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

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**ABSTRACT**

The two β-carboxylic 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 (Ks in the range of 43.2–796 nM), whereas acetazolamide, methazolamide, topiramate, ethoxzolamide, dorzolamide, and brinzolamide were medium potency inhibitors (Ks 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 Ks in the range of 48.1–92.5 nM. Acetazolamide, dorzolamide, ethoxzolamide, topiramate, sulpiride, indisolam, celecoxib, and sulthiame were medium potency CAS2 inhibitors (Ks 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. These enzymes may show applications for the removal of anthropically generated polluting gases, finding modulators of their activity may be crucial for designing environment-friendly CO₂ 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.\textsuperscript{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.\textsuperscript{2}

Recently, our groups cloned, expressed, and investigated in some detail two β-carboxylic 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 CO₂ with formation of bicarbonate and protons.\textsuperscript{2} Indeed, CAs belonging to at least two of the seven genetical families known to date, are widespread in fungi\textsuperscript{3–4}, where they are involved in crucial physiologic processes such as, among others, pH regulation and anaplerotic/bio-synthetic reactions leading to fatty acids, amino acids, nucleic acids, and other biomolecules\textsuperscript{5–9}. Furthermore, both protons and bicarbonate, the reagents products of the enzyme catalysed CO₂ 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\textsuperscript{10–15}. It is thus understandable that CAs\textsuperscript{7–10} have been extensively investigated in the last decade especially in pathogenic fungi, such as *Candida albicans*\textsuperscript{11,12}, *Candida glabrata*\textsuperscript{13,14}, *Cryptococcus neoformans*\textsuperscript{15,16}, *Malassezia globosa*\textsuperscript{17,18} and to a lower extent in *Saccharomyces cerevisiae*\textsuperscript{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*\textsuperscript{20}. *S. macrospora* also encodes for an α-CA\textsuperscript{20}. 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\textsuperscript{11–19}. In the case of CAS1 and CAS2, only anion inhibitors were investigated, which generally showed low affinity for both isozymes, as expected for this class of CA inhibitors (CAIs)\textsuperscript{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\textsuperscript{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 CO₂ 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 Na₂SO₄ (for maintaining constant the ionic strength), following the initial rates of the CA-catalysed CO₂ hydration reaction for a period of 10–100 s. The CO₂ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalysed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of 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 isomers were recombinant ones obtained in-house as reported earlier.2,9

2.3. 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 dichlorphenamide 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.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.24 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 CO₂ to bicarbonate and protons, with kinetic constants which are lower compared to those of the human (h) isomers 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 $>10^5$ s⁻¹. Furthermore, this activity is inhibited by the clinically used 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). 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 $K_I > 50$ 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 $K_I$ 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 benzene sulfonamides with compact moieties (a amine, sulfamoyl, aminomalyl, 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 hydrogensubstituted 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 sulfanalimide 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-amino-sulfonamide 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 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 $K_i$ 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 $K_i$s 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.
belong to heterogeneous chemical classes, such as the mono- or poly-substituted benzenesulfonamides (2–8, 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 $K_I$ 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

Figure 1. Continued.
Table 1. Kinetic parameters for the CO$_2$ hydration reaction catalysed by the human cytosolic isoforms hCA I and II ($\alpha$-class CAs) at 20°C and pH 7.5 in 10 mM HEPES buffer and 20 mM NaClO$_4$, and the $\beta$-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.

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

| Inhibitor/enzyme class | $K_i^a$ (nM) | hCA I$^b$ | hCA II$^b$ | CAS1$^c$ | CAS2$^c$ |
|------------------------|--------------|-----------|-----------|----------|----------|
|                        | $K_a$ | $K_d/K_mC_a$ (s$^{-1}$) | $K_i$ (acetazolamide) (nM) |
| 1                      | 28,000 | 300 | 361 | 386 |
| 2                      | 25,000 | 240 | 144 | 3480 |
| 3                      | 79     | 8   | 225 | 3630 |
| 4                      | 78,500 | 320 | 471 | 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   | 60  | 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 | 325 |
| 19                     | 109    | 33  | 475 | 6760 |
| 20                     | 6      | 2   | 363 | 9860 |
| 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   | 368 | 742 |
| BRZ                    | 45,000 | 3   | 451 | 739 |
| BZA                    | 15     | 9   | 2115 | 410 |
| TPM                    | 250    | 10  | 414 | 673 |
| ZNS                    | 56     | 35  | 1820 | 1885 |
| SLP                    | 120    | 40  | 1715 | 670 |
| IND                    | 31     | 15  | 4240 | 216 |
| VLY                    | 54,000 | 43  | 4425 | 3730 |
| CLX                    | 50,000 | 21  | 2513 | 857 |
| SLT                    | 374    | 9   | 3210 | 496 |
| SAC                    | 18,540 | 9599 | 5280 | 7076 |
| 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.

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

vii. 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, $\alpha$-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 $\beta$-CAs from S. macrospora, CAS1 and CAS2. CAS1 was efficiently inhibited by tosylamide, 3-fluorosulfanilamide and 3-chlorosulfanilamide ($K_i$ in the range of 43.2–79.6 nM), whereas acetazolamide, methazolamide, topiramate, ethoxzolamide, dorzolamide, and brinzolamide were medium potency inhibitors ($K_i$ 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-hydroxy methyl-benzenesulfonamide, with $K_i$ in the range of 48.1–92.5 nM. Acetazolamide, dorzolamide, ethoxzolamide, topiramate, sulpiride, indisolam, celecoxib, and sulthiame were medium potency CAS2 inhibitors ($K_i$ 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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