Bioorganic & Medicinal Chemistry 17 (2009) 4503–4509

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Carbonic anhydrase inhibitors. Inhibition and homology modeling studies of the fungal β-carbonic anhydrase from *Candida albicans* with sulfonamides

Alessio Innocenti [a], Rebecca A. Hall [b], Christine Schlicker [c], Andrea Scozzafava [a], Clemens Steegborn [c], Fritz A. Mühlschlegel [b], Claudiu T. Supuran [a,*]

[a] *Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino (Florence), Italy*
[b] *Department of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, United Kingdom*
[c] *Department of Physiological Chemistry, Ruhr Universität Bochum, Universitätsstrasse 150, 44801 Bochum, Germany*

ARTICLE INFO

Article history:
Received 31 March 2009
Revised 29 April 2009
Accepted 4 May 2009
Available online 8 May 2009

Keywords:
Carbonic anhydrase
β-Class enzymes
Nce103
Can2
Candida albicans
Cryptococcus neoformans
Sulfonamide

ABSTRACT

The β-carbonic anhydrase (CA, EC 4.2.1.1) from the fungal pathogen *Candida albicans* (Nce103) is involved in a CO_2 sensing pathway critical for the pathogen life cycle and amenable to drug design studies. Herein we report an inhibition study of Nce103 with a library of sulfonamides and one sulfamate, showing that Nce103, similarly to the related enzyme from *Cryptococcus neoformans* Can2, is inhibited by these compounds with K_Is in the range of 132 nM–7.6 μM. The best Nce103 inhibitors were acetazolamide, methazolamide, bromosulfanilamide, and 4-hydroxymethylbenzenesulfonamide (K_Is < 500 nM). A homology model was generated for Nce103 based on the crystal structure of Can2. The model shows that compounds with zinc-binding groups incorporating less polar moieties and compact scaffolds generate stronger Nce103 inhibitors, whereas highly polar zinc-binding groups and bulkier compounds appear more promising for the specific inhibition of Can2. Such compounds may be useful for the design of antifungal agents possessing a new mechanism of action.

1. Introduction

Candida albicans is an important opportunistic fungal pathogen of humans.[1] In healthy individuals *C. albicans* can be frequently found as a member of the gastrointestinal flora and rarely causes infections. However, when individuals become immuno-suppressed secondary to chemotherapy, AIDS, or even as a consequence of age, *C. albicans* is able to disseminate and cause chronic and potentially life threatening systemic disease.[1] One important factor that contributes to *C. albicans*' success as a pathogen is its polymorphic nature. Indeed, this ascomycete may grow as yeast, pseudohyphal, true hyphal and chlamydospore forms. The ability to switch between the yeast and hyphal forms is regulated by a wide range of host environmental factors including serum, pH, temperature, CO_2, and available carbon sources.[1–4] *C. albicans* is the predominant cause of both superficial and invasive forms of candidosis, with the last ones leading to serious complication for critically ill intensive care unit patients.[5,6] The emergence of resistance to the currently used drugs (e.g., azoles, polyenes, caspofungin and amphotericin B), as well as changes in the spectrum of *Candida* infections have led to an increased interest in susceptibility testing of new antifungal drugs, possibly possessing a different mechanism of action.[6,7] The management of these infections is complicated and highly dependent on the susceptibility profile not only of the species but also of particular strains of the infecting fungi/yeast.[7] Thus, there is a constant need for finding antifungal agents with a new mechanism of action.

CO_2 was shown to be a strong promoter of filamentation in *C. albicans*.[1,2] In systemic infections *C. albicans* disseminates into the blood stream, being thus exposed to high concentrations of CO_2 (5.5%). This phenomenon has been dubbed 'CO_2 sensing' and has opened up a wide and expansive new field into the diagnosis and drug design studies in *C. albicans* research.[2,8] *C. albicans* is the only Candida species that displays a filamentous phenotype in the presence of CO_2, allowing diagnosis and treatment within 48 hours of the sample being received.[1,2] Attempts to characterize the CO_2 sensing pathway have so far highlighted two proteins that play a significant role. The first is an adenylyl cyclase, which produces the secondary messenger cAMP, required for filamentation in *C. albicans*.[2,8] Adenylyl cyclase in *C. albicans* is a soluble enzyme which is directly activated by CO_2/HCO_3^-.[2,8] The second protein involved in CO_2 sensing in pathogenic fungi is a carbonic anhydrase (CA, EC 4.2.1.1) belonging to the β-class,[9] encoded by the gene *nce103*, termed here Nce103.[9,10] Nce103 in *C. albicans* was identified through its similarity to the ortholog protein from *Saccharomyces cerevisiae*, also denominated Nce103 but in order to avoid confusions with the *C. albicans* enzyme, the *S. cerevisiae*

* Corresponding author. Tel.: +39 055 4573005; fax: +39 055 4573385.
E-mail address: claudiu.supuran@unifi.it (C.T. Supuran).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.05.002

