representation of the superimposed X-ray crystallographic data for hCA II adducts with cyanate (cyan, pdb 4EQ³¹), bromide (magenta, pdb 1RAZ³²), azide (blue, pdb 1RAY³²), O₂ (presumably as superoxide anion or radical anion; red, pdb 5EOI³³) with a tetrahedrally coordinated active site zinc ion (grey sphere). The key active site zinc ligands (His94, 96 and 119) and "gate-keeping" residues (Thr199, Glu106) are also shown.

that the anion CAs coordinate to the metal ion in the enzyme active site in a tetrahedral or more rarely, a trigonal bipyramidal geometry²⁶⁻³⁰, as shown schematically in Figure 2 for some anion inhibitors, again using hCA II as model enzyme and high-resolution X-ray crystallography³¹⁻³³.

Clearly based on the data in Figure 2, all these anion inhibitors from significantly different chemical classes (e.g. halides, azide, cyanate and even oxygen, presumably bound as anion or radical anion³³) act as monodentate ligands to the zinc ion, which is in its usual tetrahedral geometry, with three additional ligands (residues His94, His96 and His119). All these (and other) anions have their closest atom to zinc at a distance of about 2 Å, and most of these anions show a rather low affinity (usually millimolar to micromolar²⁶) for this CA isoform. Thus, the zinc remains tetrahedrally coordinated upon inhibition by displacement of a water molecule ligand (or hydroxide) by many different anions. However, in 1993, the results of an X-ray crystallography study performed by the Liljas' group showed that³⁴ cyanate and cyanide (well known "metal poisons") do not inhibit CAs by this metal binding mechanism but instead are bound at a non-coordinating distance, presumably in the same location that the substrate CO₂ binds (n.b. the CO₂ binding mode was not precisely known at that time). Liljas presented these results in 1992 at an international bioinorganic chemistry conference in Florence prior to their publication in the following year³⁴, in which many scientists active in the CA field participated. This discovery was considered by many to be a turning point in our understanding of CA inhibition mechanisms. However, these surprising findings presented by Liljas were difficult to rationalise, especially considering the fact that Bertini and Scozzafava's groups, who continued the early research lines pioneered by Lindskog and Coleman, mentioned above^{5,29}, presented highly convincing evidence that cyanate and

cyanide coordinate to the metal ion (zinc or cobalt) in the CA active site based on data from electronic spectroscopy, EPR and NMR methods^{27,28}. One of us (CTS) proposed at the 1992 meeting that cyanate and presumably also cyanide might instead be hydrated by the CA enzyme given that these anions have a shape that is rather similar to that of the substrate CO₂, hypothesis which has been validated in an interesting computational study by Merz and Banci's groups³⁵. Although the eventual experimental validation that CAs can hydrate cyanate and cyanide was more arduous than initially thought, ultimately Maren's group and one of our groups (CTS) demonstrated that this was definitively the case³⁶. Maren's laboratory was highly skilled in measuring inhibition constants of anions, including halides, cyanate, cyanide, etc^{37,38}, as well as sulphonamides, considering that he was a co-discoverer of several sulphonamide CAs that have been used clinically for decades as well as the developer of two assay methods based on the CO₂ hydration reaction². Maren's group measured the liberation of ammonia from carbamate formed by CA-catalyzed cyanate hydrolysis³⁶. Although the ammonia formation was measured, the amount of ammonia was near the limits of detection for the method that was used and no clear-cut conclusions could be drawn. However, we proposed that cyanate once hydrolysed to carbamate within the CA active site may act as a suicide inhibitor, remaining strongly coordinated to the zinc ion³⁶, which might also explain the uncommon crystallographic results reported in ref.³⁴ The controversy was resolved years later by McKenna's group³¹ who reported a high-resolution X-ray crystal structure of hCA II with cyanate bound (Figure 2). Cyanate is directly coordinated to the metal ion just like most other anion inhibitors that have been investigated³¹. Although this definitive data was published in 2014, which should have "put the debate to rest", the egregiously incorrect conclusion from the Liljas paper of 1993³⁴ has not yet been rectified by a retraction or correction. Furthermore, the protein database files of the two adducts to hCA II with cyanate and cyanide from Liljas's group have not been deposited in the Protein Database.

However, this research line ultimately produced some interesting results. Cyanamide (H₂N-CN or NH=C=NH, as possible tautomers) is another molecule with a similar linear shape with respect to the *sp* hybridised carbon atom as cyanate and CO₂. Briganti et al.³⁹ investigated the role of cyanamide as either a substrate or inhibitor for CAs by using kinetic, electronic spectroscopic and X-ray crystallographic techniques. Again, and to the great surprise, it has been possible to demonstrate that cyanamide is initially acting as a weak inhibitor, which can then undergo a hydration reaction within the enzyme active site resulting in the formation of urea (more precisely the ureate anion), which can remain coordinated to the metal ion, as shown in Figure 3, in which other structurally-related anions, such as cyanate³¹, carbonate²⁴ and trithiocarbonate⁴⁰ are also shown superimposed.

Cyanamide (unlike cyanate) was thus proven to be a new CA substrate, which is not that surprising considering that it is isosteric and isoelectronic with CO₂³⁹. However, the most interesting result was probably that urea could not be displaced from the inhibited enzyme even by using highly effective, low nanomolar sulphonamide inhibitors, which demonstrated that ureate is probably one of the most effective CAs known to date³⁹. It should also be noted that urea (neutral molecule) is a very ineffective inhibitor (no K_i was measured up to 100 mM) probably because it cannot be readily deprotonated in aqueous solutions for enzyme measurement assays³⁹. Thus, only the deprotonated form, which is formed *in situ* through the suicide inhibition effects of cyanamide acts as a CAI, which is in fact the situation we expected

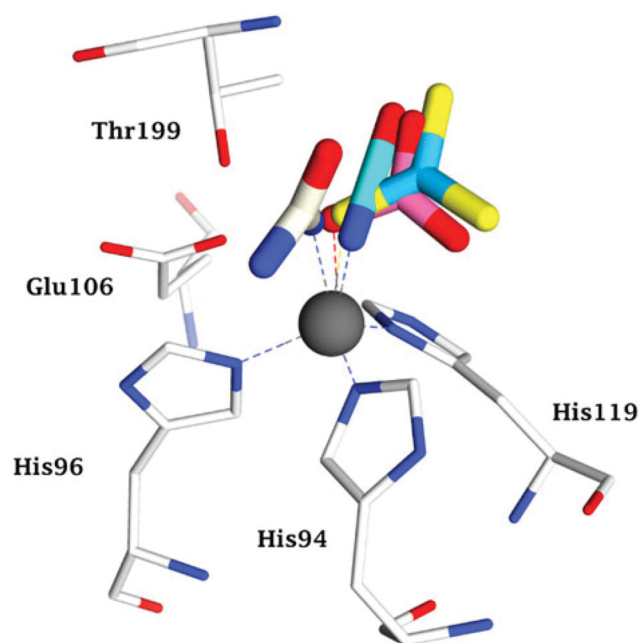


Figure 3. Representation of the superimposed hCA II adducted to cyanate (cyan, pdb 4EQ3³¹) bicarbonate (pink, pdb 2VVB²⁴) urea (as anion) (white, pdb 1BV3 [39]), and trithiocarbonate (light blue, pdb 3K7K⁴⁰). The zinc ion (grey sphere), its protein ligands (His94, 96 and 119) and gate-keeping residues (Thr199, Glu106) are also shown.

(but could not initially confirm) for cyanate. As seen in Figure 3, cyanate, carbonate and trithiocarbonate (all zinc binders) do not completely overlap in the X-ray crystal structures but they bind in a similar fashion, being monodentate ligands of the Zn(II) ion, which is in its stable tetrahedral geometry^{24,31,40}. The same geometry was observed also for the ureate adduct (Figure 3) and it was also bound in a monodentate fashion like many other small anions that have been reported to date. However, the ureate is tilted and has a very different orientation towards the gate-keeping residues Glu106-Thr199³⁹. In a subsequent study⁴¹, by using cryo-crystallography, it has been possible to "freeze" and observe the formation of the intermediates in the CA-catalyzed cyanamide hydration reaction, which revealed a new proposed reaction mechanism for this hydrolytic process.

Apart the active site metal ion, hCA II has an additional metal binding site, at its entrance⁴². Copper(II) binds rather efficiently in this additional site, being coordinated by His64, His4 and several water molecules^{33,42}. This form of the enzyme, with Zn(II) at its active site and Cu(II) at the secondary site is referred to as Zn/Cu-hCA II, in contrast to the enzyme which has zinc substituted by copper also within the catalytic centre (Cu₂-hCA II)⁴³. Recently, McKenna's group proposed that the Cu₂-hCA II also has nitrite reductase activity, with nitrite being initially coordinated (as a bidentate ligand) to the active site copper ion (Figure 4(B)). In contrast, as mentioned also above, the Zn/Cu-hCA II was observed with an O₂ molecule coordinated to zinc (Figure 4(B)), which most probably is a superoxide anion (or anion radical), as suggested by computational techniques³³. A major overall conclusion is that all serious studies performed to date on hCA II demonstrate that anions are coordinated to the metal ion in the enzyme active site.

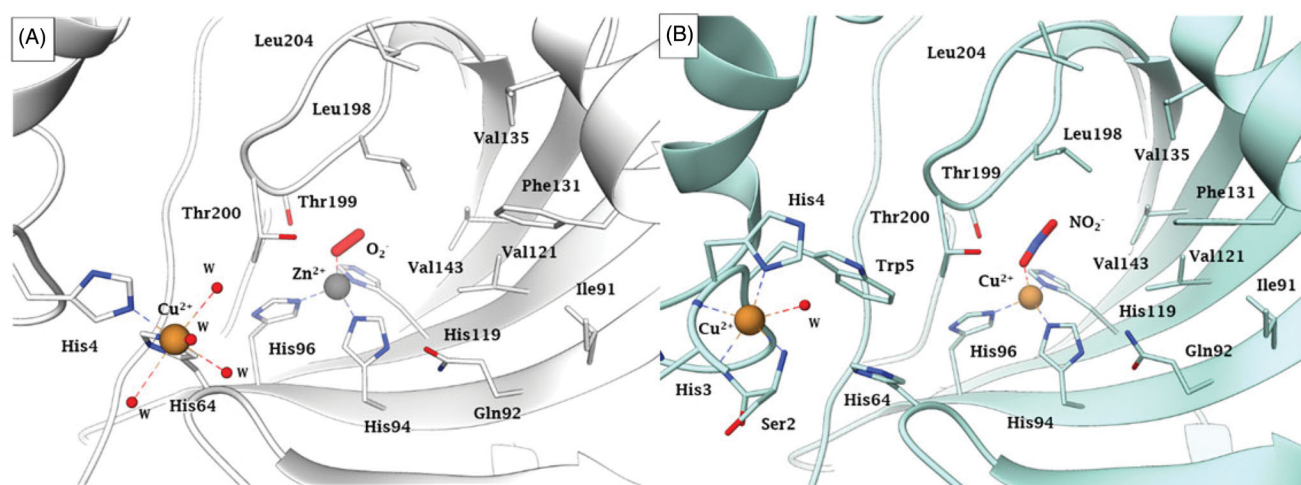


Figure 4. Representation of the binding of A) O_2^- to a $\text{Zn}^{2+}/\text{Cu}^{2+}$ hCA II (pdb 5EOI³³) and B) nitrite to Cu^{2+} substituted Cu_2 -hCA II (pdb 6PDV⁴³). Distances of the closest atom of the anion to the metal ion are: Zn-O (in the O_2 adduct) of 1.88 Å; Cu-O (nitrite adduct) of 2.14 Å.

3. Sulphonamides and other zinc binders, the tail approach

Primary sulphonamides^{44–46} were known for decades^{8–10} to potently inhibit CAs by binding as RSO_2NH^- anions to the Zn(II) ion within the enzyme active site, as shown by using various spectroscopic techniques^{5,12,13,30} as well as a crystallographic study from Liljas' group, in which acetazolamide **1**, and 3-acetoxymethyl-4-aminobenzenesulfonamide **2** (Chart 1), and the anion thiocyanate, were crystallised in complexes with hCA II⁴⁷. As for the anions mentioned in the preceding paragraphs, the sulphonamide inhibitors substituted the water/hydroxide ion acting as the fourth ligand to the tetrahedrally coordinated zinc site in a monodentate coordination with the metal ion through the deprotonated nitrogen atom of the sulphonamide moiety, resulting in a favourable hydrogen bond with Thr199, a conserved residue in most α -CAs. In contrast, thiocyanate binding was reported to result in a trigonal-bipyramidal coordination of the zinc centre because SCN^- added to the coordination sphere of the non-inhibited enzyme⁴⁷. Since then, hundreds of sulphonamides were crystallised in complexes with various CA isoforms and in all of them the sulphonamide zinc-binding group (ZBG) participates in the same interactions as mentioned above for acetazolamide (reviewed in refs.^{14,48}).

Although acetazolamide and other first generation sulphonamide CAIs (such as methazolamide, ethoxzolamide, dichlorophenamide, etc.; refer to refs.^{11,12,14} for their structures) are potent, low nanomolar CAIs, they are non-selective inhibitors¹¹, strongly inhibiting most hCA isoforms that are known to date (12 catalytically active isoforms that have been reported, hCA I-VA, VB, VI, VII, IX, XII-XIV^{11,12,14}). As a consequence, the first- and even second-generation inhibitors used clinically resulted in a range of documented side effects connected to the inhibition of off-target isoforms^{48–57}.

For such reasons, designing isoform-selective CAIs became the main issue in the revival of the CA field in the mid to late 90s for obtaining compounds with an improved safety profile and efficacy compared to the inhibitors available at that time^{58–60}. The tail approach has thus emerged in that period as an innovative modality for developing CA-selective inhibitors⁶¹. The idea is very simple: the attachment of moieties that may induce the desired physico-chemical properties (e.g. enhanced hydro- or liposolubility) to scaffolds of simple aromatic/heterocyclic sulphonamides (e.g. sulphanilamide, metanilamide, orthanilamide, and their derivatives; 5-amino-1,3,4-thiadiazole-2-sulphonamide, 4-methyl-5-

imino-1,3,4-thiadiazoline-2-sulphonamide, and many other simple sulphonamides etc.) might lead to interactions with the external part of the CA active site, and not only with residues near the zinc catalytic centre that are the most highly conserved in all hCA isoforms. Indeed, the region at the entrance of the active site cavity is the most variable between the different human isoforms⁶¹. Detailed kinetic studies were thereafter performed on over 10,000 different sulphonamide derivatives^{62–71}, which were synthesised and investigated in our laboratories and an additional 15,000 derivatives were received from academic groups and pharmaceutical companies in collaboration with the Florence group. Crystallography in complex with various isoforms was done on several hundred such derivatives. This type of research demonstrated that the hypothesis was correct and that it is possible to design sulphonamide CAIs that are selective for each isoform of interest by tailoring the dimensions (length, bulkiness, etc.) as well as the chemical nature of the various tails^{61–71}. This research led to the discovery of many new sulphonamide, sulfamate and sulfamide classes, including the ureido-benzene-sulphonamides^{70,72} as well as derivatives possessing more than one tail attached to the scaffold, such as dual-tailed or tri-tailed CAIs^{73,74}. Of the many compounds discovered in this way includes SLC-0111, compound **3**, which is a CA IX/XII-selective inhibitor that is now in clinical trials as an antitumor/antimetastatic agent (see discussion later in the text). Another notable example is the three-tailed sulphonamide **4** (Chart 1).⁷¹ Both SLC-0111 and sulphonamide **4** strongly inhibit and bind to hCA IX (Figure 5)^{71,74}.

