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Carbonic anhydrase inhibitors. Inhibition of the human cytosolic isoforms I and II and transmembrane, tumor-associated isoforms IX and XII with boronic acids

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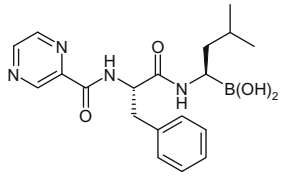
ABSTRACT

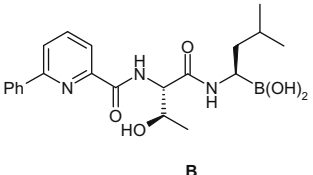
A series of aromatic, arylalkenyl- and arylalkyl boronic acids were assayed as inhibitors of four physiologically relevant carbonic anhydrase (CA, EC 4.2.1.1) isoforms, the cytosolic human (h) hCA I and II, and the transmembrane, tumor-associated hCA IX and XII. The best hCA I and II inhibitor was biphenyl boronic acid with, a K_I of 3.7–4.5 μM, whereas the remaining derivatives showed inhibition constants in the range of 6.0–1560 μM for hCA I and of 6.0–1050 μM for hCA II, respectively. hCA IX and XII were effectively inhibited by most of the aromatic boronic acids (K_Is of 7.6–12.3 μM) whereas the arylalkenyl and aryl–alkyl derivatives generally showed weaker inhibitory properties (K_Is of 34–531 μM). The nature of the moiety substituting the boronic acid group strongly influenced the CA inhibitory activity, with inhibitors possessing low micromolar to millimolar activity being detected in this small series of investigated compounds. This study proves that the $B(OH)_2$ moiety represents a new zinc-binding group for the generation of effective CA inhibitors targeting isoforms with medicinal chemistry applications. The boronic acids probably bind to the Zn(II) ion within the CA active site leading to a tetrahedral geometry of the metal ion and of the B(III) derivative.

1. Introduction

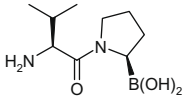
Boronic acids emerged recently as inhibitors of many enzymes involved in fundamental biological processes.[1–6] Thus, the peptidomimetic compound bortezomib **A**[1–3] is a clinically used proteasome inhibitor for the treatment of hematological malignancies, compound **B** is a second generation orally active proteasome inhibitor in clinical investigations as an antitumor drug,[4] whereas boronic acids **C** and **D** act as dipeptidyl peptidase IV inhibitors,[5] which represents a validated new target for the treatment of type 2 diabetes.[5] Boronic acids were also reported to act as inhibitors of fatty acid amide hydrolase,[6] prostate-specific antigen (a serine protease),[7] arginase,[8] or are useful as functional sensors of the physiological pH.[9] We have investigated the interaction of one such compound, phenyl boronic acid ($PhB(OH)_2$), with the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1)[10] 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.[11–13]



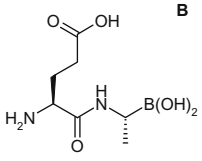
Bortezomib (A)



B



Val-boroPro (C)



Glu-boroAla (D)

Indeed, various such CA isoforms are involved in many critical physiological processes in humans,[10–13] and their inhibition mainly by sulfonamides and sulfamates,[14,15] is used therapeutically for the management of glaucoma, as diuretics, antiepileptics, antiobesity or antitumor drugs/diagnostic agents.[10,13–18] However, a main drawback of most of the clinically used sulfonamides (such as among others dichlorophenamide **DCP** and saccharin **SAC**) and sulfamates is related to their lack of selectivity for the inhibition of only therapeutically significant isoforms, among the many CAs

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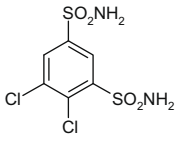
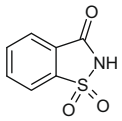
present in various tissues/organs where the inhibitor must act.[10] Thus, there is a constant search for new types of inhibitors showing a more selective profile or different pharmacological properties.[19] As most CA inhibitors (CAIs) bind to the catalytically essential Zn(II) ion from the enzyme active site,[10,19] much effort has been dedicated to the finding of novel zinc-binding groups (ZBGs) to be incorporated in the molecules of inhibitors and to the study of their inhibition mechanism by means of X-ray crystallography and kinetic experiments.[20,21] Since the boronic acids were only scarcely investigated from these points of view (as only $PhB(OH)_2$ has been tested as a CAI),[11–13] we report here a detailed inhibition study of four physiologically relevant human (h) CAs, that is, hCA I and II (cytosolic, ubiquitous isoforms) as well as hCA IX and XII (transmembrane, tumor-associated enzymes)[10] with a series of aromatic, aryl-alkenyl- and arylalkyl boronic acids. hCA II and XII are targets for obtaining anti-glaucoma drugs, whereas hCA IX and XII for designing novel antitumor therapies or diagnostic agents.[10,14]