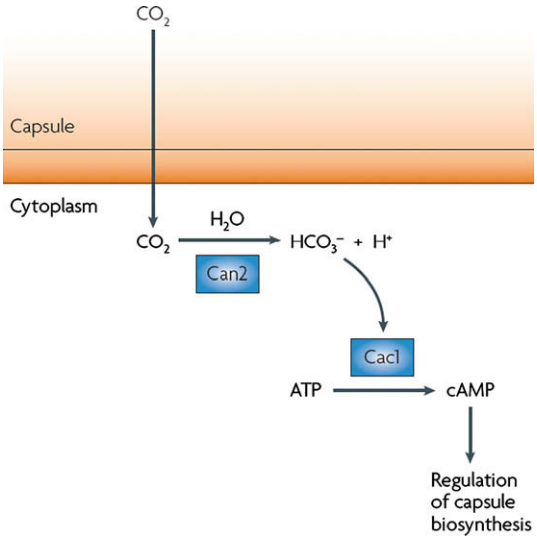


Figure 1. CO_2 sensing exemplified by the regulation of *C. neoformans* capsule.[8] The involved proteins are a soluble adenylyl cyclase (Cac1) and a β-CA (Can2), which can be probably targeted for the design of novel antifungals.[1,2,9]

protein will be denominated here as scCA.[2,8–10] It has been demonstrated by Muhlschlegel's and Heitman's groups[2,8] that physiological concentrations of CO_2/HCO_3^- induce filamentation in *C. albicans* and capsule biosynthesis in *Cryptococcus neoformans* by a direct stimulation of the adenylyl cyclase activity, as shown schematically in Figure 1. Furthermore, it has been demonstrated that CO_2/HCO_3^- equilibration by the β-CAs present in these organisms is essential for pathogenesis of *C. albicans* in niches where the available CO_2 is limited.[1,2,8] Thus, the link between cAMP signaling and CO_2/HCO_3^- sensing is conserved in fungi and reveals CO_2 sensing to be an important mediator of fungal pathogenesis, also allowing for drug design campaigns targeting some of these steps.

In previous work from our laboratories[1–3] we investigated the catalytic activity and inhibition of the β-CA from the fungal/yeast pathogens *C. neoformans* (Can2), *C. albicans* (Nce103) and *S. cerevisiae* (scCA) with inorganic anions[11] and aromatic/aliphatic carboxylates.[12] Can2 and scCA (but not Nce103) inhibition with sulfonamides has also been investigated.[13,14] These enzymes belong to the β-CA genetic family, which is not present in mammals, but is widespread in bacteria, fungi and archaea among others.[9] Finding selective inhibitors of such β-CAs may thus constitute a novel means of obtaining antifungals possessing a different mechanism of action compared to the pharmacological agents in clinical use, to which significant resistance emerged.[6–8]

We report here the first inhibition study of the Nce103 β-CA from *C. albicans* with a series of sulfonamides and one sulfamate, as well as a homology modeling study for the interaction of this inhibitor class with the enzyme, based on the recently reported[13] X-ray crystal structure of Can2.

2. Results and discussion

2.1. Catalytic activity of Nce103

We performed a kinetic investigation of purified Nce103 and Can2, comparing their kinetic parameters (k_{cat} and k_{cat}/K_m) for the physiological reaction, that is, CO_2 hydration to bicarbonate and a proton,[15] with those of thoroughly investigated CAs, such as the bacterial β-CA from *Helicobacter pylori* (hp-βCA) and α-class human CA isozymes, hCA I, II and III (cytosolic), the mitochondrial isoforms hCA VA and VB or the transmembrane, tumor-associated hCA IX (Table 1).[9,16,17] Data from Table 1 show that similarly to other CAs belonging to the α- or β-class, the fungal CAs Can2 and Nce103 possess appreciable CO_2 hydrase activity, with a k_{cat} in the range of (3.9–8.0) × 10^5 s^{-1}, and k_{cat}/K_m in the range of (4.3–9.7) × 10^7 M^{-1} s^{-1}. Thus, Nce103 shows a high catalytic efficiency, with a $k_{cat}/K_m > 5 \times 10^7$ M^{-1} s^{-1}, similarly to those of the well-established drug targets belonging to this protein family, hCA II (antiglaucoma and diuretic drugs),[9,18] hCA IX (antitumor drugs)[9,16,19] or hCA VB (antiobesity drugs).[9,20] Data of Table 1 also show that these enzymes are inhibited appreciably by the clinically used sulfonamide acetazolamide **AAZ** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), with inhibition constants in the range of 10.5–250 nM (except hCA III which is not susceptible to be inhibited by sulfonamides and also possesses a very low catalytic activity compared to other CAs).[21] It can be also observed that the *C. albicans* enzyme Nce103 is 2.3 times a better catalyst for CO_2 hydration to bicarbonate (considering the turnover number, k_{cat}/K_m) compared to the ortholog enzyme from *C. neoformans*, Can2, investigated earlier.[13]

