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Sulfonamide inhibition studies of two β-carbonic anhydrases from the ascomycete fungus *Sordaria macrospora*, CAS1 and CAS2

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ABSTRACT

The two *β*-carbonic anhydrases (CAs, EC 4.2.1.1) recently cloned and purified from the ascomycete fungus *Sordaria macrospora*, CAS1 and CAS2, were investigated for their inhibition with a panel of 39 aromatic, heterocyclic, and aliphatic sulfonamides and one sulfamate, many of which are clinically used agents. CAS1 was efficiently inhibited by tosylamide, 3-fluorosulfanilamide, and 3-chlorosulfanilamide (K_Is in the range of 43.2–79.6 nM), whereas acetazolamide, methazolamide, topiramate, ethoxzolamide, dorzolamide, and brinzolamide were medium potency inhibitors (K_Is in the range of 360–445 nM). CAS2 was less sensitive to sulfonamide inhibitors. The best CAS2 inhibitors were 5-amino-1,3,4-thiadiazole-2-sulfonamide (the deacetylated acetazolamide precursor) and 4-hydroxymethyl-benzenesulfonamide, with K_Is in the range of 48.1–92.5 nM. Acetazolamide, dorzolamide, ethoxzolamide, topiramate, sulpiride, indisulam, celecoxib, and sulthiame were medium potency CAS2 inhibitors (K_Is of 143–857 nM). Many other sulfonamides showed affinities in the high micromolar range or were ineffective as CAS1/2 inhibitors. Small changes in the structure of the inhibitor led to important differences of the activity. As these enzymes may show applications for the removal of anthropically generated polluting gases, finding modulators of their activity may be crucial for designing environmental-friendly CO_2 capture processes.

1. Introduction

Sordaria macrospora is a filamentous ascomycete and a model organism for investigating the sexual fruiting body (perithecia) formation, due to the fact that being a homothallic fungus (i.e. self-fertile), it is easily genetically tractable, and well suited for large-scale genomic, transcriptomic, and proteomic studies[1]. The proteins involved in chromatin remodelling and transcriptional regulation of the fruiting body development, as well as the primary and secondary metabolic processes involved in nutrient recycling by autophagy were also understood in greater detail by using S. *macrospora* as a model organism[1].

Recently, our groups cloned, expressed, and investigated in some detail two *β*-carbonic anhydrases (CAs, EC 4.2.1.1) encoded in the genome of this fungus, nominated CAS1 and CAS2, which showed a good catalytic activity for the physiologic reaction catalysed by these enzymes, that is, hydration of CO_2 with formation of bicarbonate and protons[2]. Indeed, CAs belonging to at least two of the seven genetical families known to date, are widespread in fungi[3,4], where they are involved in crucial physiologic processes such as, among others, pH regulation and anaplerotic/biosynthetic reactions leading to fatty acids, amino acids, nucleic acids, and other biomolecules[2–5]. Furthermore, both protons and bicarbonate, the reactions products of the enzyme catalysed CO_2 hydration, are important for chemosensing, a process which regulates fundamental physiologic processes in fungi, such as the type

of growth, the production of spores, and for the pathogenic yeasts, also virulence, survival in the host environment, and production of mycotoxins[2–6]. It is thus understandable that CAs[7–10] have been extensively investigated in the last decade especially in pathogenic fungi, such as *Candida albicans*[11,12], *Candida glabrata*[13,14], *Cryptococcus neoformans*[15,16], *Malassezia globosa*[17,18] and to a lower extent in *Saccharomyces cerevisiae*[19]. All these fungi, similar to S. *macrospora* encode for *β*-CAs, but *α*-class enzymes were also reported in some species of *Aspergillus*, such as *Aspergillus terreus, Aspergillus oryzae, Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus, Aspergillus nidulans*, and *Aspergillus clavatus*[20]. S. *macrospora* also encodes for an *α*-CA[20]c. However, the most investigated such organisms from the medicinal chemistry viewpoint, encode for *β*-CAs. For such enzymes, many inhibition studies are available, in the search of compounds which may interfere with the life cycle of these pathogens[11–19]. In the case of CAS1 and CAS2, only anion inhibitors were investigated, which generally showed low affinity for both isoforms, as expected for this class of CA inhibitors (CAIs)[21]. Thus, in this paper we report the first sulfonamide inhibition study of these enzymes, considering the fact that sulfonamides and their isosteres (sulfamates, sulfamides) are the main class of CAIs, with many such compounds possessing clinical applications for the treatment and prevention of many diseases in which CA activity and expression is dysregulated[22–25].