DCP



SAC

2. Results and discussion

2.1. Chemistry

A library of commercially available boronic acids, of types **1**–5 have been included in this study, considering $PhB(OH)_2$ **1a** investigated earlier[11–13] as lead molecule. Indeed, arylboronic acids **1** and **2** have been investigated in order to understand whether a different substitution pattern at the aromatic ring has influence on the binding of the inhibitor to the enzyme, and thus to validate $B(OH)_2$ as a new ZBG for the design of CAIs. In fact, in most enzymes investigated up to now for their interactions with boronic acids, the $B(OH)_2$ moiety present in the inhibitor molecule is crucial for the inhibition, usually reacting with nucleophilic OH moieties of Ser or Thr residues from the enzyme active site, and leading to tetrahedral B(III) species which inactivate the enzyme (Fig. 1A).[1–6] In CAs, the water molecule/hydroxide ion coordinated to the crucial Zn(II) ion is also highly nucleophilic,[10] and we hypothesized earlier[11] that **1a** acts as a weak, millimolar CAI by formation of such species, in which the tetrahedral B(III) species is coordinated to the Zn(II) ion, as shown schematically in Figure 1B. Clearly, the remaining moieties of the inhibitors also interact with amino acid residues present in the enzyme active site (data not shown in Fig. 1), explaining, for example, the low nanomolar affinity of compounds **A** and **B** for the human 20S proteasome.[1–4]

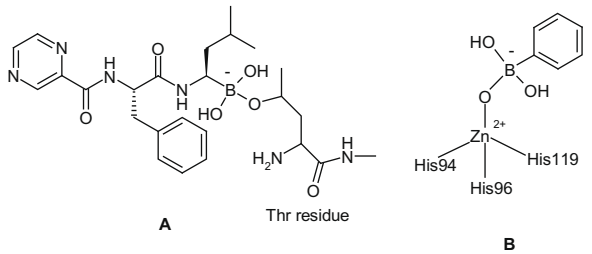


Figure 1. Enzyme inhibition mechanisms with boronic acids. (A) Inhibition of the chymotryptic-like activity of the proteasome with bortezomib **A**, as proved by X-ray crystallography (only interactions between the boronic acid moiety and a Thr residue of the enzyme are shown).[3] (B) Proposed binding of phenylboronic acid **1a** to the Zn(II) ion within the CA active site (no X-ray crystal structures of CA–boronic acid adducts are available, hCA I residues numbering system).[11]

Thus, a range of aliphatic, aromatic and halogeno-substituted phenylboronic acids **1b–1g**, as well as the 2-naphthyl boronic acid **2** were included in the study, together with a smaller subseries of substituted-phenylalkenyl- (**3a–3d**), phenylethyl- (**4**) and 3-phenylallyl-1-boronic acid **5**.

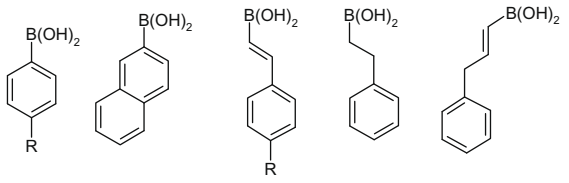
2.2. Carbonic anhydrase inhibition

Inhibition data against four catalytically active hCA isoforms, that is, hCA I, hCA II (cytosolic) and hCA IX and XII (transmembrane, tumor-associated enzymes) with the 14 boronic acids **1–5**, as well as the standard sulfonamides **DCP** and **SAC** are shown in Table 1.

The following structure–activity relationship (SAR) can be drawn by considering data of Table 1:[22]

(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 *para* position to the $B(OH)_2$ moiety leads to a dramatic increase of enzyme inhibitory activity. Thus, a 4-methyl group leads to a compound with a K_I of 278 μM (**1b**), whereas a *n*-Bu such group to a very 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**–**1g** (methoxy-, bromine-, phenyl- and phenoxy-), with the biphenyl boronic 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-naphthyl boronic acid **2** as well as the arylalkenyl/alkyl derivatives **3–5** also show effective hCA I inhibitory

Table 1
Inhibition data of human CA isozymes I, II (cytosolic), IX and XII (tumor-associated) with compounds **1–5** and the standard inhibitors dichlorophenamide **DCP** and saccharin **SAC**, by a stopped-flow, CO_2 hydration assay[22]



1 2 3 4 5

No.	R	K_I[c] (μM)			
		hCA I[a]	hCA II[a]	hCA IX[b]	hCA XII[b]
1a	H	1560	1050	1200	800
1b	Me	278	10.8	9.9	12.3
1c	*n*-Bu	7.9	8.7	10.6	6.8
1d	MeO	10.9	7.9	540	369
1e	Br	11.7	7.0	9.8	7.9
1f	Ph	3.7	4.5	11.9	7.6
1g	PhO	6.0	11.5	11.1	11.0
2	–	6.5	6.0	10.7	9.9
3a	H	12.5	534	365	10.3
3b	Me	12.1	617	254	10.9
3c	Ph	10.7	373	46.5	34.0
3d	$4\text{-}CF_3\text{–}C_6H_4$	9.5	27.6	10.8	10.5
4	–	11.4	17.9	531	348
5	–	8.6	18.1	478	11.8
DCP	–	1.2	0.038	0.050	0.050
SAC		18.5	5.9	0.103	0.633

[a] Human, recombinant isozymes.
[b] Catalytic domain, recombinant human isoforms.[11–16]
[c] Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[22]

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–3d** influences hCA I inhibitory activity but not as much as for derivatives **1a–1g** 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. All these data show that the boronic acid moiety may lead to effective hCA I 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–low micromolar, even for this small series of investigated boronic acids.

