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Sulfonamide Inhibition Studies of the β-Class Carbonic Anhydrase CAS3 from the Filamentous Ascomycete Sordaria macrospora

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Abstract: A new β-class carbonic anhydrase was cloned and purified from the filamentous ascomycete Sordaria macrospora, CAS3. This enzyme has a higher catalytic activity compared to the other two such enzymes from this fungus, CAS1 and CAS2, which were reported earlier, with the following kinetic parameters: $k_{cat}$ of $(7.9 \pm 0.2) \times 10^5 \text{ s}^{-1}$, and $k_{cat}/k_m$ of $(9.5 \pm 0.12) \times 10^7 \text{ M}^{-1}\cdot\text{s}^{-1}$. An inhibition study with a panel of sulfonamides and one sulfamate was also performed. The most effective CAS3 inhibitors were benzolamide, brinzolamide, dichlorophnamide, methazolamide, acetazolamide, ethoxzolamide, sulfanilamide, methanilamide, and benzene-1,3-disulfonamide, with $K_I$s in the range of 54–95 nM. CAS3 generally shows a higher affinity for this class of inhibitors compared to CAS1 and CAS2. As S. macrospora is a model organism for the study of fruiting body development in fungi, these data may be useful for developing antifungal compounds based on CA inhibition.

Keywords: carbonic anhydrase; fungus; sulfonamide; kinetics; antifungals; Sordaria macrospora

1. Introduction

The filamentous ascomycete Sordaria macrospora is a coprophilous fungus that naturally lives on herbivore dung. For many years S. macrospora served as a model organism to study fruiting body development in fungi [1]. Previous studies identified four carbonic anhydrase (CA, EC 4.2.1.1) genes in the genome of S. macrospora that are designated as cas1, cas2, cas3, and cas4 [2–5]. The two β-CA genes cas1 and cas2 have high sequence identity and encode enzymes with characteristics of the plant-like sub-class of β-CAs [2]. The β-CA cas3 belongs to the cab-like [6] sub-class, whereas cas4 encodes for an α-class CA. CAS1 and CAS3 are cytoplasmic enzymes, while CAS2 is located in the mitochondria, and CAS4 is a secreted protein [3–5]. The three β-CA genes cas1, cas2, and cas3 are involved in the sexual development of S. macrospora [3]. Deletion of the α-CA cas4 resulted in a significantly reduced rate of ascospore germination but showed no significant involvement in sexual development and vegetative growth [5]. Thus, the detailed physiological roles of all these enzymes are not yet entirely elucidated.

The CA metalloenzymes are ubiquitous in most life forms, with eight distinct genetic families encoding the α-, β-, γ-, δ-, ε-, η-, θ-, and i-CAs [7–13]. By catalyzing the hydration of CO₂ to bicarbonate and protons, CAs are involved in various processes, starting with pH regulation and
ending with metabolism [4,7–13]. In fungi, they play crucial roles in growth, development, virulence, and survival [10,11], and modulation of their activity with inhibitors and/or activators was proposed as a new approach for designing antifungals [10,11,14–16]. The metal ion from the enzyme active site is usually coordinated by three amino-acid residues, whereas the fourth ligand is a water molecule or hydroxide ion which acts as a nucleophile in the hydrolytic reactions [4,7–13]. In β-CAs the Zn(II) is coordinated by two Cys residues, one His residue, and one water molecule/hydroxide ion [17,18].

The two β-class CA proteins CAS1 and CAS2 representing the major CA proteins of S. macrospora were biochemically and structurally characterized by our groups [3,17,18]. Both proteins could be easily produced in Escherichia coli and obtained in high purity (5–10 mg of CAS1 and 10–20 mg of CAS2 per L of culture) and exhibited noticeable in vitro CO₂ hydration activity with \( k_{\text{cat}}/K_m \) for CAS1 of \( 1.30 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) and for CAS2 of \( 1.21 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) [17]. The crystal structures of CAS1 and CAS2 were determined to a resolution of 2.7 Å and 1.8 Å, respectively [17]. The oligomeric state of both proteins was tetrameric. With exception of the active site composition, no further major differences could be observed between the structures of CAS1 and CAS2. The activity of both CA was only weakly inhibited by nitrite and nitrate anions and some other anions, making them good candidates for industrial applications [17]. In addition, both enzymes were recently investigated for their inhibition with a panel of 39 aromatic, heterocyclic, and aliphatic sulfonamides and one sulfamate, which acted in some cases as rather efficient inhibitors [18]. Dorzolamide, brinzolamide, and acetazolamide (AAZ) were medium-potency, high-micromolar inhibitors of these enzymes, being among the most effective ones detected so far [18]. However, no studies on the purification, catalytic activity, and inhibition of CAS3 are available to date. Here, we describe the purification, kinetic characterization, and inhibition studies of the cab-like β-CA of S. macrospora CAS3, with a panel of sulfonamides/sulfamates.