Table 1
Kinetic parameters for the CO_2 hydration reaction catalyzed by the fungal β-CAs Nce103 and Can2, the bacterial β-CA from *Helicobacter pylori* hp-βCA and the human α-CA isozymes hCA I–III (cytosolic), hCA VA and VB (mitochondrial isozymes), and hCA IX (transmembrane, full length enzyme)), at 20 °C and pH 7.5 in 10 mM HEPES buffer (α-CAs) and at 20 °C, pH 8.3 in 20 mM TRIS·HCl buffer and 20 mM $NaClO_4$ (β-CAs)

Isozyme	Activity level	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_I (acetazolamide) (nM)
Nce103	High	8.0×10^5	9.7×10^7	132
Can2	Moderate	3.9×10^5	4.3×10^7	10.5
hp-βCA	Moderate	7.1×10^5	4.8×10^7	40
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
hCA III	Very low	1.0×10^4	3.0×10^5	300,000
hCA VA	Low	2.9×10^5	2.9×10^7	63
hCA VB	High	9.5×10^5	9.8×10^7	54
hCA IX	Very high	1.1×10^6	1.5×10^8	16

The inhibition data with the clinically used sulfonamide acetazolamide **AAZ** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) are also provided.[15]

In order to try to rationalize the kinetic data reported here, an alignment of the amino acid sequences of Nce103 and Can2 is shown in Figure 2. Can2 has recently been crystallized and its 3D-structure reported by this group,[13] and this structure will be used to rationalize the catalytic activity and inhibition of Nce103, which was not crystallized so far. Data from Figure 2 show that the zinc ligands of the fungal β-CA Can2, that is, Cys68, His124 and Cys127 (Can2 numbering system)[13] are also conserved in Nce103, corresponding to residues Cys106, His162 and Cys164 in the sequence of the *C. albicans* enzyme. A second pair of conserved amino acid residues in all sequenced β-CAs, known to date, is constituted by the dyad Asp70–Arg72 (Can2 numbering) corresponding to Asp108–Arg110 in Nce103 (Fig. 2). These amino acids are close[13] to the zinc-bound water molecule, which is the fourth zinc ligand in this type of open active site β-CAs, making a network of hydrogen bonds with it, which assists the deprotonation of water and formation of the nucleophilic, zinc hydroxide species of the enzyme.[12,13] Indeed, in β-CAs, unlike the α-class enzymes in which the Zn(II) ion is coordinated by three His residues and a water molecule, the formal zinc charge is zero, and as a consequence the activation of the zinc-coordinated water molecule needs the assistance of additional amino acids. The pair Asp108–Arg110 (Nce103) probably has this function, as it is conserved in all β-CAs sequenced so far.[13,17] As a consequence, the catalytic water molecule is activated both by the metal ion (as in metalloproteases and α-CAs),[9] but also by an aspartic acid residue, as in aspartic proteases.[22] This particular mechanism makes the β-CAs very different as compared to

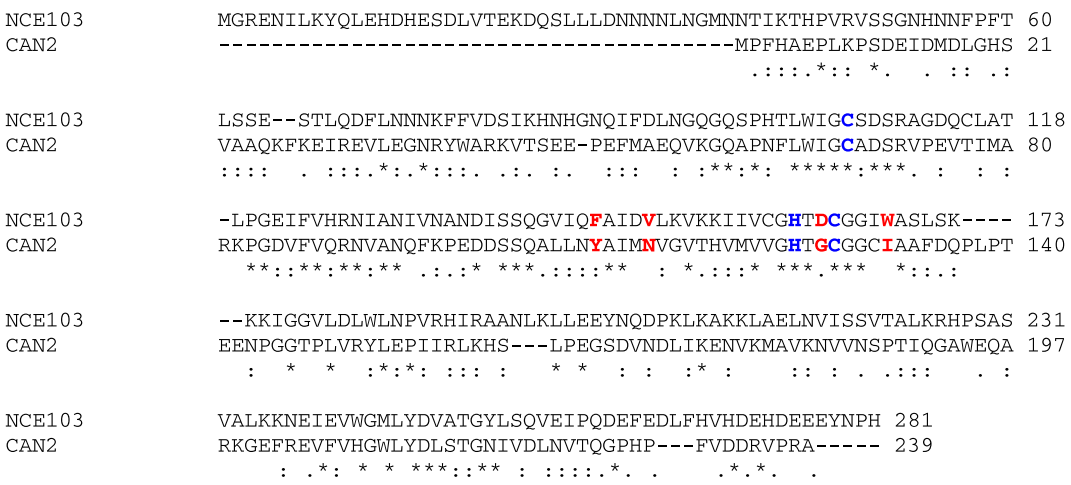


Figure 2. Alignment of the *C. albicans* Nce103 and *C. neoformans* Can2 amino acid sequences. The conserved amino acid residues are marked by an asterisk. In blue are evidenced the Zn(II) ligands (Cys68, His124 and His127, Can2 numbering system) of the two enzymes, and in red the residues thought to be involved in inhibitor binding (see also Fig. 3).

all other known enzyme classes involved in hydrolytic or hydration processes.