The tail approach was virtually the only strategy used to obtain CAIs from the moment it was disclosed, and it has been adopted by research groups all over the world^{71,75–91}. For space reasons only a limited number of such research was cited here, as the field was recently reviewed^{14,48}. The advantage of the tail approach over other strategies to obtain isoform-selective CAIs are the facility of the syntheses, the versatility and possibility to introduce a variety of tails/scaffolds with different chemical properties, and thus to generate chemical diversity. As a consequence, these procedures were extended to many other classes of CAIs, not only to the sulphonamides and their isosteres (sulfamides/sulfamates, which are not discussed here in details; see refs.^{14,48} for recent reviews). These new chemotypes will be discussed in the next paragraph, together with some new CA inhibition mechanisms which emerged more recently.

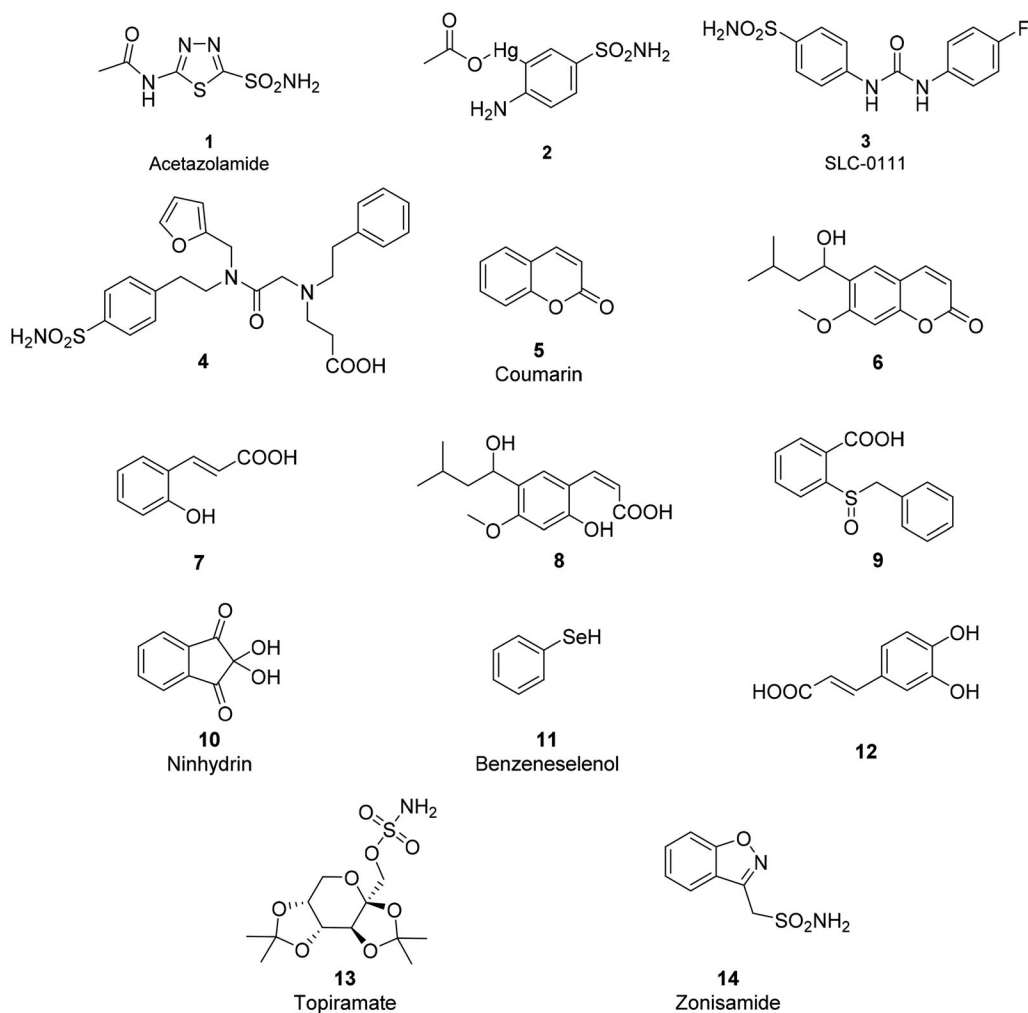


Chart 1. Structure of the compounds 1–14 discussed in this review.

4. Other chemotypes with CA inhibitory power

By comparing the sulphur-based, carbon-based⁴⁵ and phosphorus-based ZBGs, a range of novel classes of non-sulphonamide CAls which interact with the zinc ion were reported, including phosphonamidates⁹¹, dithiocarbamates⁹² and their derivatives (monothiocarbamates⁹³, xanthates and trithiocarbonates⁹⁴), selenols^{95,96}, carboxylates⁹⁷, hydroxamates⁹⁸, benzoxaboroles^{99–101}, and carbamates¹⁰². In addition, other classes of inhibitors that have been developed which exhibit a different inhibition mechanism compared to the zinc binders discussed above. It should be noted that many of the new chemotypes bind further away from the metal ion, interacting both with the hydrophobic and hydrophilic halves of the CA active site^{14,48}. These new inhibition mechanisms include:

- i. CAls that anchor to the zinc-coordinated water molecule^{103,104}. Polyamines, such as spermine and its derivatives¹⁰³, the simple phenol¹⁰⁴ and many of its derivatives^{105–108} together with polyphenols¹⁰⁹ inhibit CAls in this way. This inhibition mechanism is also shared by the sulfocoumarins and their derivatives, as demonstrated by Zalubovskis group^{110–113} using kinetic and crystallographic

studies. These classes of highly innovative CAls were developed by the Riga group^{110–113} starting from coumarins and their derivatives as a class of inhibitors with a totally new inhibition mechanism, which has been unravelled by some of us^{114–124}. Thioxocoumarins are the only exception, as they bind similar to the phenols, anchoring with their exocyclic sulphur atom to the zinc coordinated water molecule¹¹⁴.

- ii. Coumarins and many of their derivatives such as mono- and dithio-, as well as seleno/tellurium-coumarins but also many other structurally related compounds^{115–124} were shown to possess a very particular inhibition mechanism. This was demonstrated for the simple coumarin **5** and one of its derivative (compound **6**), a natural product isolated from the Australian plant *Leionema ellipticum*^{115,116}. The lactone ring of the coumarin is hydrolysed by the esterase activity of CA, leading to the formation of *cis*- or *trans*-2-hydroxycinnamic acid derivatives, of types **7** and **8** (Chart 1), which thereafter bind far from the zinc ion, at the entrance of the active site cavity (the region which is more variable among the different human isoforms, as mentioned above). As a consequence, coumarins and their derivatives behave as pro-drug inhibitors, but also show a very high selectivity for many CA isoforms, depending on the substitution pattern on their bicyclic ring system^{115–124}. Many representatives belonging

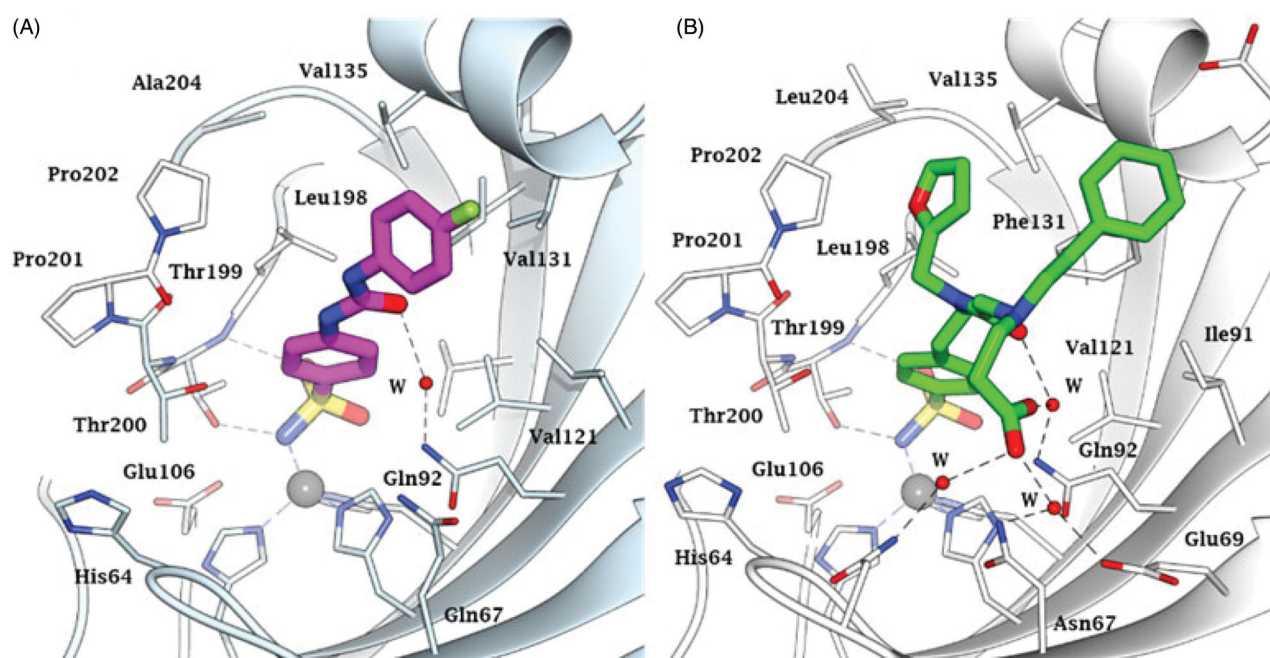


Figure 5. Active site view of A) hCA IX catalytic domain adducted to SLC-0111 (magenta)⁷⁴ and B) hCA II adducted to the three tailed inhibitor 4 (in green).⁷¹ H-bonds are represented as black dashed lines. The Zn(II) ion (grey sphere) and some important amino acid residues involved in the binding of inhibitors are shown.

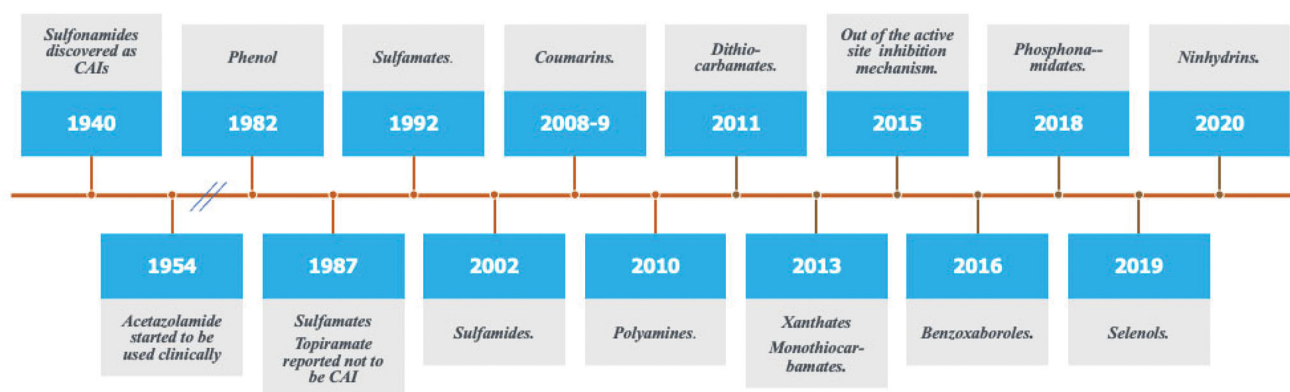


Figure 6. Historical overview on the discovery of the main chemotypes with CA inhibitory activity.

to this family of CAIs in fact possess extremely good selectivity ratios for inhibiting isoforms of interest (e.g. CA IX and XII) versus highly distributed, “house-keeping” off-target isoforms, such as CA I and II^{115–124}.

- iii. Probably one of the strangest CA inhibition mechanism is the one reported by De Simone’s and Carradori’s group for a benzoic acid derivative (compound **9**) which binds outside the active site cavity, blocking the His64 residue (the proton shuttle residue) in its *out* conformation¹²⁵. Again, X-ray crystallography was crucial for demonstration of this highly interesting CA inhibition mechanism.

An overview of the main new chemotypes acting as CAIs discovered up until now is shown in Figure 6, in which the “fresh” discovery of the ninhydrins is also mentioned¹²⁶. Indeed, ninhydrins, such as compound **10**, possess a *gem*-diol stable functionality which has been shown by means of kinetic and computational techniques to coordinate to the metal ion from the enzyme active site¹²⁶. Furthermore, Figure 7 shows the X-ray crystal structures of

some of the new chemotypes detected in the last period, such as the selenol **11**⁹⁵ (Figure 7(A)), unsubstituted ninhydrin **10**¹²⁶ (Figure 7(B)) and a catechol derivative **12**¹²⁷ (Figure 7(C)) which has been recently reported to bind to hCA II by a new inhibition mechanism. Like many phenols, the catechol **12** is anchored to the zinc-coordinated water molecule, but being a diphenol, the second OH moiety additionally binds to the “deep water” molecule in the active site. As mentioned above, selenol **11** and the ninhydrin **10** (Chart 1) directly coordinate to the zinc ion in the enzyme active site (Figure 7). Many of these inhibition mechanisms were thoroughly reviewed in ref.¹²⁸.

5. New applications of the CAIs

For the last 65 years the CAIs were used as diuretics^{2,51,53,55} and anti-glaucoma agents^{2,56–59}, and they still have a firm place for the management of these conditions^{53,57}. However, in the last decade, several other highly relevant applications emerged, probably due to the availability of much more isoform-selective

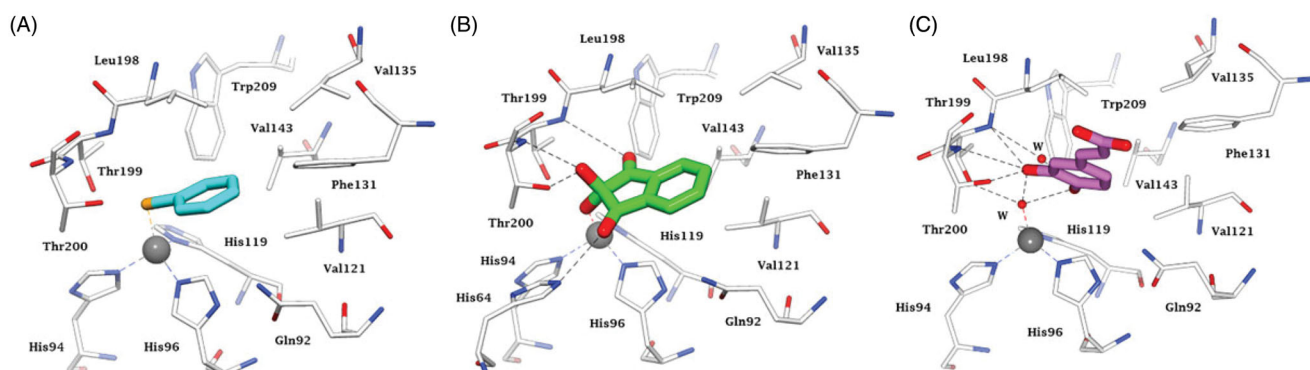


Figure 7. Active site view of hCA II complexed to A) selenol **11** (pdb 6hX5)⁹⁶, B) ninhydrin **10** (predicted *in silico*)¹²⁷ and C) catechol derivative **12** (pdb 6YRI)¹²⁸. H-bonds are represented as black dashed lines. The Zn(II) ion (grey sphere) and residues involved in its coordination and some active site residues near the binding of inhibitors are shown in CPK colours. Water molecules are shown as red spheres.

hCAs are involved in many physiological processes such as:

- Respiration
- Transportation of CO₂ and bicarbonate
- pH and CO₂ homeostasis
- Electrolyte secretion in various tissues
- Biosynthetic reactions
- Gluconeogenesis
- Adipogenesis
- Ureagenesis
- Bone resorption
- Calcification

hCAs as Therapeutic Targets for

Edema hCA I, II	Glaucoma hCA II, IV, XII	Epilepsy hCA II, VII, XIV
Obesity hCA VA/VB	Hypoxic Tumors hCA IX, XII	Inflammatory diseases hCA IV, IX, XII
Neuropathic pain hCA II, VII	Alzheimer disease unknown	Oxidative stress hCA III

Abnormal levels or activities of these enzymes have been often associated with several human diseases

Figure 8. hCAs as drug targets^{11,14,51–57}. From edoema and glaucoma, to obesity, neuropathic pain, hypoxic cancers, cerebrovascular diseases and oxidative stress, many isoforms are involved in diverse pathologies for which isoform-selective inhibitors showed a relevant potential to be translated to clinical entities.

inhibitors. They include the use of CAIs such as acetazolamide **1**, topiramate **13** and zonisamide **14** as antiepileptics^{129,130} and anti-obesity agents^{54,131,132}, targeting diverse isoforms (CA II and VII for the antiepileptic use, and CA VA/VB as antiobesity agents)^{129–132}. In fact, as seen from Figure 8, most of the CA isoforms, alone (or in combinations of few of them) possess therapeutic/pharmacological applications.