2. Results and Discussion

The protein encoded by the cas3 gene belongs to the cab-like sub-class of β-CAs and is localized to the cytoplasm [3]. Indeed, the cab-like CAs take their name from the enzyme discovered in the archaeon Methanobacterium thermoautotrophicum, which has only one such enzyme, called cab [9], and they are amongst the smallest CAs ever reported. Similar to cab, CAS3 that is reported here is composed of 174 amino-acid residues, with a calculated molecular mass of 19.2 kDa. CAS3 was synthesized in E. coli Rosetta (DE3) cells as a C-terminal His-tag fusion protein (Figure 1). After purification, 5–7.5 mg CAS3 could be obtained per L of culture. The purified enzyme was dialyzed against 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 8.3, 50 mM NaCl. To analyze the state of CAS3-His in solution, size-exclusion chromatography and multi-angle laser light scattering (SEC-MALLS) was performed (Figure 1). The calculated molar mass of CAS3 amounts to 38,650 g mol⁻¹ (±0.1%), which corresponds to 1.9 times the CAS3 monomer (20.36 kDa) (Figure 1). These findings suggest that the biological unit of CAS3 is a homo-dimer in solution, which is the same as for cab and other β-CAs from fungi or bacteria that have been investigated to date [19–23].

The catalytic activity for the physiological CO₂ hydration reaction that is catalyzed by these enzymes was investigated for the purified CAS3 by using a stopped-flow assay [24]. The activity of CAS3 was compared to those of the other two β-CAs present in the genome of this organism (CAS1 and 2), as well as with other similar enzymes from fungi/yeasts (Can2 from Cryptococcus neoformans [22], CalCA from Candida albicans [25], SceCA from Saccharomyces cerevisiae [26]), as well as the widespread human isoforms hCA I and II, belonging to the α-class [7]. As seen from data of Table 1, CAS3 has a catalytic activity that is an order of magnitude higher than CAS1 and CAS2, with the following kinetic parameters: \( k_{\text{cat}} \) of \( (7.9 \pm 0.2) \times 10^5 \text{ s}^{-1} \), and \( k_{\text{cat}}/K_m \) of \( (9.5 \pm 0.12) \times 10^7 \text{ M}^{-1} \text{s}^{-1} \). The activity of CAS3 is, thus, similar to that of CalCA and SceCA, being almost two times higher than that of the “slow” human isoform hCA I, a highly abundant enzyme in red blood cells [27]. Only hCA II among the investigated enzymes shown in Table 1 has a higher catalytic activity, but this enzyme is known to be one of the most effective catalysts in nature [7–9]. In addition, the CO₂ hydrase activity was effectively inhibited by the clinically used sulfonamide acetazolamide, one of the most investigated
Isozyme Activity Level K cat with various enzymes hCA II among the investigated enzymes shown in Table 1 has a higher catalytic activity, but this enzyme shows a lower affinity for sulfonamide inhibitors compared to CAS1 and 2, for which acetazolamide was a rather weak inhibitor.

Table 1. Kinetic parameters for the CO₂ hydration reaction [24] catalyzed by the human cytosolic enzymes hCA I and II (α-class CAs) at 20 °C and pH 7.5, and the β-CAs Can2, CalCA (from Cryptococcus neofor mans and Candida albicans, respectively), SceCA (from Saccharomyces cerevisiae), respectively, as well as the enzymes from Sordaria macrospora, CAS1–CAS3, measured at 20 °C, pH 8.3. Inhibition data with the clinically used sulfonamide acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) are also provided.

We, thus, conducted a detailed inhibition study of CAS3 with a panel of sulfonamides and one sulfamate, of types 1–24 and AAZ–HCT (Figure 2). The latter compounds are in fact clinically used drugs as diuretics, antiglaucoma, antiepileptic, and antiobesity agents. They target some of the many human isoforms involved in these pathologies [28–34].