2.2. Inhibition studies of Nce103 with sulfonamides/sulfamates

Table 2 shows Nce103 inhibition data with a panel of 36 sulfonamides and one sulfamate (obtained for the CO_2 hydration reaction catalyzed by CAs),[15] some of which are clinically used drugs,[9] such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA**, topiramate **TPM**, sulpiride **SLP**, indisulam **IND**, zonisamide **ZNS**, celecoxib **CLX**, valdecoxib **VLX**, sulthiame **SLT** and saccharin **SAC**. **DZA**, **BRZ** and **TPM** were enantiomerically pure compounds (the clinically used ones as drugs).[9] The simpler derivatives **1–22** were also included in the study as they represent the most extensively used scaffolds for designing potent or isoform-selective CAIs.[9,18–21] Data for the inhibition of the dominant human isoforms hCA I and II[9] as well as those of the related enzyme Can2 investigated recently for its interaction with sulfonamides,[13] are also included in Table 2, for comparison reasons. The following SAR can be observed from data of Table 2:

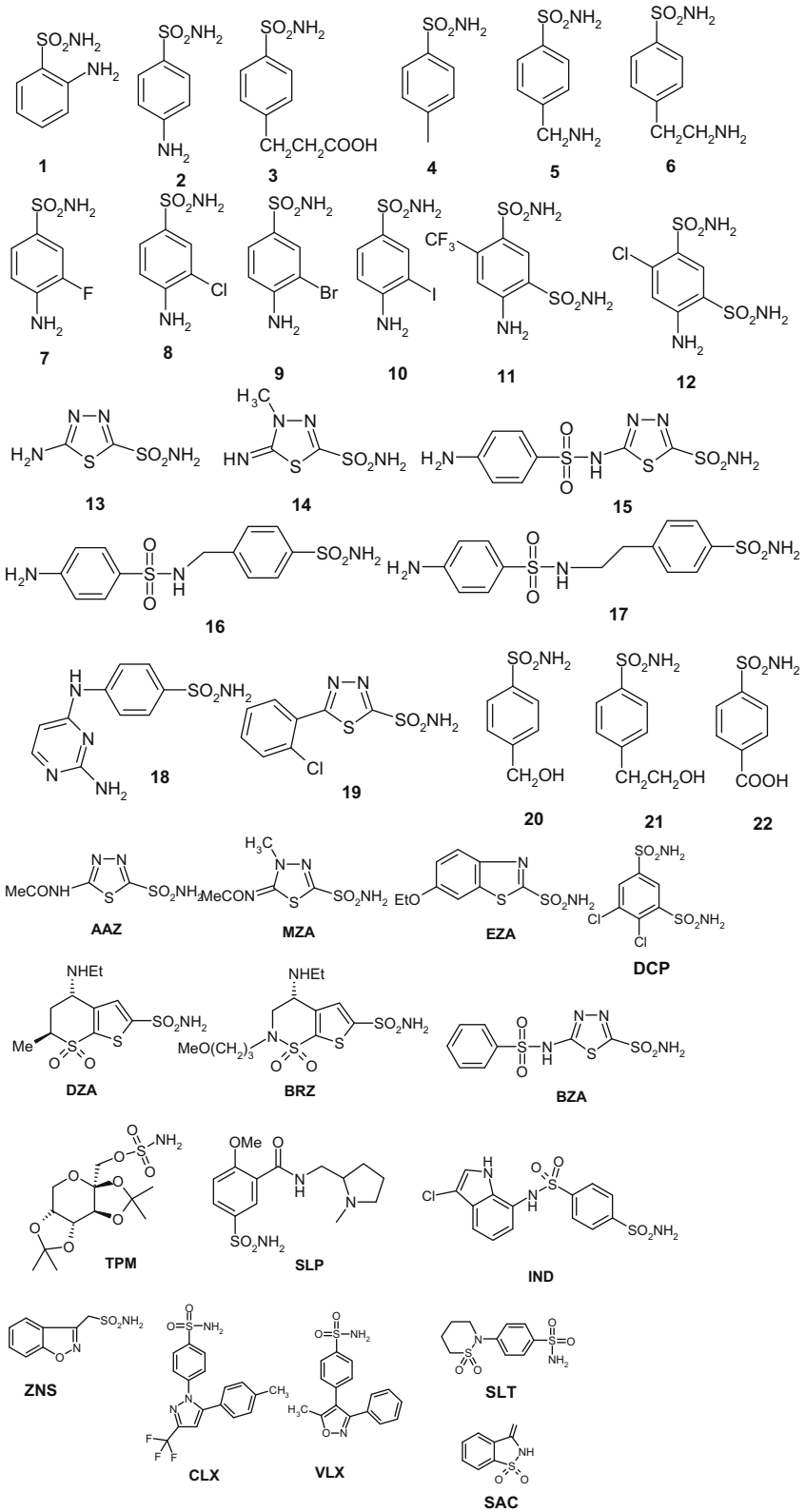
(i) Three derivatives, that is, **2**, **6** and **11**, showed very weak Nce103 inhibitory activity, with K_Is in the range of 3195–7627 nM. The three compounds are benzenesulfonamide (sulfanilamide **2** and 4-aminoethylbenzenesulfonamide **6**) or benzene-1,3-disulfonamide derivatives (**11**).