In fact, nowadays the CAIs were proposed to be used or are already used for the management of the following pathologies, in addition to the classical ones mentioned above: (i) treatment of idiopathic intracranial hypertension, by targeting hCA I and II but presumably also brain/cerebrospinal fluid isoforms¹³³; (ii) cerebral ischemia/diabetic cerebrovascular pathologies (the targeted isoforms seem to be hCA IX and XII, possibly also hCA VA)¹³⁴; (iii) neuropathic pain, by targeting the brain/peripheral nervous system isoforms hCA II and VII^{135,136}; (iv) inflammation/arthritis, by targeting hCA IX/XII and possibly hCA IV^{137–140}; (v) hypoxic tumours, by targeting the two isoforms discovered to be associated with tumours, hCA IX¹⁴¹ and hCA XII¹⁴². This is in fact the field in which the highest number of studies have been published in the last decades^{143–155}. Most of the proof-of-concept studies which validated the various isoforms for the management of such widespread diseases were conducted either in Florence or by

collaborations with well-known groups worldwide, which used the compounds developed in Florence for these studies. Highly important for the success of these investigations was the fact that the X-ray crystal structures of almost all hCA isoforms (except hCA VB, X and XI) were published in the last 15 years^{156–161}. This allowed for highly focussed drug design campaigns in order to obtain isoform-selective compounds for all these enzymes, and again using the tail approach⁶¹ and a variety of newly discovered chemotypes, discussed above, were essential for the success of these efforts.

6. CA activators and their applications

The carbonic anhydrase activators (CAAs) have been the focus of significant controversy for a long time up until 1997 when the first X-ray crystal structure of a complex of an activator (histamine) bound to hCA II was reported and the activation mechanism was revealed in molecular level detail¹⁶². The activator, which was observed to be directly bound within the enzyme active site, promotes the formation of an enzyme – activator complex, in which the proton shuttling moieties present in the activator participate in the rate-determining step of the catalytic cycle, i.e. the transfer

of a proton from the zinc-coordinated water to the external reaction medium, similar to the natural proton shuttle, which is residue His64 (in many CA isoforms), as mentioned above^{162,163}. In such enzyme-activator complexes, the proton transfer is intramolecular, being more efficient compared to the intermolecular transfer to buffer molecules, which are not bound within the enzyme cavity^{162,163}. Many X-ray crystal structures with amines and amino acid activators were reported thereafter, in addition to histamine¹⁶², including L- and D-His bound to hCA II and hCA I, L- and D-Phe, D-Trp, L-adrenaline and pyridinium derivatives of histamine bound to hCA II, which confirmed this general CA activation mechanism outlined above^{164–167}. The thirteen catalytically active mammalian CAs (e.g. CA I-VA, VB, VI, VII, IX, XII-XV) were also investigated for their interaction with a rather large library of amino acids and amines^{168,169}, whereas several drug design studies have also been performed^{170–174}. Why are these studies relevant? In the last few years it has been demonstrated that CAAs of the amino acid type (e.g. D-Phe) can enhance memory and learning, which is antagonised by the simultaneous administration of a CAI of the sulphonamide type (e.g. acetazolamide)¹⁷⁵. A recent study showed that administration of the D-Phe to rats rapidly activated the extracellular signal-regulated kinase (ERK) pathways, which are involved in critical steps of memory formation, both in the cortex and the hippocampus, two brain areas crucially involved in memory processing^{175,176}. Even more recently, the same groups (including the Florence team) demonstrated that CAAs play a crucial role in extinction of contextual fear memory¹⁷⁷, opening the way towards the validation of CAs as new targets for improving cognition, but also in therapeutic areas, such as phobias, obsessive-compulsive disorder, generalised anxiety, and post-traumatic stress disorders, for which few efficient therapies are available to date^{175–177}.

7. Discussion and conclusions

The drug design landscape for modulators of CA activity, both inhibitors and activators, appears to be a dynamic and healthy field, in which many relevant advances have been achieved in the last several decades through continuous contributions from many research groups, as outlined above. However, recently an event disturbed this situation. In an egregious polemic from Jonsson and Liljas¹⁷⁸, the measurements of some CA inhibition data were queried, in addition to some other aspects that will be discussed later in this article. This action was based on some typographical errors found in Supplementary Information from 5 papers from our group (among the > 1600 that have been published). We have replied to the above-mentioned article¹⁷⁸ in an Editorial published in the same journal¹⁷⁹ as well as two additional review articles^{153,180}. Jonsson and Liljas replied to our editorial¹⁷⁹ with a new comment published recently¹⁸¹. Furthermore, Liljas “promoted” their initial article¹⁷⁸ by writing a denigrating and arrogant letter to the president of the University of Florence about one of us (CTS), stating that he is, “bothered about how long his [Supuran’s] poorly performed science could continue without serious criticism”, and “I followed Supuran’s crystallographic investigations with horror”, but no technical comments regarding our presumed crystallographic errors were mentioned in this letter or in their papers, although Liljas himself is a crystallographer. We worked in this field in collaboration with well-established crystallographers from several laboratories in Italy, Germany, USA, Australia and Singapore over the years¹⁷⁸. Liljas continued, “the measurements are so poorly done that an undergraduate student reporting these in his works should have failed” and “we tried to publish our

manuscript in the journals where Supuran has published his work but failed. This covers a good number of journals”, meaning probably that serious journals did not take into consideration his poorly formulated polemic. Liljas ends his letter to the rector: “The work by Supuran that we criticise needs to be repeated. There may be really excellent inhibitors among the hundreds of compounds that he has studied, but his analysis does not give a trustworthy account. It is important that the field and his co-workers become fully aware of the quality of his analyses and to know that much has to be done again”. In other words, this scientist who published completely erroneous conclusions based on crystallography data as shown here for the cyanate and cyanide examples³⁴ and elsewhere by others³¹ and who has not withdrawn or corrected these published results after 27 years, finds it insufficient to publish his conceptually erroneous polemic¹⁷⁸, but also wants to defame our group to our institution. We have replied to the technical issues raised by Jonsson and Liljas, as mentioned above. In their comment on our reply¹⁸⁰ they indicated they were “happy by our prompt response” and that some clarifications were achieved¹⁸¹, however now their criticism relating to the measurement of inhibition constants was extended to the entire medicinal chemistry community, not only to our group, stating that the “original article was not intended to raise specific concerns regarding just one research group but rather was intended to raise concerns regarding medicinal chemistry publications in general, and we are glad that Prof. Supuran agrees that there is room for improvement!” CTS hardly agrees with anything from their analysis. Such a strong and prejudicial opinion from someone without experience in the medicinal chemistry field is disappointing. Notably, the “best practice” examples for the measurement of K_i s that Jonsson and Liljas provide in their reply¹⁸¹ are the highly controversial articles from Johnson & Johnson regarding topiramate **13**^{182–185}. Indeed, topiramate was initially reported to not be a CAI¹⁸², then a millimolar inhibitor¹⁸³, then a micromolar inhibitor¹⁸⁴ until finally it was established to be a sub-micromolar inhibitor¹⁸⁵ by the same group of Johnson & Johnson who initially discovered the compound. We have reported the X-ray crystal structure of this important drug bound to hCA II and demonstrated that it is a low nanomolar inhibitor for many CA isoforms¹⁸⁶. There are also other unusual comments from Jonsson and Liljas¹⁸¹ regarding the native mass spectrometry measurements. Presumably, the main issue with this method is that it can be used to measure dissociation constants that are in very good agreement with the stopped flow method used in Florence, in addition to other methods such as isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC)^{187,188}.

As noted above, there are no original contributions in the literature from Jonsson and/or Liljas regarding the measurement of inhibition constants (for CAs or any other enzyme) nor for any drug design studies targeting CAs or other druggable proteins. Jonsson is a biochemist who published several papers in the CA field and Liljas was a crystallographer (now retired for some time), who, as we mentioned earlier, contributed some important X-ray crystallographic studies in the 70s and 80s on CAs and then some questionable CA data in the 90s (as discussed above). It should be noted that there is no analysis at all of our crystallographic work in the two papers by Jonsson and Liljas^{178,181} or in the letter to the rector, from more than 250 papers that we have published in that field, as mentioned here and elsewhere^{12,14}. However, the polemic by Jonsson and Liljas¹⁷⁸ has been supported by another scientist, Matulis, based on his recent reference to this work¹⁸⁹.

Matulis entered the CA field in 2005 when he published his first paper with the Johnson & Johnson group¹⁹⁰, on a fluorescent thermal shift assay (FTSA) for measuring CA inhibition. In this method, the extent of protein unfolding is monitored as a function to temperature when heated, alone and in the presence of inhibitors, providing a way to measure the affinity of inhibitors as well as some thermodynamic parameters. This method requires high concentrations of enzyme (around 5–10 μ M), it uses a very high concentration (50 μ M) of a fluorescent dye, ANS (8-anilino-1-naphthalene-sulphonate) as well as a 50 mM phosphate buffer¹⁹⁰. We and others have demonstrated that sulphonates can be strong inhibitors of the CAs¹¹⁰, and phosphate is also an anion CA inhibitor²⁶. Just recently one of us (AA) retested phosphate as an inhibitor of hCA I and II at a concentration of 10 nM to 100 mM by the stopped-flow assay. At 50 mM, an inhibition of 13.0% of the activity of hCA I and 19.1% of the activity of hCA II was measured (unpublished results from our laboratories). Thus, the competition of the buffer, dye and inhibitor for binding to the metal ion (or elsewhere in the CA active site), coupled with the large concentrations of enzyme that is required results in this method providing information of limited usefulness regarding CA activity. FTSA was however extensively used in the subsequent Matulis papers, in combination with ITC, which as mentioned by Linkuviene et al. is one of the “most robust techniques” for measuring interactions between proteins and small molecule ligands in drug design¹⁹¹. ITC also uses high concentrations of enzymes in the assay system (6–20 μ M), and as mentioned in the Matulis papers the buffer is again 50 mM phosphate plus 100 mM NaCl. In addition to phosphate, chloride is also a CAI that binds to the metal ion active site²⁶. Chloride is even more inhibitory than phosphate, as documented earlier by many groups. The K_i of this anion is 6 mM against hCA I, 200 mM against hCA II, 1.8 mM against hCA VII, 138 mM against mCA XIII (m = murine enzyme), 33 mM against hCA IX and 0.8 mM against hCA XIV¹⁹². Thus, it is unclear how robust the inhibition data is in these ITC studies when such high concentrations of CA anion inhibitors are being used.

In the subsequent papers from Matulis' group^{193–198}, all of which were inspired by the tail approach (see above), reported by us, a combination of several CA inhibition assays were performed, including the FTSA method¹⁹⁰ and the ITC assay mentioned above, in addition to the stopped flow assay, which members of the Matulis group learned in 2014 in Florence at the request of Matulis (and we accepted them and taught them the CO₂ hydrase assay developed originally by Khalifah¹⁹⁹ which has been used in Florence since 2001). Notably, the stopped flow assay uses nanomolar concentrations of CA in the system and buffers such as Hepes or Tris at 10–20 mM (which do not interfere with the metal ion), phenol red (at a concentration of 0.2 mM as an indicator) as well as low concentrations of salts to maintain a constant ionic strength (typically 20 mM NaClO₄, which is not inhibitory to most CAs^{26,192}). Needless to say, all the methods used by Matulis gave very different results, and it is often difficult to understand which of them should be considered for evaluating the inhibitors from his laboratory.

Thus, the next step of Matulis' research was to investigate the so called “intrinsic” parameters for characterising the binding of inhibitors to various CA isoforms (there is a series of 24 papers in which “intrinsic” is the keyword, repeated with great insistence, but we will consider only the one in which the method is presented in detail²⁰⁰). The main issue with the measurements of CA inhibition from all other laboratories around the world and in all historical periods, is according to Matulis that “many protein-ligand binding reactions are linked to protonation/deprotonation

reactions or various conformational changes”, which were not considered by other researchers. N.b., conformational changes are not considered in this “intrinsic” parameter model either. Thus, the measurement of the energetic contributions for enzyme-inhibitor complex formation is considered to be correct only by considering additional contributions such as protonation/deprotonation reactions of the ligand and the protein. In this way, the observed parameter (e.g. K_i , ΔH , ΔG , etc.) must be “corrected” to obtain the “intrinsic” value (K_i^{in}) by dividing the observed parameter (K_i^{obs}) by the product of the fraction of deprotonated sulphonamide (f_{sulf}) and the fraction of enzyme in the protonated form (f_{CA})²⁰¹. In other words, $K_i^{\text{in}} = K_i^{\text{obs}} / (f_{\text{sulf}} \times f_{\text{CA}})$. The theoretical considerations for this “correction” of the observed K_i values are based on an old but correct work from Khalifah's group²⁰² in which the binding of imidazole and related compounds to hCA I was investigated. Based on this work Matulis proposes the “intrinsic” parameter model considering that “only the deprotonated form of the sulphonamide binds to the CA active site, and that, the Zn-coordinated hydroxide must be protonated before it can be replaced by the amino group of the sulphonamide”. He cites the above-mentioned Khalifah paper²⁰² (based on data for imidazole, which binds in neutral form to the enzyme and does not interact with the metal ion or the water/hydroxide coordinated to the metal ion²⁰²) to justify his model for sulphonamides, which is erroneous for several reasons which we will detail here:

- i. Apart the deprotonation of the sulphonamide moiety of the inhibitor and the protonation of the zinc hydroxide species of the enzyme, no hydrogen-bonding or hydrophobic interactions are considered at all in the “intrinsic” model. The Khalifah hypothesis²⁰², which is outdated, was appropriate for investigating imidazole and related azoles as CAIs but was never applied to sulphonamides. In fact, in a very important study from Klebe and Cavalli's groups²⁰³ it has been demonstrated by using ITC, X-ray crystallography and molecular dynamics (MD) simulations that hydrophobicity is the key factor for the binding kinetics of sulphonamide inhibitors to this enzyme. In fact, by combining the three techniques mentioned above, Klebe and Cavalli's groups²⁰³ showed that the protein-sulphonamide association rate (k_{on}) dramatically increases with the hydrophobicity of the inhibitor, thus demonstrating the existence of a pre-binding stage in which the enzyme-inhibitor adduct is stabilised by the hydrophobic half of the CA active site²⁰⁴. Such hydrophobic interactions are the only ones occurring in the so called “F conformation” of the sulphonamide inhibitor adduct, which in its neutral form (RSO₂NH₂) makes a hydrogen bond to the zinc-coordinated water, which is similar to the phenols or polyamines binding to the enzyme by the zinc-anchoring mechanism discussed above¹²⁸. Then the “S state” emerges, which is characterised by geometries that allow hydrogen bonding of the still neutral SO₂NH₂ moiety of the inhibitor with Thr199, Thr200, and finally the Zn²⁺-bound hydroxyl ion (not to zinc-water as in the Matulis model). Thus, the sulphonamide approaches hCA II in its neutral state (not deprotonated as in the Matulis model), binds to the hydrophobic patch, and in the end interacts with the Zn²⁺ ion, being deprotonated nearby it, before finally coordinating to the zinc but only if the distance between the terminal NH and the zinc ion is within about 2.50–2.75 Å, i.e. the final thermodynamic minimum²⁰³. These highly relevant data, which are in total contrast with the entire Matulis “intrinsic” theory, were thereafter validated by interesting work from