As seen from data of Table 2, sulfonamides/sulfamates 1–24 and AAZ–HCT inhibit CAS3 with various efficacies. Generally, they are active in the high nanomolar range, except
sulthiame SLT and 8 which were micromolar inhibitors (K_s in the range of 2.07–4.83 µM). Many of the investigated compounds, both among the simple derivatives 1–18 or the more complex structures 19–24 or the clinically used agents, inhibited CAS3 with K_s < 100 nM, typically in the range of 54–95 nM. To this category of effective inhibitors belong the following derivatives: 1–3 (simple amino-benzenesulfonamides and benzene-1,3-disulfonamide); 12–14 (again, a benzene-1,3-disulfonamide derivative, 12, and the deacetylated precursors of acetazolamide and methazolamide, 13 and 14); 16 (4-hydroxymethyl-benzenesulfonamide); 19–24 (an amino-pyrimidinyl-sulfanilamide, 19, and the sulfanilyl-substituted aromatic/heterocyclic sulfonamides 20–24, all of them possessing a quite elongated moiety); AAZ, MZA, EZA, DCP, BRZ, BZA, and IND (clinically used or preclinical (IND) CAIs, possessing simple or more elaborated scaffolds). Thus, the structure–activity relationship (SAR) for CAS3 inhibition is not simple, since a rather large number of structurally different compounds show this profile of effective inhibitors. However, it may be observed that, with few exceptions, CAS3 is the isoform with the highest affinity for sulfonamides among the three S. macrospora isoforms investigated to date. Only CAS2 in some cases is more effectively inhibited by some compounds (compared to CAS3) [17,18], for example, with 13, 15, 17, and SLT (Table 2).

The remaining compounds showed less effective inhibition of CAS3, with K_s ranging between 191 and 831 nM. In this category of weak–medium inhibitors were the following compounds: 4–7; 9–11; 15; 17; 18; DZA; TPM–SLP; VLX; CLX; SAC; HCT. Again, they belong to a large variety of structural classes, from simple aromatic derivatives (4–7, 15, 17, 18) to heterocyclic ones, incorporating mono- or bicyclic ring systems (DZA, ZNS, VLX, CLX) to a sugar sulfamate (TPM), and the SAR is not straightforward.

![Molecules 2020, 25, 1036 5 of 11](image-url) Figure 2. (it is continued on next page).
Figure 2. Structure of sulfonamide and sulfamate inhibitors of types 1–24 and AAZ–HCT investigated in the present study.

Table 2. Inhibition of human isoforms hCA I and hCA II, and of the β-class fungal enzymes CAS1–CAS3 with sulfonamides 1–24 and the clinically used drugs AAZ–HCT, by a stopped flow CO₂ hydrase assay [24].

| Inhibitor/Enzyme Class | hCA I α | hCA II α | CAS1 β | CAS2 β | CAS3 β |
|------------------------|---------|---------|--------|--------|--------|
| 1                      | 28,000  | 300     | 361    | 386    | 90 ± 7.1 |
| 2                      | 25,000  | 240     | 144    | 3480   | 84 ± 5.6 |
| 3                      | 79 c    | 8       | 225    | 3630   | 83 ± 6.0 |
| 4                      | 78,500  | 320     | 47.1   | 6900   | 560 ± 21 |
| 5                      | 25,000  | 170     | 323    | 8720   | 726 ± 30 |
| 6                      | 21,000  | 160     | 241    | 7650   | 441 ± 14 |
| 7                      | 8300    | 60      | 43.2   | 7360   | 585 ± 23 |
| 8                      | 9800    | 110     | 79.6   | 9120   | 2078 ± 61 |
| 9                      | 6500    | 40      | 580    | 12,000 | 712 ± 28 |
| 10                     | 7300    | 54      | >50,000| 23,500 | 350 ± 13 |
| 11                     | 5800    | 63      | 890    | 18,700 | 235 ± 16 |
| 12                     | 8400    | 75      | 3350   | >50,000| 90 ± 5.7 |
| 13                     | 8600    | 60      | 8650   | 48.1   | 88 ± 2.9 |
Table 2. Cont.