2. Materials and methods

2.1. Chemistry

Sulfonamides **1**–**24** and the clinically used agents **AAZ**–**HCT** were either commercially available, highest purity reagents from Sigma-Aldrich (Milan, Italy), or were reported earlier by one of our groups[22–25].

2.2. CA inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity[26]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM TRIS (pH 8.3) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalysed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalysed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng–Prusoff equation, as reported earlier[22–25], and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier[2,9].

3. Results and discussion

We investigated the susceptibility of CAS1 and CAS2 to inhibition with the main class of CAIs, the sulfonamides and their isosteres (sulfamates/sulfamides)[9,10,22–25]. A panel of 40 such derivatives were included in this study. Derivatives **1**–**24** and **AAZ**–**HCT** (Figure 1) are either simple aromatic/heterocyclic sulfonamides widely used as building blocks for obtaining new families of such pharmacological agents[9,10,22–25], or they are clinically used agents, among which acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, and dichlorophenamide **DCP**, are the classical, systemically acting antiglaucoma CAIs[9]. Dorzolamide **DZA** and brinzolamide **BRZ** are topically-acting antiglaucoma agents, benzolamide **BZA** is an orphan drug belonging to this class of pharmacological agents, whereas topiramate **TPM**, zonisamide **ZNS**, and sulthiame **SLT** are widely used antiepileptic drugs[9,22,25]. Sulpiride **SLP** and indisulam **IND** were also shown by our group to belong to this class of pharmacological agents, together with the COX2 selective inhibitors celecoxib **CLX** and valdecoxib **VLX**[9]. Saccharin and the diuretic hydrochlorothiazide **HCT** are also known to act as CAIs, and were included in this study[22–25].

Data of Table 1 show that both CAS1 and CAS2 possess catalytic activity for the hydration of CO_2 to bicarbonate and protons, with kinetic constants which are lower compared to those of the human (h) isoforms hCA I and II (the last enzyme is one of the best catalysts known in nature)[9]. However, even if these parameters are lower, both enzymes possess a significant catalytic activity, with k_{cat}/K_m values $>10^6 \, s^{-1}$. Furthermore, this activity is inhibited by the clinically sued sulfonamide acetazolamide, a standard CAI, as shown in Table 1, although with inhibition constants in the

high nanomolar range (K_I of 445 nM for CAS1 and of 816 nM for CAS2)[2].

Inhibition data of CAS1 and CAS2 with the sulfonamides shown in Figure 1 (compounds **1**–**24** and **AAZ**–**HCT**) are presented in Table 2, in which the hCA I/II inhibition with the same set of derivatives is also shown for comparison reasons.

The following structure–activity relationship (SAR) can be observed from the data of Table 2:

i. Several sulfonamides were ineffective as CAS1/2 inhibitors, with K_Is > 50 μM. They include **10** and **17** for CAS1, and **12**, **23**, and **24** for CAS2 (Table 2).

ii. For CAS1, a range of derivatives, among which **12**–**16**, **18**, **21**, **22**, **DCP**, **BRZ**, and **ZNS**–**HCT**, showed weak inhibitory action, with inhibition constants in the micromolar range, more precisely of 1.22–8.65 μM. They include a large variety of different chemotypes, such as the aromatic 1,3-benzene-disulfonamide **12**, the heterocyclic precursors of two clinically used agents (**AAZ**, **MZA**) **13** and **14**, as well as the elongated molecule sulfonamide of the sulfonylated-amino-sulfonamide type **21** and **22**, in addition to the clinically used agents which possess an even higher diversity of scaffolds. It is thus impossible to draw detailed SAR conclusions based on these very variable chemotypes with this modest activity.

