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Carbonic Anhydrase Inhibitors, Interaction of Boron Derivatives with Isozymes I and II: A New Binding Site for Hydrophobic Inhibitors at the Entrance of the Active Site as shown by Docking Studies

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The interaction of human carbonic anhydrase (hCA) isozymes I and II with boron derivatives was investigated by kinetic and spectroscopic studies. These derivatives, tested as new inhibitors of carbonic anhydrase, are sulfonamide and non-sulfonamide boron derivatives and some of them proved to be moderately efficient inhibitors of hCA I and hCA II, their activities being comparable to those of the unsubstituted sulfonamides, the classical inhibitors of these zinc enzymes. Ph_2BOH, one of the compounds with the highest affinity for hCA II in the present study, has been docked within the active site. After minimisation it was found situated at 7.9 Å from zinc, within the hydrophobic half of the active site, in Van der Waals contacts with the amino acid residues: Val 121, Phe 130, Val 135, Leu 141, Val 143, Val 207 and Pro 201. This is the first time that a CA inhibitor has been found to bind at the edge of the active site cavity, similarly to the CA activator histamine, which binds on the hydrophilic half. This finding may be of importance also for the design of novel types of inhibitors with increased affinity for the different CA isozymes.

Keywords: Carbonic anhydrase, Enzyme inhibitors, Boron derivatives, Sulfonamides, Phenol, Docking

INTRODUCTION

Carbonic anhydrase is a zinc-metalloenzyme which catalyzes the reversible hydratation of carbon dioxide to the bicarbonate ion.[1–6] Until now, 14 different CA isozymes have been isolated in higher vertebrates, including humans.[7–9]

The zinc ion is situated at the bottom of the enzyme's active site and is coordinated to three histidine residues and a water molecule/hydroxide ion.[10–13] The catalytic mechanism of this enzyme has been widely investigated.[14–20] It starts with the nucleophilic attack of the zinc-hydroxyl ligand on the CO_2 molecule

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bound within a hydrophobic pocket nearby.[14–20] The bicarbonate formed which is coordinated to the metal is then released into solution upon binding of a new water molecule to the Zn(II) ion. A proton transfer to the solvent regenerates the active zinc-hydroxyl species.[4]

Unsubstituted sulfonamides RSO_2NH_2 are known to be efficient inhibitors of this enzyme.[4,8] X-Ray studies have shown that sulfonamide inhibitors are bound to the Zn(II) ion in the enzyme's active site by replacing the Zn-bound H_2O/OH^-, the metal ion remaining thus in its predilect tetrahedral geometry. The stability of the enzyme/inhibitor complex is achieved by other important active site interactions, such as hydrogen bonds between the inhibitor and amino acid residues present at the entrance of the cavity.[6,14,21,22]

Sulfonamide inhibitors are widely used as diuretics or for the treatment of diseases such as glaucoma, epilepsy and many others.[6,8,23–25]

Sulfonamide derivatives incorporating germanium(IV), phosphorus(V) and antimony(V) have already been tested as CA inhibitors.[26,27] Some of them proved to be efficient inhibitors, maybe because there is an additional coordination between the enzyme and the inhibitor, promoted by the metal ion or some of its ligands.[26,27]

Taking into account the interesting activity of the Ge(IV), P(V) and Sb(V) derivatives mentioned above, we report here the synthesis of some new boron compounds, some of which are sulfonamide derivatives, which have been assayed as inhibitors of two CA isozymes, hCA I and hCA II. Due to the smaller size of boron, as compared to germanium and antimony, it was expected that the B(III) derivatives would be able to readily enter into the active cavity of the enzyme. Thus, the affinity for the enzyme would be increased and the inhibitor would be more efficient. The results presented here seem to confirm this idea, as some of these boron derivatives showed good activities as inhibitors of the two CA isozymes mentioned above. These activities are comparable to those of the unsubstituted aromatic sulfonamides, the classical inhibitors of these zinc enzymes.

Compounds **1–8** investigated for their interaction with CA isozymes in the present study are shown below.

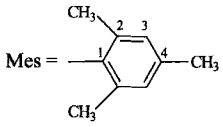
MATERIALS AND METHODS

Chemistry

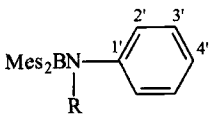
All reactions were carried out under nitrogen and with dry solvents. NMR spectra were recorded on Brücker AC 80 (^1H) and AC 200 (^{13}C and ^{11}B) spectrometers, chemical shifts being

Mes_2BF **1**

Mes_2BNH_2 **2**

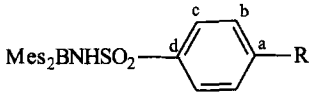


Mes_2BNHNH_2 **3**



$R = C_6H_5$ **4**

$R = CH_3$ **5**



$R = CH_3$ **6**

$R = NH_2$ **7**

Mes_2BOH **8**

reported as δ values, in ppm, relative to tetramethylsilane as internal standard. IR spectra were conducted on a Perkin-Elmer 1600 FTIR spectrometer and mass spectra on a HP 5889 in the electron impact mode (70 eV) or on a Rybermag R10–10 spectrometer operating in the electron impact mode or by chemical desorption (DCi/CH_4). Melting points were measured on a Leitz microscope.

Preparation of dimesitylfluoroborane, **1** This was prepared by the method described in reference 28.