| Inhibitor/Enzyme Class | hCA I \( ^a \) α K_i* \( ^a \) (nM) | hCA II \( ^a \) α CAS1 \( ^b \) β | CAS2 \( ^b \) β | CAS3 \( ^c \) β |
|------------------------|-------------------------|------------------|--------------------|-----------------|
| 14                     | 9300                    | 19               | 7215               | 280             | 94 ± 4.1        |
| 15                     | 5500                    | 80               | 3160               | 143             | 605 ± 23        |
| 16                     | 9500                    | 94               | 4520               | 92.5            | 82 ± 3.7        |
| 17                     | 21,000                  | 125              | >50,000            | 390             | 507 ± 26        |
| 18                     | 164                     | 46               | 4435               | 3250            | 226 ± 13        |
| 19                     | 109                     | 33               | 475                | 6760            | 91 ± 4.4        |
| 20                     | 6                       | 2                | 363                | 9880            | 85 ± 2.7        |
| 21                     | 69                      | 11               | 4550               | 4060            | 95 ± 3.9        |
| 22                     | 164                     | 46               | 1985               | 25,200          | 85 ± 2.6        |
| 23                     | 109                     | 33               | 282                | >50,000         | 89 ± 3.4        |
| 24                     | 95                      | 30               | 294                | >50,000         | 84 ± 1.5        |
| AAZ                    | 250                     | 12               | 445                | 816             | 94 ± 3.0        |
| MZA                    | 50                      | 14               | 421                | 8140            | 91 ± 6.2        |
| EZA                    | 25                      | 8                | 440                | 3170            | 95 ± 2.8        |
| DCP                    | 1200                    | 38               | 1220               | 5790            | 73 ± 3.0        |
| DZA                    | 50,000                  | 9                | 360                | 742             | 274 ± 14        |
| BRZ                    | 45,000                  | 3                | 451                | 739             | 61 ± 1.4        |
| BZA                    | 15                      | 9                | 2115               | 410             | 54 ± 0.9        |
| TPM                    | 250                     | 10               | 414                | 673             | 363 ± 23        |
| ZNS                    | 56                      | 35               | 1820               | 1885            | 710 ± 40        |
| SLP                    | 1200                    | 40               | 1715               | 670             | 493 ± 27        |
| IND                    | 31                      | 15               | 4240               | 216             | 94 ± 4.6        |
| VLX                    | 54,000                  | 43               | 4425               | 3730            | 831 ± 38        |
| CLX                    | 50,000                  | 21               | 2513               | 857             | 669 ± 29        |
| SLT                    | 374                     | 9                | 3210               | 496             | 4838 ± 257      |
| SAC                    | 18,540                  | 5959             | 5280               | 7075            | 191 ± 12        |
| HCT                    | 328                     | 290              | 3350               | 6680            | 545 ± 41        |

\( ^a \) From references [7,8]; \( ^b \) from reference [17]; \( ^c \) this work: mean ± standard error, from three different assays.

3. Materials and Methods

3.1. Construction of the cas3 Overexpression Vector

RNA from *S. macrospora* was isolated as described in Reference [3]. To remove obsolete genomic DNA, the RNA was treated with DNase I (Thermo Scientific, EN0521, Waltham, MA, USA) according to the manufacturer’s manual. The “Transcriptor High Fidelity cDNA Synthesis kit” (Roche, Basel, Switzerland) was used for the reverse transcription reaction. Template concentration was 2 µg of RNA. The complementary DNA (cDNA) of the cas3 open reading frame (ORF; 525 bp) was amplified with primer pair CAS-pet-f (GGAGATATACATAATGATGCCCGTCACCAACGAAGA) and Cas3-pet-r (GGTGGTGGTGTCGAGAACACACCTACCGTCTTGC). The obtained PCR fragment was cloned into the pET22b(+) expression plasmid (Novagen, Madison, WI, USA) linearized with *Nde*I and *Xho*I to create a 6×His fusion protein. The plasmid was named pET-CAS3.

3.2. Heterologous Expression of the cas3 Gene in *E. coli*

The production of the CAS3 protein was performed in *E. coli* strain Rosetta (DE3) (Invitrogen, Carlsbad, Germany). An overnight pre-culture was used to inoculate 4 × 0.5 L of Luria broth (LB) medium supplemented with 100 mg/L ampicillin and 0.5 mM ZnSO\(_4\). The cultures were grown to an OD\(_{600}\) of 0.1. Heterologous gene expression was then induced by the addition of 1 mM IPTG during the exponential phase of growth and lasted for 3–4 h at 30 °C. Subsequently, the cells were harvested (4000×g, 30 min, 4 °C) and flash-frozen with liquid nitrogen and stored at −20 °C.
For determination of the protein concentration, 10 µL of the protein was mixed with 990 µL of Bradford reagent \[35\] and incubated for 2 min at RT. Then, the absorption was determined at 595 nm in a “Libra S12” (biochrom, Cambridge, UK) spectrophotometer in a 1-mL cuvette. Prior to this, a calibration line with bovine serum albumin was recorded.