iii. A large number of derivatives behaved as medium potency CAS1 inhibitors, with K_Is in the range of 144–890 nM (Table 1). They include **1**–**3**, **5**, **6**, **9**, **11**, **19**, **20**, **23**, **24**, **AAZ**, **MZA**, **EZA**, **DZA**, **BRZ**, and **TPM**. All of them belong to the sulfonamide class, except **TPM** which is the only sulfamate investigated here. From the chemical viewpoint, they also possess a rather high variability, but some of these chemotypes are easier to rationalize. Thus, 3- or 4-substituted benzenesulfonamides with compact moieties (amino, sulfamoyl, aminoalkyl), such as in derivatives **1**–**3**, **5**, and **6**, lead to a rather effective CAS inhibitory action compared to the bulkier derivatives discussed above (e.g. **11**, **12**, **21**, **22**, etc.). The halogenosubstituted sulfanilamides show a good activity (especially for light halogens incorporating derivatives which will be discussed shortly), with the bromosubstituted derivative **9** being less effective than sulfanilamide **2**, whereas the fluoro- and chloro-containing derivatives **7** and **8** being much better CAS1 inhibitors than the lead **2**. It is also interesting to note the difference between the two 1,3-benzene-disulfonamides **11** and **12**, with the trifluoromethyl derivative **11** being 3.76 times a better inhibitor compared to the structurally related chlorine derivative **12**. Compounds **20**, **23**, and **24** belong to the sulfonylated-aminosulfonamide class of CAIs, as **21** and **22** discussed earlier, but in the case of **20** the presence of the 1,3,4-thiadiazole-2-sulfonamide head probably leads to the enhanced inhibitory effect, whereas for **23** and **24**, the longer spacers between the two parts of the molecule (compared to the spacer from **20**, which is in fact absent) produce the same effect. Thus, in these cases the SAR is rather well defined, demonstrating that small structural changes in the molecule of the inhibitor lead to drastic changes in the affinity for the enzyme. Among the clinically used sulfonamides/sulfamates, **AAZ**, **MZA**, **EZA**, **DZA**, **BRZ**, and **TPM** are in this category of medium potency inhibitors. It should be noted that whereas **AAZ** and **MZA** possess rather compact, monocyclic scaffolds, the ones from **EZA**, **DZA**, and **BRZ** are much bulkier, which proves that the active site of the enzyme may accommodate

even these sterically hindered sulfonamides. The same situation was observed for the even bulkier sugar sulfamate **TPM**, which has an activity quite similar to that of **MZA** (Table 2).

iv. The best CAS1 inhibitors were tosylamide **4**, 3-fluorosulfanilamide **7**, and 3-chlorosulfanilamide **8**, which had K_Is in the range of 43.2–79.6 nM. Thus, these compounds were one order of magnitude more effective as CAS1 inhibitors compared to the clinically used agents mentioned above (**AAZ**, **MZA**, **TPM**, etc.). The increase in the inhibition power of **7** and **8** over sulfanilamide **2** was in the range of 1.80–3.33-fold, demonstrating that it may be possible to obtain highly

effective and probably isoform-specific CAS1 inhibitors through a drug design program, using these derivatives as lead molecules.