(ii) The ubiquitous cytosolic isoform hCA II showed an 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–1g**, 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–3c** 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 as a hCA II inhibitor than the last one.

(iii) The SAR for inhibiting the tumor-associated isoform hCA IX with this series of boronic acids is more complicated a compared to the previously discussed isoforms. Thus, **1a** is a weak inhibitor (K_I of 1.2 mM) similarly with the methoxy-substituted derivative **1d**, the alkenyl-aryl boronic acids **3a** and **3b**, as well as derivatives **4** and **5** (K_Is in the range of 254–540 μM, Table 1). However, several simple boronic acids, such as **1b, 1c, 1e–1g, 2**, and **3d** are effective hCA IX inhibitors, K_Is in the range of 9.8–11.9 μM. The derivative **3c** showed a behavior of medium potency inhibitor, with a K_I of 46.5 μM.

(iv) hCA XII was also inhibited by all the boronic acids **1–5** investigated here. Starting from the weak inhibitor **1a** (K_I of 800 μM), the presence of the 4-substituents of the alkyl-, aryl-, aryloxy- or halogeno type, such as in compounds**1b, 1c, 1e, 1f** and **1g**, leads to a drastic enhancements of the hCA XII inhibitory properties, these derivatives behaving as effective inhibitors (K_Is in the range of 6.8–11.0 μM, Table 1): Unexpectedly, the methoxy derivative **1d** is a much weaker hCA XII inhibitor as compared to the structurally related derivatives **1b**–**1d**, with a K_I of 369 μM. Compounds 2, **3a**–**3c** and **5** also showed effective hCA XII inhibition, with inhibition constants of 9.9–34.0 μM, whereas the arylalkyl derivative **4** was a much less effective inhibitor (K_I of 348 μM).

(v) Although boronic acids **1–5** showed effective inhibitory activity against the four CA isoforms, they are generally weaker inhibitors than the sulfonamide dichlorophenamide **DCP** or saccharin **SAC**, used as standards in the enzyme inhibition experiments (Table 1). However, considering the quite simple scaffold they incorporate, we hypothesize that the introduction of longer 'tails' as substituents to the aromatic rings present in compounds **1–5** may lead to better inhibitors, eventually with a more interesting inhibition profile. Further work in this field is warranted.

3. Conclusions

A series of aromatic, arylalkenyl- and arylalkyl boronic acids were assayed as inhibitors of four physiologically relevant CA isoforms, the cytosolic hCA I and II, and the transmembrane, tumor-associated hCA IX and XII. The best hCA I and II inhibitor was biphenyl boronic acid with, a K_I of 3.7–4.5 μM, whereas the remaining derivatives showed inhibition constants in the range of 6.0–1560 μM for hCA I and of 6.0–1050 μM for hCA II, respectively. hCA IX and XII were effectively inhibited by most of the aromatic boronic acids (K_Is of 7.6–12.3 μM) whereas the arylalkenyl and aryl–alkyl derivatives generally showed weaker inhibitory properties (K_Is of 34–531 μM). The nature of the moiety substituting the boronic acid group strongly influenced the CA inhibitory activity, with inhibitors possessing low micromolar to millimolar activity being detected in this small series of investigated compounds. This study proves that the $B(OH)_2$ moiety represents a new zinc-binding group for the generation of effective CA inhibitors targeting isoforms with medicinal chemistry applications. The boronic acids probably bind to the Zn(II) ion within the CA active site leading to a tetrahedral geometry of the metal ion and of the B(III) derivative.

4. Experimental

4.1. Chemistry

Buffers, boronic acids **1–5** and other chemicals (**DCP**, **SAC**) were of highest purity available reagents from Sigma–Aldrich (Milan, Italy), and were used without further purification. All CA isozymes were recombinant ones produced and purified in our laboratory as described earlier.[11–16]

4.2. CA inhibition assay

An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic/inhibition of various CA isozymes as reported by Khalifah.[22] 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 7.4) as buffer, 0.1 M Na_2SO_4 or $NaClO_4$ (for maintaining constant the ionic strength; these anions are not inhibitory in the used concentration),[11] following the CA-catalyzed CO_2 hydration reaction for a period of 5–10 s. Saturated CO_2 solutions in water at 25 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in DMSO–water 1:1, v/v) and dilutions up to 1 nM done with the assay buffer mentioned above. At least

seven different inhibitor concentrations have been used for measuring the inhibition constant. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the *E–I* complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[11] and represent the mean from at least three different determinations. All CA isozymes used here were recombinant proteins obtained as reported earlier by our group.[11–16]

Acknowledgments

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