The completeness of CAS3 was analyzed by mass spectrometry using the “LCQ DecaXP mass spectrometer” (Thermo Scientific, Waltham, MA, USA) and by SDS polyacrylamide gel electrophoresis (PAGE) \[36\]. CAS3 was separated on a 15% SDS PAGE with the protein standard (Pageruler\textsuperscript{TM} Prestained Protein Ladder, SM0671, Thermo Fisher, Waltham, MA, USA). For visualization of the proteins after the electrophoresis, the gel was incubated for 30 min in SDS-gel staining solution (0.02% (w/v) Coomassie brilliant blue R250, 0.02% (w/v) Coomassie brilliant blue G250, 42.5% (v/v) ethanol, 0.5% (v/v) methanol, 10% (v/v) acetic acid), and afterward in SDS-gel distaining solution (45% (v/v) ethanol, 10% (v/v) acetic acid) for 30 min.

3.3. Purification of CAS3-His

For purification of CAS3, 10 g of \textit{E. coli} cells were re-suspended in 30 mL of lysis buffer (20 mM imidazole, 50 mM NaH\textsubscript{2}PO\textsubscript{4} pH 8, 300 mM NaCl, 0.02 mM MgCl\textsubscript{2}, one “Protease Inhibitor Cocktail” Tablet (Roche, Basil, Switzerland) and subsequently incubated for 30 min at 4 °C with a spatula tip of lysozyme (Serva, Heidelberg, Germany, 28262.03) and DNase I. After incubation, the cells were disrupted using a microfluidizer 110S (Microfluidics, bonn, Germany). After centrifugation (50,000× g, 30 min, 4 °C), the clarified lysate was applied on an Ni-NTA agarose (Qiagen, Hilden, Germany, 1018244) column equilibrated with lysis buffer. Unbound proteins were removed by washing gradually with three column volumes (CV) of lysis buffer containing 40 mM, 60mM, 80 mM, and 100 mM imidazole. Bound CAS3 was eluted with lysis buffer containing 250 mM imidazole. Elution fractions were analyzed by SDS-PAGE, pooled, concentrated in a “Spin-X® UF 20” (Corning, Kaiserslautern, Germany), and stored at 4 °C. After purification, 5–7.5 mg of CAS3 could be obtained per L of culture.

3.4. Size-Exclusion Chromatography Coupled with Multi-Angle Laser Light Scattering

Four hundred microliters CAS3 (3 mg/mL) were loaded separately on a 24-mL analytical “Superdex 200 (10/300)” gel filtration column using an “Äkta purifier” coupled to a “miniDAWN MALS” detector (Wyatt Technology, Santa Barbara, CA, USA). The column was pre-equilibrated with 50 mM NaH\textsubscript{2}PO\textsubscript{4} pH 8.0, 250 mM imidazole, 300 mM NaCl. Multi-angle laser light-scattering analysis was performed continuously on the column eluate at 291 K (size-exclusion chromatography coupled with multi-angle laser light scattering, SEC-MALLS). Data analysis was carried out with Astra software (Wyatt Technology, Santa Barbara, CA, USA).

3.5. CA Inhibition Assay

An Applied Photophysics stopped-flow instrument was used for assaying the CA catalyzed CO\textsubscript{2} hydration activity \[24\]. The pH indicator used was bromothymol blue at a concentration of 0.2 mM, working at the absorbance maximum of 557 nm. The buffer used was 20 mM Tris (pH 8.3), and 20 mM Na\textsubscript{2}SO\textsubscript{4} was added to this buffer for maintaining constant the ionic strength (sulfate is not inhibitory and has a K\textsubscript{I} > 200 mM against this enzyme; data not shown). The initial rates of the CA-catalyzed CO\textsubscript{2} hydration were followed for a period of 10–100 s for each assay. The CO\textsubscript{2} concentrations ranged from 1.7 to 17 mM for determining kinetic parameters of the reaction and the inhibition constants of tested inhibitors. For each measurement, six traces of the initial 5%–10% of the reaction were used for determining the initial velocity. Then, 10-fold decreasing dilution of inhibitor solutions, ranging from 1 nM and 100 µM were employed for determining the inhibition constants. Uncatalyzed rates were determined in the same manner and subtracted from total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled deionized water, and dilutions up to 1 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature before to assay for allowing for the formation of E–I complexes. The inhibition
constants were obtained by non-linear least-squares methods using the Cheng–Prusoff equation, and they represent the mean from at least three different determinations. The human isoforms hCA I, II were assayed in the same conditions as above except that the working pH was 7.4 HEPES buffer with phenol red as an indicator [21,22]. The inhibition constants measured by this method are in excellent agreement with the dissociation constants measured by native mass spectrometry [37,38].