v. CAS2 was poorly inhibited by **9–11** and **22**, with K_Is in the range of 12.0–25.2 μM. These derivatives incorporate two or three substituents on the benzenesulfonamide scaffold (as in **9–11**) or have the elongated sulfonylated-aminosulfonamide scaffold (**22**). Another rather large series of derivatives showed slightly better but still micromolar affinity for CAS2. They include **2–8**, **18–21**, **MZA**, **EZA**, **DCP**, **ZNS**, **VLX**, **SAC**, and **HCT**, and their inhibition constants range between 1.88 and 9.88 μM (Table 2). As discussed above, these inhibitors
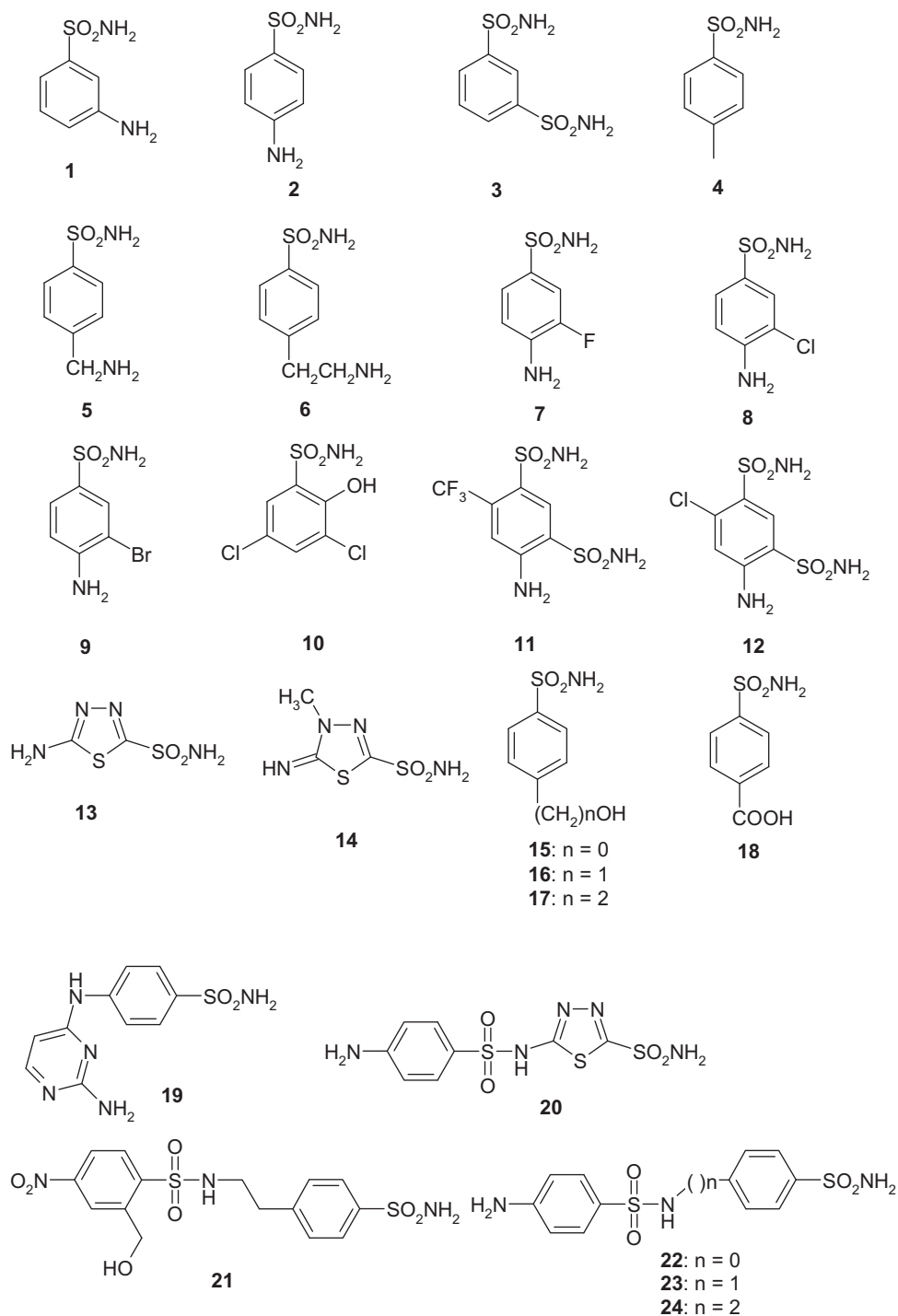