(ii) A larger number of derivatives, such as **1**, **3–5**, **7**, **8**, **14–16**, **18**, **19**, **21**, **EZA**, **BRZ**, **BZA**, **TPM**, **IND**, **CLX** and **SLT** showed much more effective Nce103 inhibitory action, with inhibition constants in the range of 1017–1510 nM. They belong to heterogeneous classes of derivatives, some of them possessing rather simple scaffolds based on the benzenesulfonamide/disulfonamide motifs (**1**, **3–5**, **7**, **8**, **16**, **18**, **21**), whereas others incorporate more complicated scaffolds, such as those present in the clinically used derivatives **EZA**, **BRZ**, **BZA**, **IND**, **CLX** and **SLT**. Quite small structural changes in the inhibitor scaffold lead to dramatic changes of enzyme inhibitory activity. This is obvious for example for the simple derivatives **1** and **2**, with the *ortho* isomer **1** being 7 times a better Nce103 inhibitor than the *para* isomer **2**, for the aminoalkyl-substituted sulfonamides **5** and **6**, with the aminomethyl derivative **5** being a 2.9 times a better inhibitor than the aminoethyl one **6**, or for the benzenedisulfonamides **11** and **12**. In this last case, the chlorine-substituted sulfonamide **12** is again a 7 times better Nce103 inhibitor compared to the structurally related trifluoromethyl-substituted derivative **11** (Table 2). The nature of the moiety substituting the benzene ring on which the sulfamoyl zinc binding group is anchored is also highly important for the enzyme inhibitory activity of these compounds. For example, the isostructural and isosteric aminoethyl (present in **6**) or hydroxyethyl (present in **21**) moieties lead to compounds differing by 2.6 times in their affinity for the enzyme, with the most active one being the hydroxyethyl-substituted sulfonamide **21**.

(iii) A third subgroup of the investigated derivatives, including **10**, **12**, **13**, **17**, **22**, **DCP**, **DZA**, **SLP**, **ZNS**, **VLX** and **SAC**, showed even more effective Nce103 inhibitory activity, with K_Is in the range of 699–949 nM (Table 2). Again they belong to heterogeneous classes of derivatives, some of them being simple substituted benzenesulfonamides/disulfonamides (such as the halogeno sulfonamides **10**, **12** and **DCP**, or the 4-carboxy-substituted derivative **22**), simple heterocyclic derivatives (**13**) or possess more complicated scaffolds as those present in **17**, and the clinically used drugs **DZA**, **SLP**, **ZNS**, **VLX** and **SAC**. Some of these compounds may be considered good leads to develop better Nce103 inhibitors, as they possess simple scaffolds which can easily be derivatized in a versatile manner, leading to compounds with different structures.

(iv) Four compounds showed effective Nce103 inhibitory activity, with K_Is < 500 nM. More precisely, **AAZ**, **9**, **20** and **MZA** showed K_Is in the range of 132–484 nM for inhibiting Nce103 (Table 2). Again quite small structural differences had large effects in the inhibitory capacity, with **AAZ** and **MZA** differing only by a CH_2 group, having the activity different by a factor of 3.7. The remaining two best inhibitors, **9** and **20**, are very simple compounds structurally related to other derivatives investigated here, which showed weaker inhibition of Nce103. Thus, among the many halogenated sulfonamides investigated here, bromosulfanilamide **9** was the best inhibitor (K_I of 408 nM) differing significantly of the corresponding chloro-**8** (3.3 times a weaker inhibitor) or iodo-**10** (1.7 times a weaker inhibitor) derivatives with which it has a very similar structure. The same is true for the 4-hydroxymethyl-benzenesulfonamide **20** (K_I of 383 nM), which is 3.15 times a better Nce103 inhibitor compared to the 4-hydroxyethyl derivative **21** and 2.9 times a better inhibitor than the corresponding 4-aminoethyl derivative **5**. Thus, again, small structural changes in the inhibitor molecule scaffold are very sensitive for the Nce103 inhibitory properties. Undoubtedly, **AAZ** is the best Nce103 inhibitor detected so far, with an inhibition constant of 132 nM (Table 2).

Table 2
hCA I, II, Can2 and Nce103 inhibition data with sulfonamides **1**–**22** and 15 clinically used derivatives **AAZ**–**SAC**



Inhibitor	K_I[a] (nM)			
	hCA I[b]	hCA II[b]	Can2[c]	Nce103[c]
1	45,400	295	379	1086
2	25,000	240	765	7627
3	6690	495	440	1277

Table 2 (*continued*)

Inhibitor	K_I[a] (nM)			
	hCA I[b]	hCA II[b]	Can2[c]	Nce103[c]
4	78,500	320	1150	1204
5	25,000	170	18,490	1109
6	21,000	160	1394	3195
7	8300	60	809	1240
8	9800	110	605	1345
9	6500	40	977	408
10	6000	70	711	719
11	5800	63	968	6115
12	8400	75	300	874
13	8600	60	791	832
14	9300	19	815	1368
15	6	2	42	1092
16	164	46	971	1310
17	185	50	624	727
18	109	33	3887	1108
19	690	12	379	1293
20	55	80	623	383
21	21,000	125	878	1209
22	23,000	133	484	861
AAZ	250	12	10.5	132
MZA	50	14	63	484
EZA	25	8	87	1070
DCP	1200	38	1203	909
DZA	50,000	9	8347	887
BRZ	45,000	3	87	1095
BZA	15	9	23	1510
TPM	250	10	367	1108
SLP	12,000	40	812	760
IND	31	15	963	1090
ZNS	56	35	971	942
CLX	50,000	21	3056	1017
VLX	54,000	43	704	699
SLT	374	9	890	1071
SAC	18,540	5950	32,000	736

Data of isoforms hCA I and II are from Ref. 21, whereas Can2 inhibition data from Ref. 13.

