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Carbonic anhydrase inhibitors. Inhibition of the fungal β-carbonic anhydrases from *Candida albicans* and *Cryptococcus neoformans* with boronic acids

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ABSTRACT

Inhibition of the β-carbonic anhydrases (CAs, EC 4.2.1.1) from the pathogenic fungi *Cryptococcus neoformans* (Can2) and *Candida albicans* (Nce103) with a series of aromatic, arylalkenyl- and arylalkylboronic acids was investigated. Aromatic, 4-phenylsubstituted- and 2-naphthylboronic acids were the best Can2 inhibitors, with inhibition constants in the range of 8.5–11.5 μM, whereas arylalkenyl and aryalkylboronic acids showed K_Is in the range of 428–3040 μM. Nce103 showed a similar inhibition profile, with the 4-phenylsubstituted- and 2-naphthylboronic acids possessing K_Is in the range of 7.8–42.3 μM, whereas the arylalkenyl and aryalkylboronic acids were weaker inhibitors (K_Is of 412–5210 μM). The host human enzymes CA I and II were also effectively inhibited by these boronic acids. The $B(OH)_2$ moiety is thus a new zinc-binding group for designing effective inhibitors of the α- and β-CAs.

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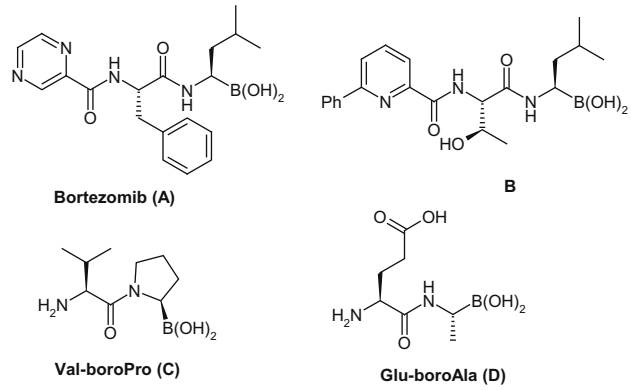
In previous work from our laboratories[1–3] we investigated the catalytic activity and inhibition of the carbonic anhydrases (CAs, EC 4.2.1.1) from the fungal pathogens *Cryptococcus neoformans* (Can2) and *Candida albicans* (Nce103) with inorganic anions,[1] sulfonamides[2] and aromatic/aliphatic carboxylates.[3] 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.[4,5] Finding selective inhibitors of the β-CAs may thus constitute a novel means of obtaining antiinfective agents (antibacterials or antifungals) possessing a different mechanism of action compared to the pharmacological agents in clinical use, to which significant resistance has emerged.[6–8] We have so far identified some aromatic/heterocyclic sulfonamides and several carboxylates with low micromolar activity against the two enzymes from the above mentioned fungal pathogens.[2,3] However, sulfonamides generally show micro-nanomolar affinity for α-CAs (of which 16 isoforms are present in mammals),[4,9] being thus not interesting leads for developing β-CA-selective inhibitors. Thus, the exploration of different chemotypes[10] is warranted in order to identify possible inhibitors with good activity and eventually selectivity for the inhibition of β- over the α-CAs.



Boronic acids emerged recently as inhibitors of many enzymes involved in fundamental biological processes.[11–16] Thus, the peptidomimetic compound bortezomib **A**[11–13] is a clinically used proteasome inhibitor for the treatment of haematological malignancies, compound **B** is a second generation orally active proteasome inhibitor in clinical investigations as an antitumor drug,[14] whereas boronic acids **C** and **D** act as dipeptidyl peptidase IV inhib-

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itors,[15] which represents a validated new target for the treatment of type 2 diabetes.[15] Boronic acids were also reported to act as inhibitors of fatty acid amide hydrolase,[16] prostate-specific antigen (a serine protease),[17] arginase,[18] or are useful as functional sensors of the physiological pH.[19] We have investigated the interaction of one such compound, phenylboronic acid ($PhB(OH)_2$), with α-CAs,[20–22] showing that this simple derivative acts as a weak, generally millimolar inhibitor against most of the 16 CA isoforms presently known in mammals, CA I–XV.[4,9,10,21]