- the groups of Klebe^{205,206} and Whitesides^{207–209}, and are supported by our analysis of the hydrophobicity of the CA active site²⁰⁴ as well as the report of several classes of inhibitors which anchor to zinc-bound hydroxide/water molecule¹²⁸. Whitesides' group elegantly demonstrated using ITC, crystallography and MD that the differences in binding between homologous sulphonamide ligands stem from changes in the number and organisation of active site localised water molecules rather than (or perhaps in addition to) the release of structured water from the apposed hydrophobic surfaces²⁰⁷. Again, the hydrophobic effects, not at all considered in the Matulis intrinsic model, are the main players in the inhibition process. In a subsequent study²¹⁰ again from the very famous laboratory of Whitesides, it was demonstrated that hydrophobicity is essential also for the binding of anion inhibitors (never investigated by Matulis). It was shown that the binding of the Hofmeister anions is determined by where, and how strongly, they associate with concavities on the surfaces of proteins and how, upon binding, they alter the structure of water within those hydrophobic concavities. It should be stressed here again that in the "intrinsic" method of Matulis, the measurements are done in 50 mM phosphate buffer and 100 mM NaCl, and that these anions are both inhibitory to CAs^{26,192}, as detailed above, because they bind the zinc ion sometimes with a rather relevant affinity, but this phenomenon is never considered properly, and leads to erroneous measurements (apart from the conceptually wrong model used, see above and below).
- ii. Matulis considers that sulphonamides bind to the zinc ion in the CA active site with the sulphonamide group in the deprotonated, negatively charged form, to the water bound form (the so-called acidic form) of the enzyme, which as shown above, is not correct. Although noting that the association rates of sulphonamides to CA are slow, he considers this as "unexpected" considering that association is driven by a strong electrostatic interaction between the negatively charged sulphonamide and the positively charged Zn^{2+} . Again, only electrostatic and acid-base equilibria are considered and not any hydrogen bonding and/or hydrophobic contacts, which are as important (or maybe even more important) than the electrostatic interactions in this context. This leads to the discovery of supposedly "kinetic – selective CAIs"²¹¹. For decades, the kinetics of association (k_{on}) and dissociation (k_{off}) of the sulphonamide inhibitors to hCA II were well known^{212,213}. Maren and his group reported the kinetic values for more than 25 sulphonamides directly by measurement of association rate constants (k_{on}) and equilibrium constants (K_i), which were used to measure the dissociation rate constants, k_{off} ^{212,213}. The values for k_{on} ranged from 0.003 to $31 \times 10^6 \text{ L}^{-1} \text{ M}^{-1}$, whereas the k_{off} range was exceedingly low, 0.01–0.05 s^{-1} . Thus, the inhibitory activity was entirely influenced by the association rate k_{on} as also detailed by the groups of Klebe, Cavalli and Whitesides^{203–210}, which depends primarily on hydrophobic effects. That is, another major error in the analysis of Matulis and colleagues is the lack of considering the hydrophobic effects when analysing the kinetics of association of the sulphonamide inhibitor within the active site. By analysing such low dissociation rates, they reported several ligands to be "kinetic-selective" against certain CA isozymes²¹¹. Such a proposal is completely at odds with the well-established selectivity of CAIs for 15 CA isoforms from vertebrate organisms that is based on extensive thermodynamic measurements by
- groups from all over the world. Will the inhibitors reach the thermodynamic equilibrium, or will they "hurry up" to selectively inhibit some isoforms for which the association rates are faster, and in this way provide novel therapeutic applications, as suggested²¹¹? We leave the answer to this question to the reader.
- iii. Matulis suggested that the observed experimental inhibition constants should not be directly translated into a structure – activity relationship as done by all medicinal chemists, instead proposing that the observed gain in inhibitor potency might be attributed to an increase in pK_a for the ionisable pharmacophore (presumably deprotonation of the sulphonamide moiety, the only pharmacophore that he has explored) and a corresponding "jump" in the observed affinity instead of to an improvement in ligand-protein interactions. Based on such a model, the explanation he gave for the difference in inhibition between $CF_3SO_2NH_2$ (a very potent CAI, K_i of 2 nM against hCA II, as measured by Maren's group and confirmed by others²¹³) and $CH_3SO_2NH_2$ (a very weak CAI, K_i of 0.1 mM against hCA II, again data from Maren and confirmed by other groups²¹³) is dubious. Matulis predicts a 6-fold difference in the K_i values between the two sulphonamides based on this theory, whereas the actual measured difference is 5 orders of magnitude. Thus, according to Matulis, only the quantification of intrinsic parameters may allow for the correct understanding of the inhibition mechanism. However, this method has another major issue: it works only with aromatic/heterocyclic primary sulphonamides (the data on aliphatic sulphonamides is illustrated above). All other inhibitor classes (and even a cyclic sulphonamide such as saccharin²¹⁴) cannot be measured with this approach (personal communication from Matulis to CTS). In fact, there are no studies from his group on other types of inhibitors apart primary sulphonamides. As showed here, there is a large variety of other chemotypes with potent CA inhibitory action, and their inhibition effects can be measured and compared well with the sulphonamides by using the stopped-flow CO_2 hydrase assay queried by Liljas and referred to by Matulis. Note that other groups (Klebe, Whitesides, etc.) which use ITC and other techniques, have not queried the measurements done in our laboratory. So, what is the real problem?
- Although we collaborated in the past with Matulis, his behaviour towards some of us should be discussed here. In 2013 at a conference organised by De Simone and one of us (CTS) in Naples, Italy, Matulis was invited as speaker and presented the ITC inhibition method. CTS asked whether the high concentration of sulphonate dye and the sulphonamide inhibitor will all compete for the same binding site and asked whether these effects were considered? There was not an adequate reply from Matulis. Later, during that conference, another author of this paper (RZ) was approached by Matulis who asked him to send him all his compounds that had been tested in Florence, so that he can check if the inhibition data were correct. RZ was shocked by the request and did not send the compounds to his group in Vilnius. The next year, in 2014, as already mentioned here, Matulis asked CTS to host some of his group members in Florence in order to learn the stopped flow assay. CTS agreed, and two students from Matulis' group spent several months in Florence and learned the assay. In early 2015 after their stopped flow assay was working in Vilnius, Matulis wrote an e-mail to RZ asking him to no longer collaborate with the Florence group because "they do the stopped-flow

measurements in an incorrect manner". RZ replied to Matulis copying CTS and indicated his refusal to collaborate. Only at that point, considering this unethical behaviour, CTS decided to stop any interaction with this colleague.

Returning to the Jonsson and Liljas polemic¹⁷⁸, although these two colleagues have not made contributions to anticancer drugs or drug design, they questioned the drug candidate, SLC-0111, which is in Phase Ib/II clinical trials for the treatment of advanced, metastatic solid tumours²¹⁵. Jonsson and Liljas state that "it is doubtful that SLC-0111 has the required K_i to advance to clinical trials" and that the "commercial interests of pharmaceutical companies and patients may be hurt"¹⁷⁸, although the paper in which we describe the compound⁷² is not correctly cited and there are no new data on this compound in their commentary. SLC-0111 was reported by the Supuran group⁷² in efforts that spanned many years during which thousands of compounds were synthesised, assayed and characterised by X-ray crystallography and other biophysical techniques. SLC-0111 belongs to a class of ureidobenzene sulphonamides which were also assessed for their "druggability" criteria, including ADME (Absorption, Distribution, Metabolism, Excretion). Extensive *in vitro* and *in vivo* analysis in appropriate cancer models were then performed in Florence and other laboratories. Although the targets of this compound, CA IX/CA XII, are highly expressed within the hypoxic niches of solid tumour cell population, these hypoxic cells have the properties for self-renewal^{216–218}, migration/invasion^{219–221}, survival in acidic tumour microenvironment^{222–227}, and significantly contribute to resistance to chemo-, radio-, and immune-therapies^{228–236}. The combination of SLC-0111 and other CAIs with chemotherapy agents^{237–239}, immunotherapy^{240–242} and radiotherapy^{243,244} has been investigated, including SLC-0111, in combination with proton pump inhibitors²³⁶, antimetabolites²³⁷, cisplatin²³⁸, APE1-Ref-1 inhibitors²³⁹ and histone deacetylase inhibitors²⁴⁰, which resulted in excellent synergistic antitumor/antimetastatic effects. Such studies showed a synergistic effect between the CAI and the second antitumor agent, but also the lack of endothelial toxicity²⁴¹, which are highly important and desirable features for a sustained therapeutic response. The extensive studies reported in these and other papers, provided solid positive pre-clinical data to warrant the initiation of Phase 1 clinical trials in 2014, of which a Phase 1 safety trial with SLC-0111 (as a monotherapeutic agent), has been completed²¹⁵ and a Phase 1b trial is currently underway to evaluate SLC-0111 in combination with gemcitabine in metastatic pancreatic cancer patients whose tumours are CA IX positive (ClinicalTrials.gov Identifier: NCT03450018).

Other groups also used SLC-0111 in various biomedical studies in which selective inhibition of some CA isoforms was needed. These include the effects on prostate cancer cells of SLC-0111 alone or in combination with daunorubicin²⁴², radiobiological effects of CA IX inhibition in human breast cancer cells²⁴³, microvascular endothelial cell pH regulation²⁴⁴, glycolysis and migration suppression in pulmonary microvascular endothelial cells^{245,246}, involvement of CA isoforms in mitochondrial biogenesis and in the control of lactate production in human Sertoli cells²⁴⁶. Very recently, SLC-0111 was combined with 3-O-acetylbetulin for the treatment of CA IX-positive tumours by Vordermark's group, again with excellent synergistic effects²⁴⁷. Dedhar's group recently reported the interconnection between CA IX inhibition and the amino acid and acid/base transporters in hypoxic tumours²⁴⁸ as well as the possibility to overcome the adaptive resistance to KRAS and MEK inhibitors by using such anticancer agents in combination with SLC-0111²⁴⁹. All these studies confirmed the

usefulness of this clinical candidate, SLC-0111, in tumours and other biomedical conditions. Although Jonsson and Liljas presumably read our reply¹⁸⁰, in their reply to our reply they continue that they, "would have liked to see a properly reported inhibition study of this compound to make it obvious that it indeed is a good enough inhibitor to enter Phase I/Phase II studies"¹⁸¹.

Thus, the CA field seems to be now in a typical ancient Greek tragedy situation: we have the *Deus ex machina* (the couple Jonsson and Liljas) who decide who is doing good and who is doing bad experiments (without however having any documented expertise in enzyme inhibition measurements) and then we have the *Computantis* (accountant, Matulis) who needs to check the measurements from other laboratories, and asking (in the rare occasions when he acted as reviewer to our papers) for a huge amount of supporting information data that are without any usefulness to anybody. Is this dogmatism or science?

Disclosure statement

All authors declare no conflict of interest except FC and CTS who are among the discoverers of SC-0111.

ORCID

Alessio Nocentini  <http://orcid.org/0000-0003-3342-702X>
 Andrea Angeli  <http://orcid.org/0000-0002-1470-7192>
 Simone Carradori  <http://orcid.org/0000-0002-8698-9440>
 Clemente Capasso  <http://orcid.org/0000-0003-3314-2411>
 Claudiu T. Supuran  <http://orcid.org/0000-0003-4262-0323>