4. Conclusions

Fungal CAs are of great interest both for biotechnological and pharmaceutical applications [10] because some of these enzymes are stable, relatively easy to produce, and may be used as model enzymes for testing inhibitors/activators. The organism *Sordaria macrospora*, used as a genetic model to study fruiting body development of filamentous fungi, encodes for at least four different CAs, two of which were thoroughly investigated earlier, CAS1 and CAS2. Here, we prove that CAS3, another representative enzyme from this organism, belonging to the β-CA class, may be of interest for better understanding the roles these proteins play in various physiologic processes of fungi. Unlike CAS1 and CAS2, which showed rather low catalytic activity for the hydration of CO$_2$ to bicarbonate and protons, CAS3 is a highly effective catalyst, showing kinetic parameters comparable to those of other fungal/mammalian enzymes, i.e., $k_{\text{cat}}$ of $(7.9 \pm 0.2) \times 10^5$ s$^{-1}$, and $k_{\text{cat}}/K_m$ of $(9.5 \pm 0.12) \times 10^7$ M$^{-1}$·s$^{-1}$. A detailed inhibition study of CAS3 with sulfonamides and one sulfamate allowed us to demonstrate that this enzyme has higher affinity for these inhibitors compared to CAS1 and CAS2, with clinically used agents such as benzolamide, brinzolamide, dichlorophnamide, methazolamide, acetazolamide, ethoxzolamide, sulfamilamide, metanilamide, and benzene-1,3-disulfonamide possessing $K_I$s under 100 nM.

Author Contributions: D.V. performed the enzyme kinetics and all the inhibition assays; R.L. performed the cloning, expression, and purification of the recombinant coral enzyme; W.A.D., C.T.S., and S.P. supervised the experiments and checked the results of performed experiments, as well as wrote and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. Teichert, I.; Nowrousian, M.; Pöggeler, S.; Kück, U. The filamentous fungus *Sordaria macrospora* as a genetic model to study fruiting body development. *Adv. Genet.* 2014, 87, 201–246.
2. Elleuche, S.; Pöggeler, S. Evolution of carbonic anhydrases in fungi. *Curr. Genet.* 2009, 55, 211–222. [CrossRef] [PubMed]
3. Elleuche, S.; Pöggeler, S. β-Carbonic anhydrases play a role in fruiting body development and ascospore germination in the filamentous fungus *Sordaria macrospora*. *PLoS ONE* 2009, 4, e5177. [CrossRef] [PubMed]
4. Elleuche, S.; Pöggeler, S. Carbonic anhydrases in fungi. *Microbiology* 2010, 156, 23–29. [CrossRef]
5. Lehneck, R.; Elleuche, S.; Pöggeler, S. The filamentous ascomycete *Sordaria macrospora* can survive in ambient air without carbonic anhydrases. *Mol. Microbiol.* 2014, 95, 931–944. [CrossRef]
6. Innocenti, A.; Zimmerman, S.; Ferry, J.G.; Scozzafava, A.; Supuran, C.T. Carbonic anhydrase inhibitors. Inhibition of the beta-class enzyme from the methanoarchaeon *Methanobacterium thermoautotrophicum* (cab) with anions. *Bioorg. Med. Chem. Lett.* 2004, 14, 4563–4567.
7. Supuran, C.T. Structure and function of carbonic anhydrases. *Biochem. J.* 2016, 473, 2023–2032. [CrossRef]
8. Supuran, C.T. Carbonic anhydrases: Novel therapeutic applications for inhibitors and activators. *Nature Rev. Drug Discov.* 2008, 7, 168–181. [CrossRef]
9. Zimmerman, S.A.; Ferry, J.G.; Supuran, C.T. Inhibition of the archael beta-class (Cab) and gamma-class (Cam) carbonic anhydrases. *Curr. Top. Med. Chem.* 2007, 7, 901–908. [CrossRef]
10. Lehneck, R.; Pöggeler, S. Fungal carbonic anhydrases and their inhibition. In *Zinc Enzyme Inhibitor -Volume 1: Enzymes from microorganisms*; Supuran, C.T., Capasso, C., Eds.; Topics in Medicinal Chemistry; Springer International Publishing AG: Cham, Switzerland, 2017; Volume 22, pp. 95–110.