Figure 1. Sulfonamides and sulfamates investigated in this article as CAS1/2 inhibitors.
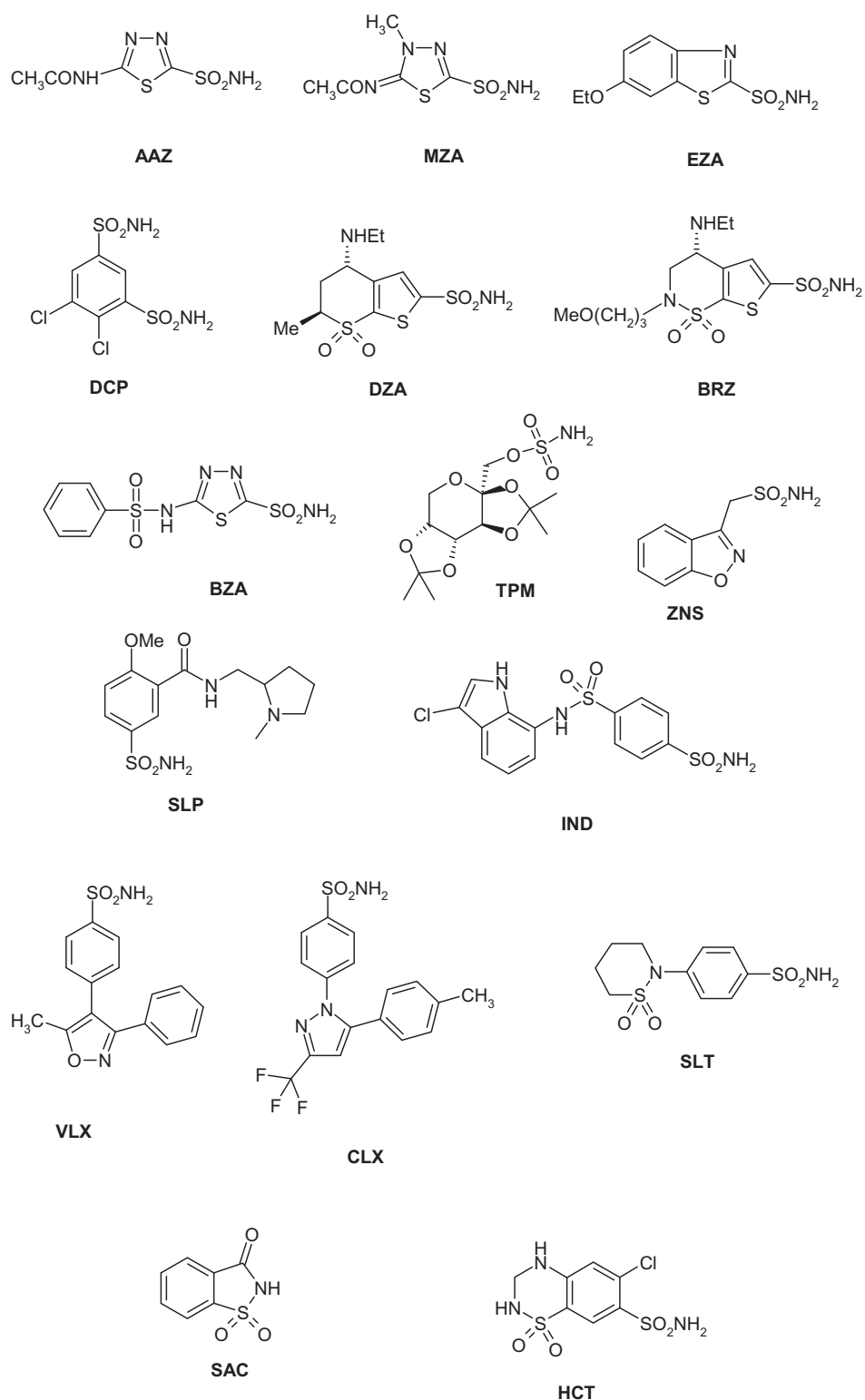
Figure 1. Continued.

belong to heterogeneous chemical classes, such as the mono- or poly-substituted benzenesulfonamides (**2–8**, **18**, **DCP**), the derivatives with bulkier scaffolds (**19–21**, **EZA**, **VLX**, **HCT**) but other derivatives such as saccharin **SAC** or zonisamide (**ZNS**) which possess rather unique structural features among the library of investigated compounds.

vi. Medium potency CAS2 inhibitors were **1**, **14**, **15**, **17**, **AAZ**, **DZA**–**TPM**, **SLP**, **IND**, **CLX**, and **SLT**, with K_Is in the range of

143–857 nM (Table 2). Again small structural changes in the molecule of the inhibitor lead to important changes of activity. For example, in the isomeric pair **1** and **2**, the amino moiety in *meta* (as in **1**) leads to a nine times better CAS2 inhibitory power compared to the *para*-amino substituted derivative **2**. Comparing the *p*-amino- and *p*-hydroxy-benzenesulfonamides **2** and **15**, the latter one is 24.33 times a better CAS2 inhibitor compared to sulfanilamide **2**, showing

Table 1. Kinetic parameters for the CO_2 hydration reaction catalysed by the human cytosolic isozymes hCA I and II (α-class CAs) at 20°C and pH 7.5 in 10 mM HEPES buffer and 20 mM NaClO$_4$, and the β-CAs CAS1 and CAS2 of *Sordaria macrospora* measured at 20°C, pH 8.3 in 20 mM TRIS buffer and 20 mM NaClO$_4$.

Isozyme	Activity level	k_{cat}	k_{cat}/K_m (s^{-1})	K_I (acetazolamide) (nM)
hCA I	Moderate	2.0×10^5	5.0×10^7	250
hCA II	Very high	1.4×10^6	1.5×10^8	12
CAS1	Low	1.2×10^4	1.30×10^6	445
CAS2	Low	1.3×10^4	$1.21 \times$	816

Inhibition data with the clinically used sulfonamide acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) are also provided.