References

1. Meldrum NU, Roughton FJ. Carbonic anhydrase. Its preparation and properties. *J Physiol* 1933;80:113–42.
2. Maren TH. Carbonic anhydrase: chemistry, physiology, and inhibition. *Physiol Rev* 1967;47:595–781.
3. Lindskog S, Malmstrom BG. Metal binding and catalytic activity in bovine carbonic anhydrase. *J Biol Chem* 1962; 237:1129–37.
4. Lindskog S, Coleman JE. The catalytic mechanism of carbonic anhydrase. *Proc Natl Acad Sci USA* 1973;70:2505–8.
5. Thorslund A, Lindskog S. Studies of the esterase activity and the anion inhibition of bovine zinc and cobalt carbonic anhydrases. *Eur J Biochem* 1967;3:117–23.
6. Strandberg B, Tilander B, Fridborg K, et al. The crystallization and x-ray investigation of one form of human carbonic anhydrase. *J Mol Biol* 1962;5:583–4.
7. Liljas A, Kannan KK, Bergstén PC, et al. Crystal structure of human carbonic anhydrase C. *Nat New Biol* 1972;235: 131–7.
8. Keilin D, Mann T. Carbonic anhydrase. Purification and nature of the enzyme. *Biochem J* 1940;34:1163–76.
9. Krebs HA. Inhibition of carbonic anhydrase by sulphonamides. *Biochem J* 1948;43:525–8.
10. Krebs HA, Roughton FJ. Carbonic anhydrase as a tool in studying the mechanism of reactions involving H(2)CO(3), CO(2) or HCO(3)'. *Biochem J* 1948;43:550–5.
11. Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat Rev Drug Discov* 2008;7:168–81.
12. 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.
13. Krishnamurthy VM, Kaufman GK, Urbach AR, et al. Carbonic anhydrase as a model for biophysical and physical-organic studies of proteins and protein-ligand binding. *Chem Rev* 2008;108:946–1051.
 14. Mishra CB, Tiwari M, Supuran CT. Progress in the development of human carbonic anhydrase inhibitors and their pharmacological applications: where are we today? *Med Res Rev* 2020;40:2485–565.
 15. Neri D, Supuran CT. Interfering with pH regulation in tumours as a therapeutic strategy. *Nat Rev Drug Discov* 2011;10:767–77.
 16. (a) Supuran CT. Structure and function of carbonic anhydrases. *Biochem J* 2016;473:2023–32. (b) Supuran CT. Carbonic anhydrases and metabolism. *Metabolites* 2018;8:25.
 17. (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) Ozensoy Guler O, Capasso C, Supuran CT. A magnificent enzyme superfamily: carbonic anhydrases, their purification and characterization. *J Enzyme Inhib Med Chem* 2016;31:689–94. (c) Bertucci A, Moya A, Tambutté S, et al. Carbonic anhydrases in anthozoan corals—a review. *Bioorg Med Chem* 2013;21:1437–50.
 18. (a) Kikutani S, Nakajima K, Nagasato C, et al. Thylakoid luminal theta-carbonic anhydrase critical for growth and photosynthesis in the marine diatom *Phaeodactylum tricornutum*. *Proc Natl Acad Sci USA* 2016;113:9828–33. (b) Jin S, Vullo D, Bua S, et al. Structural and biochemical characterization of novel carbonic anhydrases from *Phaeodactylum tricornutum*. *Acta Crystallogr D Struct Biol* 2020;76:676–86. (c) Del Prete S, Bua S, Supuran CT, Capasso C. *Escherichia coli* γ -carbonic anhydrase: characterisation and effects of simple aromatic/heterocyclic sulphonamide inhibitors. *J Enzyme Inhib Med Chem* 2020;35:1545–54. (d) Kaur J, Cao X, Abutaleb NS, et al. Optimization of acetazolamide-based scaffold as potent inhibitors of vancomycin-resistant enterococcus. *J Med Chem* 2020;63:9540–62.
 19. (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) Cox EH, McLendon GL, Morel FM, et al. The active site structure of *Thalassiosira weissflogii* carbonic anhydrase 1. *Biochemistry* 2000;39:12128–30. (c) 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.
 20. (a) Capasso C, Supuran CT. Bacterial, fungal and protozoan carbonic anhydrases as drug targets. *Expert Opin Ther Targets* 2015;19:1689–704. (b) Supuran CT, Capasso C. The eta-class carbonic anhydrases as drug targets for antimalarial agents. *Expert Opin Ther Targets* 2015;19:551–63. (c) Supuran CT, Capasso C. Antibacterial carbonic anhydrase inhibitors: an update on the recent literature. *Expert Opin Ther Pat* 2020;30:963–82. (d) Angeli A, Pinteala M, Maier SS, et al. Inhibition of α -, β -, γ -, δ -, ζ - and η -class carbonic anhydrases from bacteria, fungi, algae, diatoms and protozoans with famotidine. *J Enzyme Inhib Med Chem* 2019;34:644–50.
 21. (a) Jensen EL, Clement R, Kosta A, et al. A new widespread subclass of carbonic anhydrase in marine phytoplankton. *Isme J* 2019;13:2094–106. (b) Del Prete S, Nocentini A, Supuran CT, Capasso C. Bacterial ι -carbonic anhydrase: a new active class of carbonic anhydrase identified in the genome of the Gram-negative bacterium *Burkholderia territorii*. *J Enzyme Inhib Med Chem* 2020;35:1060–8. (c) Petreni A, De Luca V, Scaloni A, et al. Anion inhibition studies of the Zn(II)-bound ι -carbonic anhydrase from the Gram-negative bacterium *Burkholderia territorii*. *J Enzyme Inhib Med Chem* 2021;36:372–6. (d) De Luca V, Petreni A, Nocentini A, et al. Effect of sulfonamides and their structurally related derivatives on the activity of ι -carbonic anhydrase from *Burkholderia territorii*. *Int J Mol Sci* 2021;22:571.
 22. (a) Tripp BC, Bell CB, 3rd, Cruz F, et al. A role for iron in an ancient carbonic anhydrase. *J Biol Chem* 2004;279:6683–7. (b) Zimmerman SA, Ferry JG, Supuran CT. Inhibition of the archaeal beta-class (Cab) and gamma-class (Cam) carbonic anhydrases. *Curr Top Med Chem* 2007;7:901–8.
 23. (a) Del Prete S, Vullo D, Fisher GM, et al. Discovery of a new family of carbonic anhydrases in the malaria pathogen *Plasmodium falciparum*—the η -carbonic anhydrases. *Bioorg Med Chem Lett* 2014;24:4389–96. (b) De Simone G, Di Fiore A, Capasso C, Supuran CT. The zinc coordination pattern in the η -carbonic anhydrase from *Plasmodium falciparum* is different from all other carbonic anhydrase genetic families. *Bioorg Med Chem Lett* 2015;25:1385–9.
 24. (a) Domsic JF, Avvaru BS, Kim CU, et al. Entrapment of carbon dioxide in the active site of carbonic anhydrase II. *J Biol Chem* 2008;283:30766–71. (b) Sjöblom B, Polentarutti M, Djinic-Carugo K. Structural study of X-ray induced activation of carbonic anhydrase. *Proc Natl Acad Sci USA* 2009;106:10609–13.
 25. Aggarwal M, Chua TK, Pinard MA, et al. Carbon dioxide "trapped" in a β -carbonic anhydrase. *Biochemistry* 2015;54:6631–8.
 26. De Simone G, Supuran CT. (In)organic anions as carbonic anhydrase inhibitors. *J Inorg Biochem* 2012;111:117–29.
 27. (a) Bertini I, Luchinat C, Scozzafava A. Binding affinity of bicarboxylate ions for cobalt (II) bovine carbonic anhydrase. *Bioinorg Chem* 1978;9:93–100. (b) Bertini I, Luchinat C, Scozzafava A. Interaction of cobalt-bovine carbonic anhydrase with the acetate ion. *Biochim Biophys Acta* 1976;452:239–44.
 28. (a) Bertini I, Luchinat C, Pierattelli R, Vila AJ. The interaction of acetate and formate with cobalt carbonic anhydrase. An NMR study. *Eur J Biochem* 1992;208:607–15. (b) Bertini I, Luchinat C, Scozzafava A. A ^{31}P NMR study of phosphate in presence of cobalt(II)- and copper(II)- substituted bovine carbonic anhydrase B. *FEBS Lett* 1978;93:251–4.
 29. (a) Taylor JS, Coleman JE. Electron spin resonance of ^{63}Cu and ^{65}Cu carbonic anhydrases. Resolution of nitrogen ligand superhyperfine structure. *J Biol Chem* 1973;248:749–55. (b) Coleman JE, Coleman RV. Magnetic circular dichroism of Co (II) carbonic anhydrase. *J Biol Chem* 1972;247:4718–28.
 30. Coleman JE. Mechanism of action of carbonic anhydrase. Substrate, sulfonamide, and anion binding. *J Biol Chem* 1967;242:5212–9.
 31. West D, Pinard MA, Tu C, et al. Human carbonic anhydrase II-cyanate inhibitor complex: putting the debate to rest. *Acta Crystallogr F Struct Biol Commun* 2014;70:1324–7.

32. Jönsson BM, Håkansson K, Liljas A. The structure of human carbonic anhydrase II in complex with bromide and azide. *FEBS Lett* 1993;322:186–90.
33. Ferraroni M, Gaspari R, Scozzafava A, et al. Dioxigen, an unexpected carbonic anhydrase ligand. *J Enzyme Inhib Med Chem* 2018;33:999–1005.
34. Lindahl M, Svensson LA, Liljas A. Metal poison inhibition of carbonic anhydrase. *Proteins* 1993;15:177–82.
35. Peng Z, Merz KM, Jr, Banci L. Binding of cyanide, cyanate, and thiocyanate to human carbonic anhydrase II. *Proteins* 1993;17:203–16.
36. Supuran CT, Conroy CW, Maren TH. Is cyanate a carbonic anhydrase substrate? *Proteins* 1997;27:272–8.
37. Maren TH. Kinetics, equilibrium and inhibition in the Hansson histochemical procedure for carbonic anhydrase: a validation of the method. *Histochem J* 1980;12:183–90.
38. Maren TH. Inhibition by anions of human red cell carbonic anhydrase B: physiological and biochemical implications. *Science* 1976;194:746–7.
39. Briganti F, Mangani S, Scozzafava A, et al. Carbonic anhydrase catalyzes cyanamide hydration to urea: is it mimicking the physiological reaction? *J Biol Inorg Chem* 1999;4: 528–36.
40. Temperini C, Scozzafava A, Supuran CT. Carbonic anhydrase inhibitors. X-ray crystal studies of the carbonic anhydrase II-trithiocarbonate adduct-an inhibitor mimicking the sulfonamide and urea binding to the enzyme. *Bioorg Med Chem Lett* 2010;20:474–8.
41. Guerri A, Briganti F, Scozzafava A, et al. Mechanism of cyanamide hydration catalyzed by carbonic anhydrase II suggested by cryogenic X-ray diffraction. *Biochemistry* 2000;39:12391–7.
42. Nettles WL, Song H, Farquhar ER, et al. Characterization of the Copper(II) Binding Sites in Human Carbonic Anhydrase II. *Inorg Chem* 2015;54:5671–80.
43. Andring JT, Kim CU, McKenna R. Structure and mechanism of copper-carbonic anhydrase II: a nitrite reductase. *IUCrJ* 2020;7:287–93.
44. Carta F, Scozzafava A, Supuran CT. Sulfonamides: a patent review (2008–2012). *Expert Opin Ther Pat* 2012;22:747–58.
45. Supuran CT. Carbon- versus sulphur-based zinc binding groups for carbonic anhydrase inhibitors? *J Enzyme Inhib Med Chem* 2018;33:485–95.
46. Scozzafava A, Carta F, Supuran CT. Secondary and tertiary sulfonamides: a patent review (2008–2012). *Expert Opin Ther Pat* 2013;23:203–13.
47. Eriksson AE, Kylsten PM, Jones TA, Liljas A. Crystallographic studies of inhibitor binding sites in human carbonic anhydrase II: a pentacoordinated binding of the SCN⁻ ion to the zinc at high pH. *Proteins* 1988;4:283–93.
48. Supuran CT. Exploring the multiple binding modes of inhibitors to carbonic anhydrases for novel drug discovery. *Expert Opin Drug Discov* 2020;15:671–86.
49. Supuran CT. Carbonic anhydrase inhibition and the management of hypoxic tumors. *Metabolites* 2017;7:48.
50. Supuran CT. Advances in structure-based drug discovery of carbonic anhydrase inhibitors. *Expert Opin Drug Discov* 2017;12:61–88.
51. Carta F, Supuran CT. Diuretics with carbonic anhydrase inhibitory action: a patent and literature review (2005–2013). *Expert Opin Ther Pat* 2013;23:681–91.
52. Nocentini A, Supuran CT. Advances in the structural annotation of human carbonic anhydrases and impact on future drug discovery. *Expert Opin Drug Discov* 2019;14:1175–97.
53. Supuran CT. Carbonic anhydrase inhibitors and their potential in a range of therapeutic areas. *Expert Opin Ther Pat* 2018;28:709–12.
54. Scozzafava A, Supuran CT, Carta F. Antiobesity carbonic anhydrase inhibitors: a literature and patent review. *Expert Opin Ther Pat* 2013;23:725–35.
55. Supuran CT. Applications of carbonic anhydrases inhibitors in renal and central nervous system diseases. *Expert Opin Ther Pat* 2018;28:713–21.
56. Supuran CT, Altamimi ASA, Carta F. Carbonic anhydrase inhibition and the management of glaucoma: a literature and patent review 2013-2019. *Expert Opin Ther Pat* 2019; 29:781–92.
57. Supuran CT. The management of glaucoma and macular degeneration. *Expert Opin Ther Pat* 2019;29:745–7.
58. Wistrand PJ. Carbonic anhydrase research: a clinical perspective, past and future. *EXS* 2000;90:597–609.
59. Maren TH. Sulfonamides and secretion of aqueous humor. *J Exp Zool* 1997;279:490–7.
60. Supuran CT, Nicolae A, Popescu A. Carbonic anhydrase inhibitors. Part 35. Synthesis of Schiff bases derived from sulfanilamide and aromatic aldehydes: the first inhibitors with equally high affinity towards cytosolic and membrane-bound isozymes. *Eur J Med Chem* 1996;31:431–8.
61. Scozzafava A, Menabuoni L, Mincione F, et al. Carbonic anhydrase inhibitors. Synthesis of water-soluble, topically effective, intraocular pressure-lowering aromatic/heterocyclic sulfonamides containing cationic or anionic moieties: is the tail more important than the ring? *J Med Chem* 1999; 42:2641–50.
62. D'Ascenzio M, Secci D, Carradori S, et al. 1,3-Dipolar Cycloaddition, HPLC Enantioseparation, and Docking Studies of Saccharin/Isoxazole and Saccharin/Isoxazoline Derivatives as Selective Carbonic Anhydrase IX and XII Inhibitors. *J Med Chem* 2020;63:2470–88.
63. Güzel O, Temperini C, Innocenti A, et al. Carbonic anhydrase inhibitors. Interaction of 2-(hydrazinocarbonyl)-3-phenyl-1H-indole-5-sulfonamide with 12 mammalian isoforms: kinetic and X-ray crystallographic studies. *Bioorg Med Chem Lett* 2008;18:152–8.
64. Biswas S, Aggarwal M, Güzel Ö, et al. Conformational variability of different sulfonamide inhibitors with thienyl-acetamido moieties attributes to differential binding in the active site of cytosolic human carbonic anhydrase isoforms. *Bioorg Med Chem* 2011;19:3732–8.
65. Wagner J, Avvaru BS, Robbins AH, et al. Coumarinyl-substituted sulfonamides strongly inhibit several human carbonic anhydrase isoforms: solution and crystallographic investigations. *Bioorg Med Chem* 2010;18:4873–8.
66. Avvaru BS, Wagner JM, Maresca A, et al. Carbonic anhydrase inhibitors. The X-ray crystal structure of human isoform II in adduct with an adamantyl analogue of acetazolamide resides in a less utilized binding pocket than most hydrophobic inhibitors. *Bioorg Med Chem Lett* 2010;20:4376–81.
67. Carta F, Garaj V, Maresca A, et al. Sulfonamides incorporating 1,3,5-triazine moieties selectively and potently inhibit carbonic anhydrase transmembrane isoforms IX, XII and XIV over cytosolic isoforms I and II: solution and X-ray crystallographic studies. *Bioorg Med Chem* 2011;19:3105–19.