[a] Errors in the range of 5–10 % of the shown data, from three different assays.

[b] Human recombinant isozymes, stopped flow CO_2 hydrase assay method, pH 7.5, 20 mM TRIS·HCl buffer.[21]

[c] Fungal recombinant enzymes, at 20 °C, pH 8.3 in 20 mM TRIS·HCL buffer and 20 mM $NaClO_4$.

(v) Comparing the Nce103 data of Table 1 with those for the inhibition of the ortholog enzyme from *C. neoformans* (Can2) investigated earlier,[13] it can be observed that these two β-CAs have a diverse inhibition profile, which is to be expected considering the different length and sequence of amino acid residues, as discussed above and shown in Figure 2. Indeed, many compounds showing excellent Can2 inhibitory activity, such as, for example, **15**, **MZA**, **EZA**, **BRZ**, **BZA**, are only moderate-weak Nce103 inhibitors. Generally speaking, Can2 seems to have an overall higher affinity for sulfonamides/sulfamates as compared to Nce103, with the best inhibitor of both enzymes being acetazolamide **AAZ**. However, **AAZ** is a 12.5 times a better Can2 than Nce103 inhibitor. In order to understand the different behavior of these two β-CAs for their interaction with sulfonamides, an considering that the X-ray structure of Nce103 is not available, a homology modeling study has been performed for the interaction of **AAZ** with Can2 and Nce103, based on the recently reported 3D structure of the first enzyme[13] (see later in the text).

(vi) The affinity of these inhibitors for the α-CAs of the human host, that is, hCA I and II, compared to that of Nce103 is also discussed here. It may be observed from data of Table 2 that hCA I has generally a low affinity for most of these compounds, except for compounds **15**, **20**, **MZA**, **EZA**, **BZA**, **IND** and **ZNS** which possess K_Is < 60 nM. On the contrary, hC AII is much more inhibited by this class of derivatives, most of the compounds **1**–**22** and **AAZ**–**SLT** having K_Is < 300 nM (only SAC is a weak hCA II inhibitor with a K_I of 5950 nM, Table 2). Thus, sulfonamides/sulfamates may be considered as excellent hCA II inhibitors, whereas their affinities for hCA I, Can2 and Nce103 are orders of magnitude lower.

2.3. Modeling of sulfonamide binding to Nce103

In order to compare the different inhibition profiles of Nce103 and Can2, a homology model was generated for Nce103 based on the crystal structure of Can2.[13] The Nce103 model was superimposed with the Can2 dimer and an **AAZ** molecule positioned in the active site analogous to the modeled benzolamide **BZA** complex of Can2.[13] There are four major differences in the active sites and ligand binding channels of Can2 and Nce103, as observed from Figure 3. Near the sulfonamide group of the **AAZ** molecule, two hydrophilic amino acids in the Can2 structure (Asn113 and Tyr109) are replaced by hydrophobic amino acids in Nce103 (Val101 and Phe97). This substitution might be the reason for the generally higher affinity of the hydrophilic sulfonamides/sulfamates to Can2 than to Nce103. Close to the other end of the **AAZ** molecule, two small amino acids in Can2 (Gly126 and Ile131) are replaced by two bulkier amino acids in Nce103 (Asp114 and Trp119). These side chains might be oriented toward the ligand binding channel and thereby narrow it in Nce103, so that bulkier inhibitors are harder to accommodate in the Nce103 active site. This structural comparison thus suggests to focus on compounds with zinc-binding groups having a less polar functionality and more compact scaffolds for Nce103 inhibition, whereas highly polar zinc-binding groups and bulkier compounds appear more

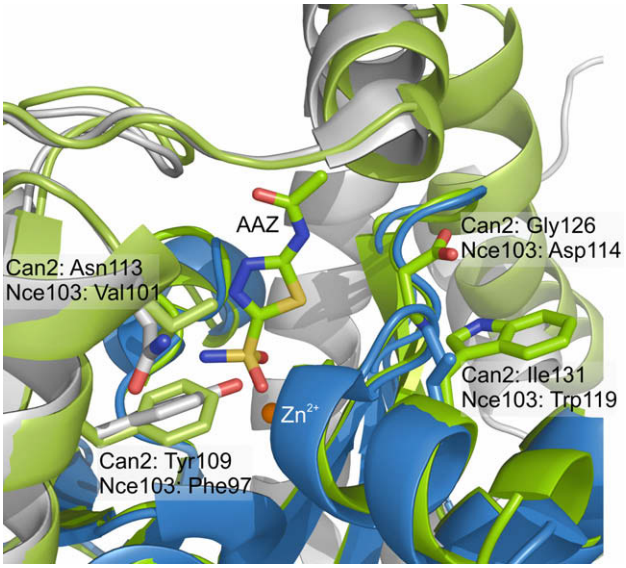


Figure 3. Modeling o AAZ binding to Can2 (dimer colored in blue and gray) and Nce103 (dimer colored in green) based on Can2 crystal structure.[13] The Zn(II) ion is shown as an orange sphere. The different residues in the Can2 and Nce103 structures discussed in the text are shown in stick presentation.