In this contribution, we report an inhibition study of the β-CAs from the fungal pathogens *C. neoformans* (Can2) and *C. albicans* (Nce103) with a series of aromatic, aryl–alkenyl- and arylalkylboronic acids. The two ubiquitous human isoforms hCA I and II were also included in this study in order to investigate whether this new class of CA inhibitors (CAIs) may show some selectivity for the fungal over the host enzymes.[23,24]

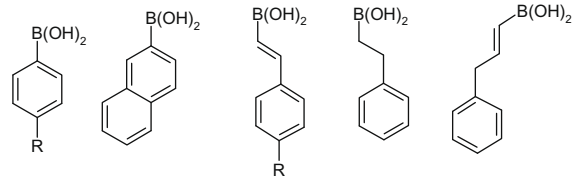
The following structure–activity relationship (SAR) can be drawn from data of Table 1:

(i) Phenylboronic acid **1a** acts as a very weak hCA I inhibitor, with an inhibition constant of 1.56 mM. However, the presence of various substituents in the *para* position to the $B(OH)_2$ moiety in the aromatic boronic acids **1b–g**, leads to a drastic increase of enzyme inhibitory activity. Thus, a 4-methyl group leads to a compound with a K_I of 278 μM (**1b**), whereas an *n*-Bu such group to a more efficient inhibitor, with a K_I of 7.9 μM (**1c**). Beneficial substitution patterns for hCA I inhibition are also those present in compounds **1d–g** (methoxy-, bromine-, phenyl- and phenoxy-), with the biphenylboronic acid **1f** being the best hCA I inhibitor detected in this study, with a K_I of 3.7 μM (an increase of potency compared to the lead **1a** of 421.6 times). The β-naphthylboronic acid **2** as well as the arylalkenyl/alkyl derivatives **3–5** also show effective hCA I inhibitory properties, with inhibition constants in the range of 6.5–12.5 μM. It is interesting to note that for the elongated derivatives **3a** and **3b**, the introduction of the methyl group only slightly increases the inhibitory properties, whereas for the lead **1a** and its 4-methyl-substituted derivative, the difference in inhibition is very important, with the methyl derivative **1b** being 5.6 times a better hCA I inhibitor as compared to **1a**. The nature of the R moiety present in derivatives **3a–d** influences hCA I inhibitor activity but not as much as for derivatives **1a–g** discussed above. There are also small differences of activity between the alkenyl boronic acid **3a** and the corresponding saturated compound **4** or between the alkenyl compounds **3a** and **5** which differ only by the presence of an additional CH_2 group in the last derivative.

(ii) The ubiquitous cytosolic isoform hCA II showed and inhibition profile similar to that of hCA I with boronic acids **1, 2, 4**, and **5**, whereas the alkenyl derivatives **3** presented a different profile (Table 1). Thus, the lead **1a** is a very weak CAI, with a K_I of 1050 μM, but the presence of various groups in *para* to the $B(OH)_2$ moiety, such as in derivatives **1b–g**, leads to a strong enhancement of the inhibitory properties, these compounds having K_Is in the range of 4.5–11.5 μM. Again the best hCA II inhibitor was (as for hCA I) biphenyl boronic acid **1f**. The β-naphthyl derivative **2** was also an effective hCA II inhibitor (K_I of 6.0 μM). However, the alkenyl boronic acids **3a–c** showed quite weak hCA II inhibitory activity, with inhibition constants in the range of 373–617 μM, and only the trifluoromethyl-substituted derivative **3d** showed a better inhibitory activity (K_I of 27.6 μM), in the same range as the structurally related derivatives **4** and **5** (K_Is of 17.9–18.1 μM, Table 1). Thus, again small structural changes in the molecule of the inhibitor had dramatic consequences for the affinity to the enzyme. For example, the alkenyl derivative **3a** and the dihydrogenated corresponding compound **4** differ by a factor of 29.8 in inhibiting this enzyme, even if the structural differences between the two compounds are minimal. The same is true for the two homologues **3a** and **5** which differ by the presence of an extra CH_2 moiety in the second compound, which is 29.5 times more potent a hCA II inhibitor than the last one.