Table 2. Inhibition of human isoforms hCA I and hCA II, and of the β-class fungal enzymes CAS1 and CAS2 with sulfonamides **1–24** and the clinically used drugs **AAZ–HCT**.

	$K_I{}^a$ (nM)			
	hCA I^b	hCA II^b	CAS1^c	CAS2^c
Inhibitor/enzyme class	α	α	β	β
1	28,000	300	361	386
2	25,000	240	144	3480
3	79^c	8	225	3630
4	78,500	320	47.1	6900
5	25,000	170	323	8720
6	21,000	160	241	7650
7	8300	60	43.2	7360
8	9800	110	79.6	9120
9	6500	40	580	12,000
10	7300	54	>50,000	23,500
11	5800	63	890	18,700
12	8400	75	3350	>50,000
13	8600	60	8650	48.1
14	9300	19	7215	280
15	5500	80	3160	143
16	9500	94	4520	92.5
17	21,000	125	>50,000	390
18	164	46	4435	3250
19	109	33	475	6760
20	6	2	363	9880
21	69	11	4550	4060
22	164	46	1985	25,200
23	109	33	282	>50,000
24	95	30	294	>50,000
AAZ	250	12	445	816
MZA	50	14	421	8140
EZA	25	8	440	3170
DCP	1200	38	1220	5790
DZA	50,000	9	360	742
BRZ	45,000	3	451	739
BZA	15	9	2115	410
TPM	250	10	414	673
ZNS	56	35	1820	1885
SLP	1200	40	1715	670
IND	31	15	4240	216
VLX	54,000	43	4425	3730
CLX	50,000	21	2513	857
SLT	374	9	3210	496
SAC	18,540	5959	5280	7075
HCT	328	290	3350	6680

^{a}Errors in the range of 5–10% of the reported data, from three different assays (data not shown).
^{b}Human recombinant isozymes, stopped flow CO_2 hydrase assay method, from Refs. [9,22–25].
^{c}Recombinant fungal enzyme, stopped flow CO_2 hydrase assay method, this work.

again that quite similar compounds from the structural viewpoint may interact in a very diverse manner with the enzyme. Another striking example is constituted by the deacetylated acetazolamide precursor **13** (the best CAS2 inhibitor detected here), which is almost 17 times a better inhibitor compared to **AAZ**.

vii. The best CAS2 inhibitors detected here were 5-amino-1,3,4-thiadiazole-2-sulfonamide (the deacetylated acetazolamide precursor **13**) and 4-hydroxymethyl-benzenesulfonamide **16**, which showed K_Is in the range of 48.1–92.5 nM.

viii. The inhibition profiles with sulfonamides and one sulfamate of CAS1 and CAS2 were very different between the two fungal isoforms, and also when compared to the inhibition of the human, α-class enzymes hCA I and II; for which many of the investigated derivatives acted with efficiencies in the low nanomolar range (Table 2). This is in fact to be expected, considering that the fungal and the human isoforms belong to two distinct genetic families.

4. Conclusions

We report the first sulfonamide inhibition study of two fungal β-CAs from *S. macrospora*, CAS1 and CAS2. CAS1 was efficiently inhibited by tosylamide, 3-fluorosulfanilamide and 3-chlorosulfanilamide (K_Is in the range of 43.2–79.6 nM), whereas acetazolamide, methazolamide, topiramate, ethoxzolamide, dorzolamide, and brinzolamide were medium potency inhibitors (K_Is in the range of 360–445 nM). CAS2 was less sensitive to sulfonamide inhibitors. The best CAS2 inhibitors were 5-amino-1,3,4-thiadiazole-2-sulfonamide (the deacetylated acetazolamide precursor) and 4-hydroxymethyl-benzenesulfonamide, with K_Is in the range of 48.1–92.5 nM. Acetazolamide, dorzolamide, ethoxzolamide, topiramate, sulpiride, indisulam, celecoxib, and sulthiame were medium potency CAS2 inhibitors (K_Is of 143–857 nM). Many other sulfonamides showed affinities in the high micromolar range or were ineffective as CAS1/2 inhibitors. Small changes in the structure of the inhibitor led to important differences of activity. As these enzymes may show applications for the removal of anthropically generated polluting gases, finding modulators of their activity may be crucial for designing environmental-friendly CO_2 capture processes.

Disclosure statement

The authors declare that there is no conflict of interest with the reported data in this article.

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