68. Carta F, Birkmann A, Pfaff T, et al. Lead development of thiazolylsulfonamides with carbonic anhydrase inhibitory action. *J Med Chem* 2017;60:3154–64.
69. Alterio V, Vitale RM, Monti SM, et al. Carbonic anhydrase inhibitors: X-ray and molecular modeling study for the interaction of a fluorescent antitumor sulfonamide with isozyme II and IX. *J Am Chem Soc* 2006;128:8329–35.
70. 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-benzenesulfonamides and correlate to inhibitor potency. *Chem Commun (Camb)* 2010;46:8371–3.
71. Mboge MY, Mahon BP, Lamas N, et al. Structure activity study of carbonic anhydrase IX: selective inhibition with ureido-substituted benzenesulfonamides. *Eur J Med Chem* 2017;132:184–91.
72. Pacchiano F, Carta F, McDonald PC, et al. Ureido-substituted benzenesulfonamides potently inhibit carbonic anhydrase IX and show antimetastatic activity in a model of breast cancer metastasis. *J Med Chem* 2011;54:1896–902.
73. Tanpure RP, Ren B, Peat TS, et al. Carbonic anhydrase inhibitors with dual-tail moieties to match the hydrophobic and hydrophilic halves of the carbonic anhydrase active site. *J Med Chem* 2015;58:1494–501.
74. Bonardi A, Nocentini A, Bua S, et al. Sulfonamide inhibitors of human carbonic anhydrases designed through a three-tails approach: improving ligand/isoform matching and selectivity of action. *J Med Chem* 2020;63:7422–44.
75. Gieling RG, Babur M, Mamnani L, et al. Antimetastatic effect of sulfamate carbonic anhydrase IX inhibitors in breast carcinoma xenografts. *J Med Chem* 2012;55:5591–600.
76. Bozdog M, Carta F, Ceruso M, et al. Discovery of 4-hydroxy-3-(3-(phenylureido)benzenesulfonamides as SLC-0111 analogues for the treatment of hypoxic tumors overexpressing carbonic anhydrase IX. *J Med Chem* 2018;61:6328–38.
77. Akocak S, Lolak N, Bua S, et al. Synthesis and biological evaluation of novel N,N'-diaryl cyanoguanidines acting as potent and selective carbonic anhydrase II inhibitors. *Bioorg Chem* 2018;77:245–51.
78. Lolak N, Akocak S, Bua S, et al. Design and synthesis of novel 1,3-diarylthiazene-substituted sulfonamides as potent and selective carbonic anhydrase II inhibitors. *Bioorg Chem* 2018;77:542–7.
79. Lomelino CL, Mahon BP, Carta F, et al. Kinetic and X-ray crystallographic investigations on carbonic anhydrase isoforms I, II, IX and XII of a thioureido analog of SLC-0111. *Bioorg Med Chem* 2016;24:976–81.
80. Angeli A, Tanini D, Peat TS, et al. Discovery of new selenoureido analogues of 4-(4-Fluorophenylureido) benzenesulfonamide as Carbonic Anhydrase Inhibitors. *ACS Med Chem Lett* 2017;8:963–8.
81. Eldehna WM, Abo-Ashour MF, Berrino E, et al. SLC-0111 enaminone analogs, 3/4-(3-aryl-3-oxopropenyl) aminobenzenesulfonamides, as novel selective subnanomolar inhibitors of the tumor-associated carbonic anhydrase isoform IX. *Bioorg Chem* 2019;83:549–58.
82. Congiu C, Onnis V, Deplano A, et al. Synthesis of sulfonamides incorporating piperazinyl-ureido moieties and their carbonic anhydrase I, II, IX and XII inhibitory activity. *Bioorg Med Chem Lett* 2015;25:3850–3.
83. Moi D, Nocentini A, Deplano A, et al. Appliance of the piperidinyl-hydrazidoureido linker to benzenesulfonamide compounds: synthesis, in vitro and in silico evaluation of potent carbonic anhydrase II, IX and XII inhibitors. *Bioorg Chem* 2020;98:103728.
84. Abo-Ashour MF, Eldehna WM, Nocentini A, et al. Novel synthesized SLC-0111 thiazole and thiadiazole analogues: determination of their carbonic anhydrase inhibitory activity and molecular modeling studies. *Bioorg Chem* 2019;87:794–802.
85. Alkhalidi AAM, Al-Sanea MM, Nocentini A, et al. 3-Methylthiazolo[3,2-a]benzimidazole-benzenesulfonamide conjugates as novel carbonic anhydrase inhibitors endowed with anticancer activity: design, synthesis, biological and molecular modeling studies. *Eur J Med Chem* 2020;207:112745.
86. Iikuni S, Ono M, Watanabe H, et al. Cancer radiotheranostics targeting carbonic anhydrase-IX with ¹¹¹In- and ⁹⁰Y-labeled ureidosulfonamide scaffold for SPECT imaging and radionuclide-based therapy. *Theranostics* 2018;8:2992–3006.
87. Eldehna WM, Fares M, Ceruso M, et al. Amido/ureidosubstituted benzenesulfonamides-isatin conjugates as low nanomolar/subnanomolar inhibitors of the tumor-associated carbonic anhydrase isoform XII. *Eur J Med Chem* 2016;110:259–66.
88. Eldehna WM, Abo-Ashour MF, Nocentini A, et al. Enhancement of the tail hydrophobic interactions within the carbonic anhydrase IX active site via structural extension: design and synthesis of novel N-substituted isatins-SLC-0111 hybrids as carbonic anhydrase inhibitors and antitumor agents. *Eur J Med Chem* 2019;162:147–60.
89. Carta F, Vullo D, Osman SM, et al. Synthesis and carbonic anhydrase inhibition of a series of SLC-0111 analogs. *Bioorg Med Chem* 2017;25:2569–76.
90. Akgul O, Singh S, Andring JT, et al. Handling drug-target selectivity: a study on ureido containing Carbonic Anhydrase inhibitors. *Eur J Med Chem* 2020;113035.DOI:10.1016/j.ejmech.2020.113035
91. Nocentini A, Gratteri P, Supuran CT. Phosphorus versus sulfur: discovery of benzenephosphonamidates as versatile sulfonamide-mimic chemotypes acting as carbonic anhydrase inhibitors. *Chemistry* 2019;25:1188–92.
92. 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.
93. Vullo D, Durante M, Di Leva FS, et al. Monothiocarbamates strongly inhibit carbonic anhydrases in vitro and possess intraocular pressure lowering activity in an animal model of glaucoma. *J Med Chem* 2016;59:5857–67.
94. Carta F, Akdemir A, Scozzafava A, et al. Xanthates and tri-thiocarbonates strongly inhibit carbonic anhydrases and show antiglaucoma effects in vivo. *J Med Chem* 2013;56:4691–700.
95. Angeli A, Tanini D, Nocentini A, et al. Selenols: a new class of carbonic anhydrase inhibitors. *Chem Commun (Camb)* 2019;55:648–51.
96. Tanini D, Capperucci A, Ferraroni M, et al. Direct and straightforward access to substituted alkyl selenols as novel carbonic anhydrase inhibitors. *Eur J Med Chem* 2020;185:111811.
97. Langella E, D'Ambrosio K, D'Ascenzio M, et al. A Combined crystallographic and theoretical study explains the capability of carboxylic acids to adopt multiple binding modes in

- the active site of carbonic anhydrases. *Chemistry* 2016;22: 97–100.
98. Di Fiore A, Maresca A, Supuran CT, et al. Hydroxamate represents a versatile zinc binding group for the development of new carbonic anhydrase inhibitors. *Chem Commun (Camb)* 2012;48:8838–40.
 99. Alterio V, Cadoni R, Esposito D, et al. Benzoxaborole as a new chemotype for carbonic anhydrase inhibition. *Chem Commun (Camb)* 2016;52:11983–6.
 100. Langella E, Alterio V, D'Ambrosio K, et al. Exploring benzoxaborole derivatives as carbonic anhydrase inhibitors: a structural and computational analysis reveals their conformational variability as a tool to increase enzyme selectivity. *J Enzyme Inhib Med Chem* 2019;34:1498–505.
 101. Nocentini A, Supuran CT, Winum JY. Benzoxaborole compounds for therapeutic uses: a patent review (2010–2018). *Expert Opin Ther Pat* 2018;28:493–504.
 102. De Simone G, Angeli A, Bozdog M, et al. Inhibition of carbonic anhydrases by a substrate analog: benzyl carbamate directly coordinates the catalytic zinc ion mimicking bicarbonate binding. *Chem Commun (Camb)* 2018;54:10312–5.
 103. Carta F, Temperini C, Innocenti A, et al. Polyamines inhibit carbonic anhydrases by anchoring to the zinc-coordinated water molecule. *J Med Chem* 2010;53:5511–22.
 104. Nair SK, Ludwig PA, Christianson DW. Two-site binding of phenol in the active site of human carbonic anhydrase II: structural implications for substrate association. *J Am Chem Soc* 1994;116:3659–60.
 105. Innocenti A, Vullo D, Scozzafava A, et al. Carbonic anhydrase inhibitors: interactions of phenols with the 12 catalytically active mammalian isoforms (CA I–XIV). *Bioorg Med Chem Lett* 2008;18:1583–7.
 106. Innocenti A, Vullo D, Scozzafava A, et al. Carbonic anhydrase inhibitors: inhibition of mammalian isoforms I–XIV with a series of substituted phenols including paracetamol and salicylic acid. *Bioorg Med Chem* 2008;16:7424–8.
 107. (a) Bayram E, Senturk M, Kufrevioglu OI, et al. In vitro inhibition of salicylic acid derivatives on human cytosolic carbonic anhydrase isozymes I and II. *Bioorg Med Chem* 2008;16:9101–5. (b) Innocenti A, Gülçin I, Scozzafava A, Supuran CT. Carbonic anhydrase inhibitors. Antioxidant polyphenols effectively inhibit mammalian isoforms I–XV. *Bioorg Med Chem Lett* 2010;20:5050–3.
 108. Nocentini A, Bonardi A, Gratteri P, et al. Steroids interfere with human carbonic anhydrase activity by using alternative binding mechanisms. *J Enzyme Inhib Med Chem* 2018; 33:1453–9.
 109. Karioti A, Carta F, Supuran CT. Phenols and polyphenols as carbonic anhydrase inhibitors. *Molecules* 2016;21:1649.
 110. Tars K, Vullo D, Kazaks A, et al. Sulfocoumarins (1,2-benzoxathiine-2,2-dioxides): a class of potent and isoform-selective inhibitors of tumor-associated carbonic anhydrases. *J Med Chem* 2013;56:293–300.
 111. Grandane A, Tanc M, Žalubovskis R, Supuran CT. Synthesis of 6-aryl-substituted sulfocoumarins and investigation of their carbonic anhydrase inhibitory action. *Bioorg Med Chem* 2015;23:1430–6.
 112. Pustenko A, Stepanovs D, Žalubovskis R, et al. 3H-1,2-benzothiepine 2,2-dioxides: a new class of isoform-selective carbonic anhydrase inhibitors. *J Enzyme Inhib Med Chem* 2017;32:767–75.
 113. Grandane A, Nocentini A, Werner T, et al. Benzoxepinones: a new isoform-selective class of tumor associated carbonic anhydrase inhibitors. *Bioorg Med Chem* 2020;28:115496.
 114. Ferraroni M, Carta F, Scozzafava A, et al. Thioxocoumarins show an alternative carbonic anhydrase inhibition mechanism compared to coumarins. *J Med Chem* 2016;59:462–73.
 115. Maresca A, Temperini C, Vu H, et al. Non-zinc mediated inhibition of carbonic anhydrases: coumarins are a new class of suicide inhibitors. *J Am Chem Soc* 2009;131: 3057–62.
 116. Maresca A, Temperini C, Pochet L, et al. Deciphering the mechanism of carbonic anhydrase inhibition with coumarins and thiocoumarins. *J Med Chem* 2010;53:335–44.
 117. Temperini C, Innocenti A, Scozzafava A, et al. The coumarin-binding site in carbonic anhydrase accommodates structurally diverse inhibitors: the antiepileptic lacosamide as an example and lead molecule for novel classes of carbonic anhydrase inhibitors. *J Med Chem* 2010;53:850–4.
 118. Supuran CT. Coumarin carbonic anhydrase inhibitors from natural sources. *J Enzyme Inhib Med Chem* 2020;35: 1462–70.
 119. Touisni N, Maresca A, McDonald PC, et al. Glycosyl coumarin carbonic anhydrase IX and XII inhibitors strongly attenuate the growth of primary breast tumors. *J Med Chem* 2011;54:8271–7.
 120. Davis RA, Vullo D, Maresca A, et al. Natural product coumarins that inhibit human carbonic anhydrases. *Bioorg Med Chem* 2013;21:1539–43.
 121. De Luca L, Mancuso F, Ferro S, et al. Inhibitory effects and structural insights for a novel series of coumarin-based compounds that selectively target human CA IX and CA XII carbonic anhydrases. *Eur J Med Chem* 2018;143:276–82.
 122. Maresca A, Scozzafava A, Supuran CT. 7,8-disubstituted-but not 6,7-disubstituted coumarins selectively inhibit the transmembrane, tumor-associated carbonic anhydrase isoforms IX and XII over the cytosolic ones I and II in the low nanomolar/subnanomolar range. *Bioorg Med Chem Lett* 2010;20:7255–8.
 123. Maresca A, Supuran CT. Coumarins incorporating hydroxy- and chloro-moieties selectively inhibit the transmembrane, tumor-associated carbonic anhydrase isoforms IX and XII over the cytosolic ones I and II. *Bioorg Med Chem Lett* 2010;20:4511–4.
 124. Bonneau A, Maresca A, Winum JY, Supuran CT. Metronidazole-coumarin conjugates and 3-cyano-7-hydroxy-coumarin act as isoform-selective carbonic anhydrase inhibitors. *J Enzyme Inhib Med Chem* 2013;28: 397–401.
 125. D'Ambrosio K, Carradori S, Monti SM, et al. Out of the active site binding pocket for carbonic anhydrase inhibitors. *Chem Commun (Camb)* 2015;51:302–5.
 126. Bouzina A, Berredjem M, Nocentini A, et al. Ninhydrins inhibit carbonic anhydrases directly binding to the metal ion. *Eur J Med Chem* 2021;209:112875.
 127. D'Ambrosio K, Carradori S, Cesa S, et al. Catechols: a new class of carbonic anhydrase inhibitors. *Chem Commun (Camb)* 2020;56:13033–6.
 128. (a) Supuran CT. How many carbonic anhydrase inhibition mechanisms exist? *J Enzyme Inhib Med Chem* 2016;31: 345–60. (b) Supuran CT. Structure-based drug discovery of carbonic anhydrase inhibitors. *J Enzyme Inhib Med Chem* 2012;27:759–72.