promising for the specific inhibition of Can2. Further work is warranted to check these findings.

3. Conclusions

The β-carbonic anhydrase (CA, EC 4.2.1.1) from the fungal pathogen *C. albicans* (Nce103) is involved in a CO_2 sensing pathway critical for the pathogen life cycle and amenable to drug design studies. This enzyme shows a high catalytic activity for the physiologic, CO_2 hydration reaction to bicarbonate and protons, with a k_{cat} of 8.0×10^5 s^{-1} and a k_{cat}/K_m of 9.7×10^7 M^{-1} s^{-1}. Herein we report an inhibition study of Nce103 with a library of sulfonamides and one sulfamate, showing that Nce103, similarly to the related enzyme from *C. neoformans* Can2, is inhibited by these compounds with K_Is in the range of 132 nM–7.6 μM. The best Nce103 inhibitors were acetazolamide, methazolamide, bromosulfanilamide, and 4-hydroxymethyl-benzenesulfonamide (K_Is < 500 nM). A homology model was generated for Nce103 based on the crystal structure of Can2 recently reported by this group. The model showed that compounds with zinc-binding groups incorporating less polar moieties and compact scaffolds generate stronger Nce103 inhibitors, whereas highly polar zinc-binding groups and bulkier compounds appear more promising for the specific inhibition of Can2. Such compounds may be useful for the design of antifungal agents possessing a novel mechanism of action.

4. Experimental

4.1. Materials

Sulfonamides **1–22** and **AAZ–SAC** are commercially available compounds (from Sigma–Aldrich, Milan, Italy). The CA isozymes used in the experiments were recombinant ones obtained and purified as reported earlier by this group.[11–13]

4.2. CA activity and inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[15] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) or TRIS (pH 8.3) as buffer, and 20 mM Na_2SO_4 or 20 mM $NaClO_4$ (for maintaining constant the ionic strength), following the CA-catalyzed 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 uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled–deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter with distilled–deionized water. 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, from Lineweaver–Burk plots, as reported earlier,[10–14] and represent the mean from at least three different determinations.

4.3. Modeling of sulfonamide binding to Nce103

For homology modeling of the Nce103 structure, an alignment of Nce103 and Can2 was prepared with ClustalW and corrected manually (Fig. 2). Modeling using the alignment and the crystal structure of Can2 (pdb code 2w3q) was done with the program modeller9v1.[23] An NCE103 complex with **AAZ** in the active site was generated by superimposing the NCE103 model with a Can2 dimer structure containing a modeled benzolamide **BZA** as ligand.[13]

Acknowledgments

We thank Barbara Kachholz and Kara Turner for technical assistance. This research was financed in part by a grant of the 6th Framework Programme of the European Union (DeZnIT project to CTS), by Grant STE1701/2 of Deutsche Forschungsgemeinschaft (to CS) and by MRC and bbsrc grants (to FAM).