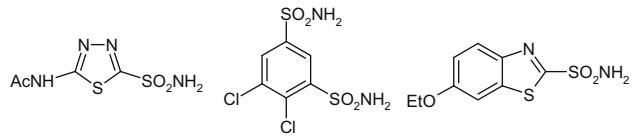
(iii) Can2 was weakly inhibited by the lead **1a** as well as by derivatives **3a, 3b, 3d, 4** and **5**, which showed inhibition constants in the range of 428–3040 μM. For the subseries of 4-substituted phenylboronic acids **1b–g**, the presence of the 4-substituents in *para* to the boronic acid moiety was highly beneficial for the Can2 inhibitory activity, all these compounds being low micromolar inhibitors (K_Is in the range of 8.9–11.4 μM), unlike the lead **1a** mentioned above. Thus, aliphatic, alkoxy, halogeno, aryl or aryloxy groups as substituents in the *para* position of the phenylboronic acid lead **1a**, were enhancing dramatically (70–91 times) the Can2 inhibitory effects of derivatives **1b–g**, compared to **1a**. The β-naphthyl derivative **2** and the biphenyl-ethenylboronic acid **3c** were also effective Can2 inhibitors (K_Is in the range of 8.5–11 μM), but not the remaining alkenyl- or aralkyl derivatives **3a, 3b, 3d, 4** and **5**. All these data show that the boronic acid moiety may lead to effective Can2 inhibitors and that the substitution pattern of the aryl-, arylalkyl- and arylalkenyl- moieties present in the inhibitor molecule fine tunes the inhibitory capacity, with a well defined SAR, evidencing compounds with inhibitory properties between milli and low micromolar affinity, even for this small series of boronic acids.

Table 1
Inhibition data of human CA isozymes I, II (cytosolic), fungal β-CAs Can2 and Nce103 with compounds **1–5** and standard sulfonamide inhibitors (acetazolamide **AZA**, dichlorophenamide **DCP** and ethoxzolamide **EZA**), by a stopped-flow, CO_2 hydration assay[24]



No.	R	K_I* (μM)			
		hCA I[a]	hCA II[a]	Can2[b]	Nce103[b]
1a	H	1560	1050	810	30850
1b	Me	278	10.8	8.9	9.0
1c	*n*-Bu	7.9	8.7	10.9	8.0
1d	MeO	10.9	7.9	11.4	15.6
1e	Br	11.7	7.0	9.6	15.9
1f	Ph	3.7	4.5	9.9	7.8
1g	PhO	6.0	11.5	11.5	8.6
2	–	6.5	6.0	11.0	9.3
3a	H	12.5	534	490	779
3b	Me	12.1	617	521	460
3c	Ph	10.7	373	8.5	42.3
3d	4-CF_3–C_6H_4	9.5	27.6	3040	5210
4	–	11.4	17.9	428	412
5	–	8.6	18.1	506	633
AZA		0.25	0.012	0.010	0.132
DCP	–	1.20	0.038	1.20	0.91
EZA	–	0.025	0.008	0.087	1.07

* Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[24]
[a] Human, recombinant isozymes.
[b] Recombinant fungal enzymes.[1–3]



(iv) The lead **1a** was an exceedingly weak Nce103 inhibitor, with an inhibition constant of 30.85 mM. However, as for all CAs investigated here, its derivatives incorporating various substituents in the *para* position of the phenylboronic scaffold, of types **1b–g**, as well as the β-naphthyl derivative **2**, showed better inhibitory profiles, with K_Is in the range of 7.8–15.9 μM (Table 1). A slightly less effective inhibitor was **3c** (K_I of 42.3 μM), whereas the remaining derivatives **3, 4** and **5** showed weak Nce103 inhibitory activity, with inhibition constants in the range of 412–5210 μM. Also Nce103 was thus inhibited appreciably by some of these derivatives, with a rather well-defined SAR evidenced, but the strongest inhibitors were only in the low micromolar range, with no submicromolar derivatives evidenced so far. Furthermore, the sulfonamide derivatives in clinical use acetazolamide **AZA**, dichlorophenamide **DCP** and ethoxzolamide **EZA** showed more effective inhibition of both Can2 and Nce103 (in the range of 0.010–1.20 μM) as compared to the boronic acid, proving that the sulfonamide zinc binding group is more effective than the boronic acid one for generating potent CAIs against both α- and β-CAs.