129. (a) Aggarwal M, Kondeti B, McKenna R. Anticonvulsant/anti-epileptic carbonic anhydrase inhibitors: a patent review. *Expert Opin Ther Pat* 2013;23:717–24. (b) Thiry A, Dogné JM, Supuran CT, Masereel B. Anticonvulsant sulfonamides/sulfamates/sulfamides with carbonic anhydrase inhibitory activity: drug design and mechanism of action. *Curr Pharm Des* 2008;14:661–71.
130. (a) Hen N, Bialer M, Yagen B, et al. Anticonvulsant 4-aminobenzenesulfonamide derivatives with branched-alkylamide moieties: X-ray crystallography and inhibition studies of human carbonic anhydrase isoforms I, II, VII, and XIV. *J Med Chem* 2011;54:3977–81. (b) Thiry A, Dogné JM, Supuran CT, Masereel B. Carbonic anhydrase inhibitors as anticonvulsant agents. *Curr Top Med Chem* 2007;7:855–64.
131. (a) Muñoz W, Lamm A, Poppers D, Lamm S. Acetazolamide promotes decreased consumption of carbonated drinks and weight loss. *Oxf Med Case Reports* 2018;2018:omy081. (b) Bejaoui M, Pantazi E, De Luca V, et al. Acetazolamide protects steatotic liver grafts against cold ischemia reperfusion injury. *J Pharmacol Exp Ther* 2015;355:191–8. (c) Supuran CT. Carbonic anhydrase inhibitors as emerging drugs for the treatment of obesity. *Expert Opin Emerg Drugs* 2012;17:11–5. (d) Supuran CT, Di Fiore A, De Simone G. Carbonic anhydrase inhibitors as emerging drugs for the treatment of obesity. *Expert Opin Emerg Drugs* 2008;13:383–92.
132. (a) Costa G, Carta F, Ambrosio FA, et al. A computer-assisted discovery of novel potential anti-obesity compounds as selective carbonic anhydrase VA inhibitors. *Eur J Med Chem* 2019;181:111565. (b) Costa G, Gidaro MC, Vullo D, et al. Active components of essential oils as anti-obesity potential drugs investigated by in silico techniques. *J Agric Food Chem* 2016;64:5295–300. (c) Arechederra RL, Waheed A, Sly WS, et al. Effect of sulfonamides as carbonic anhydrase VA and VB inhibitors on mitochondrial metabolic energy conversion. *Bioorg Med Chem* 2013;21:1544–8.
133. (a) Supuran CT. For the treatment of idiopathic intracranial hypertension. *Expert Rev Neurother*. 2015;15:851–856; (b) Supuran CT. Drug interaction considerations in the therapeutic use of carbonic anhydrase inhibitors. *Expert Opin Drug Metab Toxicol* 2016;12:423–31.
134. (a) Di Cesare Mannelli L, Micheli L, Carta F, et al. Carbonic anhydrase inhibition for the management of cerebral ischemia: in vivo evaluation of sulfonamide and coumarin inhibitors. *Enzyme Inhib Med Chem* 2016;31:894–9. (b) Angeli A, di Cesare Mannelli L, Trallori E, et al. Design, synthesis, and X-ray of selenides as new class of agents for prevention of diabetic cerebrovascular pathology. *ACS Med Chem Lett* 2018;9:462–7.
135. (a) Carta F, Di Cesare Mannelli L, Pinard M, et al. A class of sulfonamide carbonic anhydrase inhibitors with neuropathic pain modulating effects. *Bioorg Med Chem* 2015;23:1828–40. (b) Vullo D, Voipio J, Innocenti A, et al. Carbonic anhydrase inhibitors. Inhibition of the human cytosolic isozyme VII with aromatic and heterocyclic sulfonamides. *Bioorg Med Chem Lett* 2005;15:971–6.
136. Supuran CT. Carbonic anhydrase inhibition and the management of neuropathic pain. *Expert Rev Neurother* 2016;16:961–8.
137. Margheri F, Ceruso M, Carta F, et al. Overexpression of the transmembrane carbonic anhydrase isoforms IX and XII in the inflamed synovium. *J Enzyme Inhib Med Chem* 2016;31:60–3.
138. (a) Bua S, Di Cesare Mannelli L, Vullo D, et al. Design and synthesis of novel nonsteroidal anti-inflammatory drugs and carbonic anhydrase inhibitors hybrids (NSAIDs-CAIs) for the treatment of rheumatoid arthritis. *J Med Chem* 2017;60:1159–70. (b) Bua S, Lucarini L, Micheli L, et al. Bioisosteric development of multitarget nonsteroidal anti-inflammatory drug-carbonic anhydrases inhibitor hybrids for the management of rheumatoid arthritis. *J Med Chem* 2020;63:2325–42. (c) Akgul O, Di Cesare Mannelli L, Vullo D, et al. Discovery of novel nonsteroidal anti-inflammatory drugs and carbonic anhydrase inhibitors hybrids (NSAIDs-CAIs) for the management of rheumatoid arthritis. *J Med Chem* 2018;61:4961–77.
139. (a) Micheli L, Bozdog M, Akgul O, et al. Pain relieving effect of NSAIDs-CAIs hybrid molecules: systemic and intra-articular treatments against rheumatoid arthritis. *Int J Mol Sci* 2019;20:1923. (b) Lucarini L, Durante M, Sgambellone S, et al. Effects of new NSAID-CAI hybrid compounds in inflammation and lung fibrosis. *Biomolecules* 2020;10:1307.
140. Berrino E, Milazzo L, Micheli L, et al. Synthesis and evaluation of carbonic anhydrase inhibitors with carbon monoxide releasing properties for the management of rheumatoid arthritis. *J Med Chem* 2019;62:7233–49.
141. Pastorek J, Pastoreková S, Callebaut I, et al. Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment. *Oncogene* 1994;9:2877–88.
142. Türeci O, Sahin U, Vollmar E, et al. Human carbonic anhydrase XII: cDNA cloning, expression, and chromosomal localization of a carbonic anhydrase gene that is overexpressed in some renal cell cancers. *Proc Natl Acad Sci USA* 1998;95:7608–13.
143. Svastová E, Hulíková A, Rafajová M, et al. Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH. *FEBS Lett* 2004;577:439–45.
144. Cecchi A, Hulikova A, Pastorek J, et al. Carbonic anhydrase inhibitors. Design of fluorescent sulfonamides as probes of tumor-associated carbonic anhydrase IX that inhibit isozyme IX-mediated acidification of hypoxic tumors. *J Med Chem* 2005;48:4834–41.
145. Ahlskog JK, Dumelin CE, Trüssel S, et al. In vivo targeting of tumor-associated carbonic anhydrases using acetazolamide derivatives. *Bioorg Med Chem Lett* 2009;19:4851–6.
146. Buller F, Steiner M, Frey K, et al. Selection of carbonic anhydrase IX inhibitors from one million DNA-encoded compounds. *ACS Chem Biol* 2011;6:336–44.
147. Lou Y, McDonald PC, Oloumi A, et al. Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. *Cancer Res* 2011;71:3364–76.
148. Krall N, Pretto F, Decurtins W, et al. A small-molecule drug conjugate for the treatment of carbonic anhydrase IX expressing tumors. *Angew Chem Int Ed Engl* 2014;53:4231–5.
149. Ebbesen P, Pettersen EO, Gorr TA, et al. Taking advantage of tumor cell adaptations to hypoxia for developing new tumor markers and treatment strategies. *J Enzyme Inhib Med Chem* 2009;24: 1–39.
150. Pettersen EO, Ebbesen P, Gieling RG, et al. Targeting tumour hypoxia to prevent cancer metastasis. *From*

- biology, biosensing and technology to drug development: the METOXIA consortium. *J Enzyme Inhib Med Chem* 2015; 30:689–721.
151. Supuran CT, Alterio V, Di Fiore A, et al. Inhibition of carbonic anhydrase IX targets primary tumors, metastases, and cancer stem cells: three for the price of one. *Med Res Rev* 2018;38:1799–836.
 152. McDonald PC, Winum JY, Supuran CT, Dedhar S. Recent developments in targeting carbonic anhydrase IX for cancer therapeutics. *Oncotarget* 2012;3:84–97.
 153. Angeli A, Carta F, Nocentini A, et al. Carbonic anhydrase inhibitors targeting metabolism and tumor microenvironment. *Metabolites* 2020;10:412.
 154. Burianova V, Kalinin S, Supuran CT, Krasavin M. Radiotracers for positron emission tomography (PET) targeting tumour-associated carbonic anhydrase isoforms. *Eur J Med Chem* 2020;2021:113046.
 155. Andring JT, Fouch M, Akocak S, et al. Structural basis of nanomolar inhibition of tumor-associated carbonic anhydrase IX: X-ray crystallographic and inhibition study of lipophilic inhibitors with acetazolamide backbone. *J Med Chem* 2020;63:13064–75.
 156. Alterio V, Hilvo M, Di Fiore A, et al. Crystal structure of the catalytic domain of the tumor-associated human carbonic anhydrase IX. *Proc Natl Acad Sci USA* 2009;106:16233–8.
 157. Whittington DA, Waheed A, Ulmasov B, et al. Crystal structure of the dimeric extracellular domain of human carbonic anhydrase XII, a bitopic membrane protein overexpressed in certain cancer tumor cells. *Proc Natl Acad Sci USA* 2001; 98:9545–50.
 158. Di Fiore A, Truppo E, Supuran CT, et al. Crystal structure of the C183S/C217S mutant of human CA VII in complex with acetazolamide. *Bioorg Med Chem Lett* 2010;20:5023–6.
 159. Di Fiore A, Monti SM, Hilvo M, et al. Crystal structure of human carbonic anhydrase XIII and its complex with the inhibitor acetazolamide. *Proteins* 2009;74:164–75.
 160. Alterio V, Pan P, Parkkila S, et al. The structural comparison between membrane-associated human carbonic anhydrases provides insights into drug design of selective inhibitors. *Biopolymers* 2014;101:769–78.
 161. Pilka ES, Kochan G, Oppermann U, Yue WW. Crystal structure of the secretory isozyme of mammalian carbonic anhydrases CA VI: implications for biological assembly and inhibitor development. *Biochem Biophys Res Commun* 2012;419:485–9.
 162. Briganti F, Mangani S, Orioli P, et al. Carbonic anhydrase activators: X-ray crystallographic and spectroscopic investigations for the interaction of isozymes I and II with histamine. *Biochemistry* 1997;36:10384–92.
 163. (a) Supuran CT. Carbonic anhydrase activators. *Future Med Chem* 2018;10:561–73. (b) Supuran CT, Balaban AT. Carbonic anhydrase activators. Part 8. pKa-activation relationship in a series of amino acid derivatives activators of isozyme II. *Rev Roum Chim* 1994;39:107–13. (c) Duda D, Tu C, Qian M, et al. Structural and kinetic analysis of the chemical rescue of the proton transfer function of carbonic anhydrase II. *Biochemistry* 2001;40:1741–8.
 164. Temperini C, Scozzafava A, Vullo D, Supuran CT. Carbonic anhydrase activators. Activation of isozymes I, II, IV, VA, VII, and XIV with l- and d-histidine and crystallographic analysis of their adducts with isoform II: engineering proton-transfer processes within the active site of an enzyme. *Chemistry* 2006;12:7057–66.
 165. (a) Temperini C, Scozzafava A, Supuran CT. Carbonic anhydrase activation and the drug design. *Curr Pharm Des* 2008;14:708–15. (b) Temperini C, Innocenti A, Scozzafava A, et al. Carbonic anhydrase activators: L-Adrenaline plugs the active site entrance of isozyme II, activating better isoforms I, IV, VA, VII, and XIV. *Bioorg Med Chem Lett* 2007;17: 628–35.
 166. (a) Temperini C, Scozzafava A, Vullo D, et al. Carbonic anhydrase activators. Activation of isoforms I, II, IV, VA, VII, and XIV with L- and D-phenylalanine and crystallographic analysis of their adducts with isozyme II: stereospecific recognition within the active site of an enzyme and its consequences for the drug design. *J Med Chem* 2006;49: 3019–27. (b) Temperini C, Innocenti A, Scozzafava A, et al. Carbonic anhydrase activators: kinetic and x-ray crystallographic study for the interaction of D- and L-tryptophan with the mammalian isoforms I–XIV. *Bioorg Med Chem* 2008;16:8373–837.
 167. (a) Dave K, Ilies MA, Scozzafava A, et al. An inhibitor-like binding mode of a carbonic anhydrase activator within the active site of isoform II. *Bioorg Med Chem Lett* 2011;21: 2764–8. (b) Bhatt A, Mondal UK, Supuran CT, et al. crystal structure of carbonic anhydrase II in complex with an activating ligand: implications in neuronal function. *Mol Neurobiol* 2018;55:7431–7.
 168. (a) Vullo D, Innocenti A, Nishimori I, et al. Carbonic anhydrase activators: activation of the human isoforms VII (cytosolic) and XIV (transmembrane) with amino acids and amines. *Bioorg Med Chem Lett* 2007;17:4107–12. (b) Pastorekova S, Vullo D, Nishimori I, et al. Carbonic anhydrase activators: activation of the human tumor-associated isozymes IX and XII with amino acids and amines. *Bioorg Med Chem* 2008;16:3530–6.
 169. Vullo D, Nishimori I, Innocenti A, et al. Carbonic anhydrase activators: an activation study of the human mitochondrial isoforms VA and VB with amino acids and amines. *Bioorg Med Chem Lett* 2007;17:1336–40.
 170. (a) Saada MC, Vullo D, Montero JL, et al. Carbonic anhydrase I and II activation with mono- and dihalogenated histamine derivatives. *Bioorg Med Chem Lett* 2011;21:4884–7. (b) Saada MC, Vullo D, Montero JL, et al. Mono- and dihalogenated histamine, histidine and carnosine derivatives are potent carbonic anhydrase I, II, VII, XII and XIV activators. *Bioorg Med Chem* 2014;22:4752–8. (c) Vistoli G, Aldini G, Fumagalli L, et al. Activation effects of carnosine- and histidine-containing dipeptides on human carbonic anhydrases: a comprehensive study. *Int J Mol Sci* 2020;21:1761.
 171. (a) Supuran CT, Dinculescu A, Balaban AT. Carbonic anhydrase activators. Part 5. CA II activation by 2,4,6-trisubstituted pyridinium cations with 1-(ω -aminoalkyl) side chains. *Rev Roum Chim* 1993;38:343–9. (b) Draghici B, Vullo D, Akocak S, et al. Ethylene bis-imidazoles are highly potent and selective activators for isozymes VA and VII of carbonic anhydrase, with a potential nootropic effect. *Chem Commun (Camb)* 2014;50:5980–3.
 172. (a) Akocak S, Lolak N, Bua S, et al. Activation of human α -carbonic anhydrase isoforms I, II, IV and VII with bis-histamine schiff bases and bis-spinaceamine substituted derivatives. *J Enzyme Inhib Med Chem* 2019;34:1193–8. (b) Akocak S, Lolak N, Bua S, et al. α -Carbonic anhydrases are strongly activated by spinaceamine derivatives. *Bioorg Med Chem* 2019;27:800–4.