References and notes

1. (a) Eckert, S. E.; Mühlschlegel, F. A. *FEMS Yeast Res.* **2009**, *9*, 2; (b) Hall, R. A.; Cottier, F.; Mühlschlegel, F. A. *Adv. Appl. Microbiol.* **2009**, *67*, 191.
2. (a) Klengel, T.; Liang, W.-J.; Chaloupka, J.; Ruoff, C.; Schroppel, K.; Naglik, J. R.; Eckert, S. E.; Mogensen, E. G.; Haynes, K.; Tuite, M. F.; Levin, L. R.; Buck, J.; Muhlschlegel, F. A. *Curr. Biol.* **2005**, *15*, 2021; (b) Sheth, C. C.; Baker, M. E.; Haynes, K.; Mühlschlegel, F. A. *Med. Mycol.* **2005**, *43*, 735.
3. Penalva, M. A.; Arst, H. N., Jr. *Microbiol. Mol. Biol. Rev.* **2002**, *66*, 426.
4. Barnesa, R. A.; Vale, L. *J. Hosp. Infect.* **2005**, *60*, 78.
5. (a) Richardson, M.; Rautemaa, R. *Front. Biosci.* **2009**, *14*, 4363; (b) Playford, E. G.; Eggimann, P.; Calandra, T. *Curr. Opin. Infect. Dis.* **2008**, *21*, 610.
6. (a) Playford, E. G.; Sorrell, T. C. *Semin. Respir. Crit. Care Med.* **2007**, *28*, 678; (b) Gupta, A. K.; Tomas, E. *Dermatol. Clin.* **2003**, *21*, 565.
7. (a) Gómez-López, A.; Zaragoza, O.; Rodríguez-Tudela, J. L.; Cuenca-Estrella, M. *Exp. Opin. Pharmacother.* **2008**, *9*, 2801; (b) Perfect, J. R. *Oncology* **2004**, *18*, 15.
8. (a) Mogensen, E. G.; Janbon, G.; Chaloupka, J.; Steegborn, C.; Fu, M. S.; Moyrand, F.; Klengel, T.; Pearson, D. S.; Geeves, M. A.; Buck, J.; Levin, L. R.; Mühlschlegel, F. A. *Eukaryotic Cell* **2006**, *5*, 103; (b) Bahn, Y.-S.; Cox, G. M.; Perfect, J. R.; Heitman, J. *Curr. Biol.* **2005**, *15*, 2013; (c) Bahn, Y. S.; Mühlschlegel, F. A. *Curr. Opin. Microbiol.* **2006**, *9*, 572.
9. Supuran, C. T. *Nat. Rev. Drug Discov.* **2008**, *7*, 168.
10. (a) Isik, S.; Kockar, F.; Arslan, O.; Ozensoy Guler, O.; Innocenti, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6327; (b) Isik, S.; Kockar, F.; Aydin, M.; Arslan, O.; Ozensoy Guler, O.; Innocenti, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1662.
11. Innocenti, A.; Mühlschlegel, F. A.; Hall, R. A.; Steegborn, C.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5066.
12. Innocenti, A.; Hall, R. A.; Schlicker, C.; Mühlschlegel, F. A.; Supuran, C. T. *Bioorg. Med. Chem.* **2009**, *17*, 2654.
13. Schlicker, C.; Hall, R. A.; Vullo, D.; Middelhaufe, S.; Gertz, M.; Supuran, C. T.; Muhlschlegel, F. A.; Steegborn, C. *J. Mol. Biol.* **2009**, *385*, 1207.
14. Isik, S.; Kockar, F.; Aydin, M.; Arslan, O.; Ozensoy Guler, O.; Innocenti, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem.* **2009**, *17*, 1158.

15. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561.
16. Hilvo, M.; Baranauskiene, L.; Salzano, A. M.; Scaloni, A.; Matulis, D.; Innocenti, A.; Scozzafava, A.; Monti, S. M.; Di Fiore, A.; De Simone, G.; Lindfors, M.; Janis, J.; Valjakka, J.; Pastorekova, S.; Pastorek, J.; Kulomaa, M. S.; Nordlund, H. R.; Supuran, C. T.; Parkkila, S. *J. Biol. Chem.* **2008**, *283*, 27799.
17. Nishimori, I.; Onishi, S.; Takeuchi, H.; Supuran, C. T. *Curr. Pharm. Des.* **2008**, *14*, 622.
18. (a) Supuran, C. T. *Curr. Pharm. Des.* **2008**, *14*, 641; (b) Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 199; (c) Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146; (d) Krishnamurthy, V. M.; Kaufman, G. K.; Urbach, A. R.; Gitlin, I.; Gudiksen, K. L.; Weibel, D. B.; Whitesides, G. M. *Chem. Rev.* **2008**, *108*, 946.
19. (a) Thiry, A.; Dogné, J. M.; Masereel, B.; Supuran, C. T. *Trends Pharmacol. Sci.* **2006**, *27*, 566; (b) Stiti, M.; Cecchi, A.; Rami, M.; Abdaoui, M.; Barragan-Montero, V.; Scozzafava, A.; Guari, Y.; Winum, J. Y.; Supuran, C. T. *J. Am. Chem. Soc.* **2008**, *130*, 16130; (c) D'Ambrosio, K.; Vitale, R. M.; Dogné, J. M.; Masereel, B.; Innocenti, A.; Scozzafava, A.; De Simone, G.; Supuran, C. T. *J. Med. Chem.* **2008**, *51*, 3230; (d) Thiry, A.; Supuran, C. T.; Masereel, B.; Dogné, J. M. *J. Med. Chem.* **2008**, *51*, 3051.
20. (a) Supuran, C. T. *Exp. Opin. Ther. Pat.* **2003**, *13*, 1545; (b) Supuran, C. T.; Di Fiore, A.; De Simone, G. *Exp. Opin. Emerg. Drugs* **2008**, *13*, 383; (c) De Simone, G.; Di Fiore, A.; Supuran, C. T. *Curr. Pharm. Des.* **2008**, *14*, 655.
21. Nishimori, I.; Minakuchi, T.; Onishi, S.; Vullo, D.; Cecchi, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem.* **2007**, *15*, 7229.
22. Mastrolorenzo, A.; Rusconi, S.; Scozzafava, A.; Barbaro, G.; Supuran, C. T. *Curr. Med. Chem.* **2007**, *14*, 2734.
23. Eswar, N.; Webb, B.; Marti-Renom, M. A.; Madhusudhan, M. S.; Eramian, D.; Shen, M. Y. *Curr. Prot. Protein Sci.* **2007**, *2*, 2.