A schematic inhibition mechanism of the α- and β-CAs with boronic acids is proposed in Figure 1. Bothe these enzymes contain a highly nucleophilic zinc hydroxide species within the enzyme active site, which is responsible for the catalytic activity and binding of most classes of inhibitors.[2,4] However, the protein zinc ligands differ significantly between the two enzyme classes, with three His ligands for α-CAs and one His and two Cys ligands for most of the β-CAs.[2,4] The electrophilic boronic acid may thus react with the zinc hydroxide species of the enzymes leading to the tetrahedral boron(III) and tetrahedral Zn(II) species depicted in Figure 1A and B, similarly with the binding of boronic acids to serine/threonine proteases, documented by means of X-ray crystallography.[11–15] Further studies to confirm or reject this hypothesis are thus surely warranted.

Although β-CAs from pathogenic fungi are not yet validated targets for the development of antifungals, in a proof-of-concept study, Mogensen et al.[25] showed that the sulfonamide inhibitor ethoxzolamide (**EZA**) significantly reduced the growth of *C. neoformans* in the presence of 0.033% CO_2 (the atmospheric concentration of this gas). The same effect has been observed for *C. albicans* pathogenesis in niches where the available CO_2 is limited, such as for example the growth of the pathogen on skin tissues.[26] These studies[25,26] represent a solid proof-of-concept regarding the validity of these targets and their potential for the pharmacological development of novel antifungals.

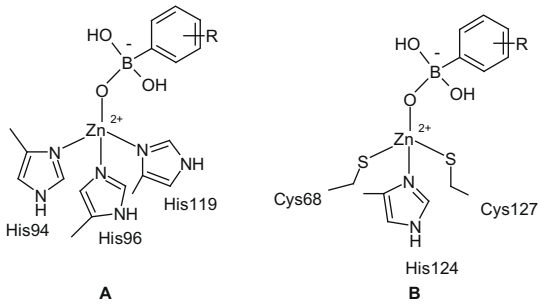


Figure 1. Proposed binding of aromatic boronic acids to α- (A) and β-CAs (B). The α-class enzymes have the Zn(II) ion coordinated by three His residues (hCA I numbering system),[4] the fourth ligand being a water molecule/hydroxide ion, which being a strong nucleophile may react with the electrophilic boronic acid leading to the adduct A. In β-CAs, the Zn(II) is coordinated by one His and two Cys residues (Can2 numbering system used in B),[2] and the fourth ligand is similar to the α-CAs one, leading thus to a similar tetrahedral inhibited species of the enzyme, depicted schematically in B.

In conclusion we report here the first inhibition study of the β-CAs from the fungal pathogens *C. neoformans* and *C. albicans* with a series of boronic acids. Aromatic, 4-phenylsubstituted- and 2-naphthylboronic acids were the best Can2 inhibitors, with inhibition constants in the range of 8.5–11.5 μM, whereas arylalkenyl and aryalkylboronic acids showed K_Is in the range of 428–3040 μM. Nce103 showed a similar inhibition profile, with the 4-phenylsubstituted- and 2-naphthylboronic acids possessing K_Is in the range of 7.8–42.3 μM, and the arylalkenyl and aryalkylboronic acids being weaker inhibitors (K_Is of 412–5210 μM). The host enzymes CA I and II were also effectively inhibited by these boronic acids. The $B(OH)_2$ moiety is thus a new zinc-binding group for designing effective inhibitors of the α- and β-CAs. This initial study gave us important hints how a millimolar lead molecule ($PhB(OH)_2$) can lead to low micromolar CA inhibitors targeting both α- and β-CAs, even for a small set of boronic acids. Manipulation of the scaffolds of compounds **1–5**, by introducing various substituents at the aromatic ring or aliphatic fragments of the molecules, may in principle lead to compounds with different inhibition profiles, since the active sites of the two classes of enzymes (α- and β-CAs) are very different. Work is in progress in these laboratories for finding such inhibitors with a more selective profile for the inhibition of the fungal over host enzymes.

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