173. a). Chiamonte N, Maach S, Biliotti C, et al. Synthesis and carbonic anhydrase activating properties of a series of 2-amino-imidazolines structurally related to clonidine. *J Enzyme Inhib Med Chem* 2020;35:1003–10. (b) Angeli A, Vaiano F, Mari F, et al. Psychoactive substances belonging to the amphetamine class potently activate brain carbonic anhydrase isoforms VA, VB, VII, and XII. *J Enzyme Inhib Med Chem* 2017;32:1253–9.
174. (a) Zhang Y, Supuran CT, Barboiu M. Exponential activation of carbonic anhydrase by encapsulation in dyanmeric host matrices with chiral discrimination. *Chemistry* 2018;24:715–20. (b) Le Duc Y, Licsandru E, Vullo D, et al. Carbonic anhydrases activation with 3-amino-1H-1,2,4-triazole-1-carboxamides: discovery of subnanomolar isoform II activators. *Bioorg Med Chem* 2017;25:1681–6. (c) Licsandru E, Tanc M, Kocsis I, et al. A class of carbonic anhydrase I - selective activators. *J Enzyme Inhib Med Chem* 2017;32:37–46. (d) Zhang Y, Legrand YM, Petit E, et al. Dynamic encapsulation and activation of carbonic anhydrase in multivalent dyanmeric host matrices. *Chem Commun (Camb)* 2016;52:4053–5. (e) Abdelrahim MY, Tanc M, Winum JY, et al. Dominant behaviours in the expression of human carbonic anhydrase hCA I activity. *Chem Commun (Camb)* 2014;50:8043–6.
175. Canto de Souza L, Provensi G, Vullo D, et al. Carbonic anhydrase activation enhances object recognition memory in mice through phosphorylation of the extracellular signal-regulated kinase in the cortex and the hippocampus. *Neuropharmacology* 2017;118:148–56.
176. Blandina P, Provensi G, Passani MB, et al. Carbonic anhydrase modulation of emotional memory. Implications for the treatment of cognitive disorders. *J Enzyme Inhib Med Chem* 2020;35:1206–14.
177. Schmidt SD, Costa A, Rani B, et al. The role of carbonic anhydrases in extinction of contextual fear memory. *Proc Natl Acad Sci USA* 2020;117:16000–8.
178. Jonsson BH, Liljas A. Perspectives on the classical enzyme carbonic anhydrase and the search for inhibitors. *Biophys J* 2020;119:1275–80.
179. Angeli A, Carta F, Nocentini A, et al. Response to perspectives on the classical enzyme carbonic anhydrase and the search for inhibitors. *Biophys J* 2021;120:178–81.
180. Supuran CT. Experimental carbonic anhydrase inhibitors for the treatment of hypoxic tumors. *J Exp Pharmacol* 2020;12:603–17.
181. Jonsson BH, Liljas A. Comments to the editor due to the response by the supuran group to our article. *Biophys J* 2021;120:182–3.
182. Maryanoff BE, Nortey SO, Gardocki JF, et al. Anticonvulsant O-alkyl sulfamates. 2,3:4,5-Bis-O-(1-methylethylidene)-beta-D-fructopyranose sulfamate and related compounds. *J Med Chem* 1987;30:880–7.
183. Dodgson SJ, Shank RP, Maryanoff BE. Topiramate as an inhibitor of carbonic anhydrase isoenzymes. *Epilepsia* 2000;41:35–9.
184. Klinger AL, McComsey DF, Smith-Swintosky V, et al. Inhibition of carbonic anhydrase-II by sulfamate and sulfamide groups: an investigation involving direct thermodynamic binding measurements. *J Med Chem* 2006;49:3496–500.
185. Shank RP, Smith-Swintosky VL, Maryanoff BE. Carbonic anhydrase inhibition. Insight into the characteristics of zonisamide, topiramate, and the sulfamide cognate of topiramate. *J Enzyme Inhib Med Chem* 2008;23:271–6.
186. Casini A, Antel J, Abbate F, et al. Carbonic anhydrase inhibitors: SAR and X-ray crystallographic study for the interaction of sugar sulfamates/sulfamides with isozymes I, II and IV. *Bioorg Med Chem Lett* 2003;13:841–5.
187. Nguyen GTH, Tran TN, Podgorski MN, et al. Nanoscale ion emitters in native mass spectrometry for measuring ligand-protein binding affinities. *ACS Cent Sci* 2019;5:308–18.
188. (a) Nguyen GTH, Nocentini A, Angeli A, et al. Perfluoroalkyl substances of significant environmental concern can strongly inhibit human carbonic anhydrase isozymes. *Anal Chem* 2020;92:4614–22. (b) Nguyen GTH, Leung WY, Tran TN, et al. Mechanism for the binding of netropsin to hairpin DNA revealed using nanoscale ion emitters in native mass spectrometry. *Anal Chem* 2020;92:1130–7.
189. Matulis D. Structural details of the enzymatic catalysis of carbonic anhydrase II via a mutation of valine to isoleucine. *IUCrJ* 2020;7:953–4.
190. Matulis D, Kranz JK, Salemme FR, Todd MJ. Thermodynamic stability of carbonic anhydrase: measurements of binding affinity and stoichiometry using ThermoFluor. *Biochemistry* 2005;44:5258–66.
191. Linkuvienė V, Krainer G, Chen WY, Matulis D. Isothermal titration calorimetry for drug design: precision of the enthalpy and binding constant measurements and comparison of the instruments. *Anal Biochem* 2016;515:61–4.
192. (a) Vullo D, Ruusuvoori E, Kaila K, et al. Carbonic anhydrase inhibitors: inhibition of the cytosolic human isozyme VII with anions. *Bioorg Med Chem Lett* 2006;16:3139–43. (b) Vullo D, Franchi M, Gallori E, et al. Carbonic anhydrase inhibitors. inhibition of cytosolic isozymes I and II and transmembrane, cancer-associated isozyme IX with anions. *J Enzyme Inhib Med Chem* 2003;18:403–6. (c) Innocenti A, Vullo D, Pastorek J, et al. Carbonic anhydrase inhibitors. Inhibition of transmembrane isozymes XII (cancer-associated) and XIV with anions. *Bioorg Med Chem Lett* 2007;17:1532–7. (d) Innocenti A, Lehtonen JM, Parkkila S, et al. Carbonic anhydrase inhibitors. Inhibition of the newly isolated murine isozyme XIII with anions. *Bioorg Med Chem Lett* 2004;14:5435–9.
193. (a) Sūdžius J, Baranauskienė L, Golovenko D, et al. 4-[N-(substituted 4-pyrimidinyl)amino]benzenesulfonamides as inhibitors of carbonic anhydrase isozymes I, II, VII, and XIII. *Bioorg Med Chem* 2010;18:7413–21. (b) Čapkauskaitė E, Zubrienė A, Baranauskienė L, et al. Design of [(2-pyrimidinylthio)acetyl]benzenesulfonamides as inhibitors of human carbonic anhydrases. *Eur J Med Chem* 2012;51:259–70.
194. Zubrienė A, Čapkauskaitė E, Gyltė J, et al. Benzenesulfonamides with benzimidazole moieties as inhibitors of carbonic anhydrases I, II, VII, XII and XIII. *J Enzyme Inhib Med Chem* 2014;29:124–31.
195. Dudutienė V, Matulienė J, Smirnov A, et al. Discovery and characterization of novel selective inhibitors of carbonic anhydrase IX. *J Med Chem* 2014;57:9435–46.
196. Dudutienė V, Zubrienė A, Smirnov A, et al. Functionalization of fluorinated benzenesulfonamides and their inhibitory properties toward carbonic anhydrases. *ChemMedChem* 2015;10:662–87.
197. Zakšauskas A, Čapkauskaitė E, Jezepčikas L, et al. Design of two-tail compounds with rotationally fixed benzenesulfonamide ring as inhibitors of carbonic anhydrases. *Eur J Med Chem* 2018;156:61–78.

198. Zakšauskas A, Čapkauskaitė E, Jezepčikas L, et al. Halogenated and di-substituted benzenesulfonamides as selective inhibitors of carbonic anhydrase isoforms. *Eur J Med Chem* 2020;185:111825.
199. Khalifah RG. The carbon dioxide hydration activity of carbonic anhydrase. *J Bio. Chem* 1971;246:2561–73.
200. Paketurytė V, Zubrienė A, Ladbury JE, Matulis D. Intrinsic thermodynamics of protein-ligand binding by isothermal titration calorimetry as aid to drug design. *Methods Mol Biol.* 2019;1964:61–74.
201. Kišonaitė M, Zubrienė A, Capkauskaitė E, et al. Intrinsic thermodynamics and structure correlation of benzenesulfonamides with a pyrimidine moiety binding to carbonic anhydrases I, II, VII, XII, and XIII. *PLoS One* 2014;9:e114106.
202. (a) Khalifah RG, Zhang F, Parr JS, Rowe ES. Thermodynamics of binding of the CO₂-competitive inhibitor imidazole and related compounds to human carbonic anhydrase I: an isothermal titration calorimetry approach to studying weak binding by displacement with strong inhibitors. *Biochemistry* 1993;32:3058–66. (b) An H, Tu C, Duda D, et al. Chemical rescue in catalysis by human carbonic anhydrases II and III. *Biochemistry* 2002;41:3235–42.
203. Gaspari R, Rechlin C, Heine A, et al. Kinetic and structural insights into the mechanism of binding of sulfonamides to human carbonic anhydrase by computational and experimental studies. *J Med Chem* 2016;59:4245–56.
204. De Simone G, Alterio V, Supuran CT. Exploiting the hydrophobic and hydrophilic binding sites for designing carbonic anhydrase inhibitors. *Expert Opin Drug Discov* 2013; 8:793–810.
205. Glöckner S, Ngo K, Sager CP, et al. Conformational changes in alkyl chains determine the thermodynamic and kinetic binding profiles of carbonic anhydrase inhibitors. *ACS Chem Biol* 2020;15:675–85.
206. Glöckner S, Ngo K, Wagner B, et al. The influence of varying fluorination patterns on the thermodynamics and kinetics of benzenesulfonamide binding to human carbonic anhydrase II. *Biomolecules* 2020;10:509.
207. Snyder PW, Mecinovic J, Moustakas DT, et al. Mechanism of the hydrophobic effect in the biomolecular recognition of arylsulfonamides by carbonic anhydrase. *Proc Natl Acad Sci USA* 2011;108:17889–94.
208. Fox JM, Kang K, Sastry M, et al. Water-restructuring mutations can reverse the thermodynamic signature of ligand binding to human carbonic anhydrase. *Angew Chem Int Ed Engl* 2017;56:3833–7.
209. Breiten B, Lockett MR, Sherman W, et al. Water networks contribute to enthalpy/entropy compensation in protein-ligand binding. *J Am Chem Soc* 2013;135:15579–84.
210. Fox JM, Kang K, Sherman W, et al. Interactions between Hofmeister anions and the binding pocket of a protein. *J Am Chem Soc* 2015;137:3859–66.
211. Talibov VO, Linkuvienė V, Matulis D, Danielson UH. Kinetically selective inhibitors of human carbonic anhydrase isozymes I, II, VII, IX, XII, and XIII. *J Med Chem* 2016; 59:2083–93.
212. Maren TH. Direct measurements of the rate constants of sulfonamides with carbonic anhydrase. *Mol Pharmacol* 1992;41:419–26.
213. (a) Maren TH, Clare BW, Supuran CT. Structure-activity studies of sulfonamide carbonic anhydrase inhibitors. *Roum Chem Quart Rev* 1994;2:259–82. (b) Maren TH, Conroy CW. A new class of carbonic anhydrase inhibitor. *J Biol Chem* 1993;268:26233–9.
214. Köhler K, Hillebrecht A, Schulze Wischeler J, et al. Saccharin inhibits carbonic anhydrases: possible explanation for its unpleasant metallic aftertaste. *Angew Chem Int Ed Engl* 2007;46:7697–9.
215. McDonald PC, Chia S, Bedard PL, et al. A phase 1 study of SLC-0111, a novel inhibitor of carbonic anhydrase IX, in patients with advanced solid tumors. *Am J Clin Oncol* 2020;43:484–90.
216. Lock FE, McDonald PC, Lou Y, et al. Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche. *Oncogene* 2013;32:5210–9.
217. Kreuzer M, Banerjee A, Birts CN, et al. Glycolysis, via NADH-dependent dimerisation of CtBPs, regulates hypoxia-induced expression of CAIX and stem-like breast cancer cell survival. *FEBS Lett* 2020;594:2988–3001.
218. Gibadulinova A, Bullova P, Strnad H, et al. CAIX-mediated control of LIN28/let-7 axis contributes to metabolic adaptation of breast cancer cells to hypoxia. *Int J Mol Sci* 2020;21: 4299.
219. Swayampakula M, McDonald PC, Vallejo M, et al. The inter-actome of metabolic enzyme carbonic anhydrase IX reveals novel roles in tumor cell migration and invadopodia/MMP14-mediated invasion. *Oncogene* 2017;36:6244–61.
220. Lee SH, Griffiths JR. How and why are cancers acidic? Carbonic anhydrase IX and the homeostatic control of tumour extracellular pH. *Cancers (Basel)* 2020;12:1616.
221. Ciccone V, Filippelli A, Angeli A, et al. Pharmacological inhibition of CA-IX impairs tumor cell proliferation, migration and invasiveness. *Int J Mol Sci* 2020;21:2983.
222. Pastorekova S, Gillies RJ. The role of carbonic anhydrase IX in cancer development: links to hypoxia, acidosis, and beyond. *Cancer Metastasis Rev* 2019;38:65–77.
223. Lee SH, McIntyre D, Honess D, et al. Carbonic anhydrase IX is a pH-stat that sets an acidic tumour extracellular pH in vivo. *Br J Cancer* 2018;119:622–30.
224. McDonald PC, Chafe SC, Brown WS, et al. Regulation of pH by carbonic anhydrase 9 mediates survival of pancreatic cancer cells with activated KRAS in response to hypoxia. *Gastroenterology* 2019;157:823–37.
225. Persi E, Duran-Frigola M, Damaghi M, et al. Systems analysis of intracellular pH vulnerabilities for cancer therapy. *Nat Commun* 2018;9:2997.
226. Boyd NH, Walker K, Fried J, et al. Addition of carbonic anhydrase 9 inhibitor SLC-0111 to temozolomide treatment delays glioblastoma growth in vivo. *JCI Insight* 2017;2: 92928.
227. Iessi E, Logozzi M, Mizzoni D, et al. Rethinking the combination of proton exchanger inhibitors in cancer therapy. *Metabolites* 2017;8:2.
228. McDonald PC, Swayampakula M, Dedhar S. Coordinated regulation of metabolic transporters and migration/invasion by carbonic anhydrase IX. *Metabolites* 2018;8:20.
229. Chafe SC, McDonald PC, Saberi S, et al. Targeting hypoxia-induced carbonic anhydrase IX enhances immune-checkpoint blockade locally and systemically. *Cancer Immunol Res* 2019;7:1064–78.
230. Damgaci S, Ibrahim-Hashim A, Enriquez-Navas PM, et al. Hypoxia and acidosis: immune suppressors and therapeutic targets. *Immunology* 2018;154:354–62.

231. Kuchuk O, Tuccitto A, Citterio D, et al. pH regulators to target the tumor immune microenvironment in human hepatocellular carcinoma. *Oncoimmunology* 2018;7:e1445452.
232. Ward C, Meehan J, Gray M, et al. Carbonic anhydrase IX (CAIX), cancer, and radiation responsiveness. *Metabolites* 2018;8:13.
233. Doyen J, Parks SK, Marcié S, et al. Knock-down of hypoxia-induced carbonic anhydrases IX and XII radiosensitizes tumor cells by increasing intracellular acidosis. *Front Oncol* 2012;2:199.
234. Serbian I, Schwarzenberger P, Loesche A, et al. Ureidobenzenesulfonamides as efficient inhibitors of carbonic anhydrase II. *Bioorg Chem* 2019;91:103123.
235. Vanchanagiri K, Emmerich D, Brusckhe M, et al. Synthesis and biological investigation of new carbonic anhydrase IX (CAIX) inhibitors. *Chem Biol Interact* 2018;284:12–23.
236. Federici C, Lugini L, Marino ML, et al. Lansoprazole and carbonic anhydrase IX inhibitors synergize against human melanoma cells. *J Enzyme Inhib Med Chem* 2016;31:119–25.
237. Andreucci E, Peppicelli S, Carta F, et al. Carbonic anhydrase IX inhibition affects viability of cancer cells adapted to extracellular acidosis. *J Mol Med (Berl)* 2017;95:1341–53.
238. Bryant JL, Gieling RG, Meredith SL, et al. Novel carbonic anhydrase IX-targeted therapy enhances the anti-tumour effects of cisplatin in small cell lung cancer. *Int J Cancer* 2018;142:191–201.
239. Logsdon DP, Grimard M, Luo M, et al. Regulation of HIF1 α under hypoxia by APE1/Ref-1 impacts CA9 expression: dual targeting in patient-derived 3D pancreatic cancer models. *Mol Cancer Ther* 2016;15:2722–32.
240. Peppicelli S, Andreucci E, Ruzzolini J, et al. The Carbonic Anhydrase IX inhibitor SLC-0111 as emerging agent against the mesenchymal stem cell-derived pro-survival effects on melanoma cells. *J Enzyme Inhib Med Chem* 2020;35:1185–93.
241. Genah S, Angeli A, Supuran CT, Morbidelli L. Effect of carbonic anhydrase IX inhibitors on human endothelial cell survival. *Pharmacol Res* 2020;159:104964.
242. Riemann A, Güttler A, Haupt V, et al. Inhibition of carbonic anhydrase IX by ureidosulfonamide inhibitor U104 reduces prostate cancer cell growth, but does not modulate daunorubicin or cisplatin cytotoxicity. *Oncol Res* 2018;26:191–200.
243. Güttler A, Theuerkorn K, Riemann A, et al. Cellular and radiobiological effects of carbonic anhydrase IX in human breast cancer cells. *Oncol Rep* 2019;41:2585–94.
244. Lee JY, Alexeyev M, Kozhukhar N, et al. Carbonic anhydrase IX is a critical determinant of pulmonary microvascular endothelial cell pH regulation and angiogenesis during acidosis. *Am J Physiol Lung Cell Mol Physiol* 2018;315:L41–L51.
245. Lee JY, Onanyan M, Garrison I, et al. Extrinsic acidosis suppresses glycolysis and migration while increasing network formation in pulmonary microvascular endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2019;317:L188–L201.
246. Bernardino RL, Dias TR, Moreira BP, et al. Carbonic anhydrases are involved in mitochondrial biogenesis and control the production of lactate by human Sertoli cells. *Febs J* 2019;286:1393–406.
247. Petrenko M, Güttler A, Funtan A, et al. Combined 3-O-acetylbetulin treatment and carbonic anhydrase IX inhibition results in additive effects on human breast cancer cells. *Chem Biol Interact* 2021;333:109326.
248. Venkateswaran G, Dedhar S. Interplay of carbonic anhydrase IX with amino acid and acid/base transporters in the hypoxic tumor microenvironment. *Front Cell Dev Biol* 2020;8:602668.
249. Brown WS, McDonald PC, Nemirovsky O, et al. Overcoming adaptive resistance to KRAS and MEK inhibitors by co-targeting mTORC1/2 complexes in pancreatic cancer. *Cell Rep Med* 2020;1:100131.