11. Supuran, C.T. Advances in structure-based drug discovery of carbonic anhydrase inhibitors. *Expert Opin. Drug Discov.* 2017, 12, 61–88. [CrossRef]

12. Supuran, C.T.; Capasso, C. Biomedical applications of prokaryotic carbonic anhydrases. *Expert Opin. Ther. Pat.* 2018, 28, 745–754. [CrossRef] [PubMed]

13. Nocentini, A.; Supuran, C.T. Advances in the structural annotation of human carbonic anhydrases and impact on future drug discovery. *Expert Opin. Drug Discov.* 2019, 14, 1175–1197. [CrossRef] [PubMed]

14. Annunziato, G.; Giovati, L.; Angeli, A.; Pavone, M.; Del Prete, S.; Pieroni, M.; Capasso, C.; Bruno, A.; Conti, S.; Magliani, W.; et al. Discovering a new class of antifungal agents that selectively inhibits microbial carbonic anhydrases. *J. Enzyme Inhib. Med. Chem.* 2018, 33, 1537–1544. [CrossRef] [PubMed]

15. Nocentini, A.; Vullo, D.; Del Prete, S.; Osman, S.M.; Alasmary, F.A.S.; AlOthman, Z.; Capasso, C.; Carta, F.; Gratteri, P.; Supuran, C.T. Inhibition of the beta-carbonic anhydrase from the dandruff-producing fungus *Malassezia globosa* with monothiocarbamates. *J. Enzyme Inhib. Med. Chem.* 2017, 32, 1064–1070. [CrossRef]

16. Del Prete, S.; Angeli, A.; Ghobril, C.; Hitce, J.; Clavaud, C.; Marat, X.; Supuran, C.T.; Capasso, C. Sulfonamide Inhibition Profile of the β-Carbonic Anhydrase from *Malassezia restricta*, An Opportunistic Pathogen Triggering Scalp Conditions. *Metabolites* 2020, 10, E39. [CrossRef]

17. Lehneck, R.; Neumann, P.; Vullo, D.; Elleuche, S.; Supuran, C.T.; Ficner, R.; Pöggeler, S. Crystal structures of two tetrameric β-carbonic anhydrases from the filamentous ascomycete *Sordaria macrospora*. *FEBS J.* 2014, 281, 1759–1772. [CrossRef]

18. Vullo, D.; Lehneck, R.; Pöggeler, S.; Supuran, C.T. Sulfonamide inhibition studies of two β-carbonic anhydrases from the ascomycete fungus *Sordaria macrospora*, CAS1 and CAS2. *J. Enzyme Inhib. Med. Chem.* 2018, 33, 390–396. [CrossRef]

19. Del Prete, S.; De Luca, V.; Vullo, D.; Osman, S.M.; AlOthman, Z.; Carginale, V.; Supuran, C.T.; Capasso, C. A new procedure for the cloning, expression and purification of the beta-carbonic anhydrase from the pathogenic yeast *Malassezia globosa*, an anti-dandruff drug target. *J. Enzyme Inhib. Med. Chem.* 2016, 31, 1156–1161. [CrossRef]

20. Ferraroni, M.; Del Prete, S.; Vullo, D.; Capasso, C.; Supuran, C.T. Crystal structure and kinetic studies of a tetrameric type II beta-carbonic anhydrase from the pathogenic bacterium *Vibrio cholerae*. *Acta Crystallogr. D Biol. Crystallogr.* 2015, 71, 2449–2456. [CrossRef]

21. Nishimori, I.; Minakuchi, T.; Vullo, D.; Scozzafava, A.; Innocenti, A.; Supuran, C.T. Carbonic anhydrase inhibitors. Cloning, characterization, and inhibition studies of a new beta-carbonic anhydrase from *Mycobacterium tuberculosis*. *J. Med. Chem.* 2009, 52, 3116–3120. [CrossRef]

22. Schlicker, C.; Hall, R.A.; Vullo, D.; Middelhaufe, S.; Gertz, M.; Supuran, C.T.; Mühlschlegel, F.A.; Steegborn, C. Structure and inhibition of the CO2-sensing carbonic anhydrase Can2 from the pathogenic fungus *Cryptococcus neoformans*. *J. Mol. Biol.* 2009, 385, 1207–1220. [CrossRef] [PubMed]

23. Supuran, C.T.; Capasso, C. An Overview of the Bacterial Carbonic Anhydrases. *Metabolites*. 2017, 7, E56. [CrossRef]

24. Khalifah, R.G. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. *J. Biol. Chem.* 1971, 246, 2561–2573. [PubMed]

25. Innocenti, A.; Hall, R.A.; Schlicker, C.; Scozzafava, A.; Steegborn, C.; Mühlschlegel, F.A.; Supuran, C.T. Carbonic anhydrase inhibitors. Inhibition and homology modeling studies of the fungal beta-carbonic anhydrase from *Candida albicans* with sulfonamides. *Bioorg. Med. Chem.* 2009, 17, 4503–4509. [PubMed]

26. Isik, S.; Kockar, F.; Arslan, O.; Guler, O.O.; Innocenti, A.; Supuran, C.T. Carbonic anhydrase inhibitors. Inhibition of the beta-class enzyme from the yeast *Saccharomyces cerevisiae* with anions. *Bioorg. Med. Chem. Lett.* 2008, 18, 6327–6331. [CrossRef]

27. Durdagi, S.; Vullo, D.; Pan, P.; Kähkönen, N.; Määttä, J.A.; Hytönen, V.P.; Scozzafava, A.; Parkkila, S.; Supuran, C.T. Protein-protein interactions: Inhibition of mammalian carbonic anhydrases I–XV by the murine inhibitor of carbonic anhydrase and other members of the transferrin family. *J. Med. Chem.* 2012, 55, 5529–5535. [CrossRef]

28. Carta, F.; Scozzafava, A.; Supuran, C.T. Sulfonamides: A patent review (2008–2012). *Expert Opin. Ther. Pat.* 2012, 22, 747–758. [CrossRef]
29. Supuran, C.T. Applications of carbonic anhydrases inhibitors in renal and central nervous system diseases. *Expert Opin. Ther. Pat.* 2018, 28, 713–721. [CrossRef]

30. Supuran, C.T. Carbonic anhydrase inhibitors and their potential in a range of therapeutic areas. *Expert Opin. Ther. Pat.* 2018, 28, 709–712. [CrossRef] [PubMed]

31. Supuran, C.T. Carbonic anhydrase inhibitors as emerging drugs for the treatment of obesity. *Expert Opin. Emerg. Drugs* 2012, 17, 11–15. [CrossRef]

32. Supuran, C.T.; Altamimi, A.S.A.; Carta, F. Carbonic anhydrase inhibition and the management of glaucoma: A literature and patent review 2013–2019. *Expert Opin. Ther. Pat.* 2019, 29, 781–792. [CrossRef] [PubMed]

33. Supuran, C.T. Carbonic anhydrase inhibitors as emerging agents for the treatment and imaging of hypoxic tumors. *Expert Opin. Investig. Drugs* 2018, 27, 963–970. [CrossRef] [PubMed]

34. Supuran, C.T. The management of glaucoma and macular degeneration. *Expert Opin. Ther. Pat.* 2019, 29, 745–747. [CrossRef] [PubMed]

35. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, 72, 248–254. [CrossRef]

36. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227, 680–685. [CrossRef] [PubMed]

37. Nguyen, G.T.H.; Tran, T.N.; Podgorski, M.N.; Bell, S.G.; Supuran, C.T.; Donald, W.A. Nanoscale Ion Emitters in Native Mass Spectrometry for Measuring Ligand-Protein Binding Affinities. *ACS. Cent. Sci.* 2019, 5, 308–318. [CrossRef]

38. Nocentini, A.; Donald, W.A.; Supuran, C.T. Human carbonic anhydrases: Tissue distribution, physiological role, and druggability. In *Carbonic Anhydrases–Biochemistry and Pharmacology of an Evergreen Pharmaceutical Target*; Supuran, C.T., Nocentini, A., Eds.; Elsevier–Academic Press: London, UK, 2019; pp. 151–185.

**Sample Availability:** Samples of the inhibitors are potentially available upon request from the authors.

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