To magnesium (4.92 g; 0.20 mol) in 15 ml THF, was added under stirring MesBr (39.88 g; 0.20 mol) in 150 ml THF at room temperature. After 2 h stirring at 20 °C, $BF_3.Me_2O$ (12.4 g; 0.10 mol) was added dropwise over 30 minutes. After 12 h further stirring at room temperature, THF was replaced by pentane and the magnesium salts were filtered off. The white residue obtained by evaporation of the solvent in vacuum was recrystallized in pentane to give 24.96 g of a white powder of Mes_2BF. Yield: 93%. m.p.: 76 °C. ^{1}H-NMR ($CDCl_3$) ppm: 2.30 (s, 6H, *p*-CH_3), 2.34 (s, 12H, *o*-CH_3), 6.87 (s, 4H, C_6H_2). ^{13}C-NMR ($CDCl_3$) ppm: 21.42 (*p*-CH_3), 22.41 (*o*-CH_3), 137.79, 137.97 (C_1), 142.47, 142.54 (C_2), 128.64 (C_3), 140.67 (C_4). ^{11}B-NMR ($CDCl_3$) ppm: 53.00. MS $C_{18}H_{22}BF$ (EI): $M^{+\bullet}$: 268 (48%); $M^{+\bullet}$ – CH_3: 253 (100%); $M^{+\bullet}$ – MesH: 148 (82%).

Preparation of dimesitylaminoborane, **2**[29] Mes_2BF (1.30 g; 4.80 mmol) in 5 ml C_6H_6 was added at room temperature to $NaNH_2$ (0.19 g; 4.80 mmol) in 3 ml C_6H_6. After 18 h stirring at room temperature, the NaF was centrifuged off. Evaporation of the solvent under vacuum gave 1.12 g of a white powder of Mes_2BNH_2. Yield: 88%. m.p.: 170 °C. IR ($CDCl_3$): 3375 and 3453 cm^{-1}(NH_2). ^{1}H-NMR ($CDCl_3$) ppm: 2.33 (s, 18H, *o*-CH_3 + *p*-CH_3), 6.85 (s, 4H, C_6H_2), 4.44 (s, 2H, NH_2). ^{13}C-NMR ($CDCl_3$) ppm: 22.81 (*p*-CH_3), 21.17 (*o*-CH_3), 140.69 (C_2), 128.22 (C_3), 137.47 (C_4). ^{11}B-NMR ($CDCl_3$) ppm: 44.14. MS $C_{18}H_{24}BN$ (EI): $M^{+\bullet}$: 265 (13%); $M^{+\bullet}$ – MesH: 145 (100%).

Preparation of dimesitylhydrazinoborane, **3** To hydrazine (5.00 mmol; 1 M in THF) in 5 ml THF, was added at −70 °C with stirring BuLi (4.64 mmol; 1.6 M in hexane). After 1 h stirring at room temperature, Mes_2BF (1.24 g; 4.64 mmol) was added dropwise. After 2 h further stirring at room temperature, THF was replaced by benzene, LiF was centrifuged off and benzene replaced by pentane. The white residue obtained by evaporation of the solvent in vacuum was washed twice with pentane yielding 0.93 g of a white powder of Mes_2BNHNH_2. Yield: 73%. m.p.: 130–132 °C. IR ($CDCl_3$): 3322 and 3385 cm^{-1} (NH_2), 3340 cm^{-1} (NH). ^{1}H-NMR ($CDCl_3$) ppm: 2.28 (s, 18H, CH_3), 6.79 (s, 2H, C_6H_2), 6.83 (s, 2H, C_6H_2), 3.68 (s, 2H, NH_2), 5.73 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$) ppm: 21.10, 21.17 (*p*-CH_3), 22.07, 22.85 (*o*-CH_3), 140.56, 141.42 (C_2), 127.96, 128.38 (C_3), 137.62, 137.71 (C_4). MS $C_{18}H_{25}BN_2$ (EI): $M^{+\bullet}$: 280 (62%); $M^{+\bullet}$ – MesH: 160 (60%); $M^{+\bullet}$ – NH_2: 264 (3%).

Preparation of dimesityl-diphenylaminoborane, **4**[30] As previously described, Mes_2BF (1.07 g; 4 mmol) in 3 ml THF added at room temperature to diphenylaminolithium prepared from Ph_2NH (0.68 g; 4 mmol) and *t*-BuLi (4 mmol; 1.7 M in pentane), gave 1.10 g of Mes_2BNPh_2 as a white solid. Yield: 66%. ^{1}H-NMR ($CDCl_3$) ppm: 2.20 (s, 6H, *p*-CH_3), 2.18 (s, 12H, *o*-CH_3), 6.60 (s, 4H, C_6H_2), 6.99–7.02 (m, 10H, C_6H_5). ^{13}C-NMR ($CDCl_3$) ppm: 21.09 (*p*-CH_3), 22.5 (*o*-CH_3), 138.52 (C_1), 140.93 (C_2), 127.90 (C_3), 137.11 (C_4), 149.30 ($C_{1'}$), 127.41 ($C_{2'}$), 127.76 ($C_{3'}$), 127.74 ($C_{4'}$). ^{11}B-NMR ($CDCl_3$) ppm: 48.91. MS $C_{30}H_{32}BN$ (EI): $M^{+\bullet}$: 417 (61%); $M^{+\bullet}$ – MesH: 297 (100%).

Preparation of dimesityl-N-methyl-N-phenylaminoborane, **5**[30] Mes_2BF (1.60 g; 6 mmol) reacted with Ph(Me)NLi prepared from *N*-methylaniline (0.64 g; 6 mmol) and *t*-BuLi (6.00 mmol; 1.7 M in pentane) at room temperature in THF leading to 1.57 g of a yellow solid identified as $Mes_2BN(Me)Ph$. Yield: 74%. m.p.: 120 °C. ^{1}H-NMR ($CDCl_3$) ppm: 2.07 (s, 6H, *p*-CH_3), 2.28 (s, 12H, *o*-CH_3), 7.06 (s, 4H, C_6H_2), 3.25 (s, 3H, NCH_3), 6.54–6.81 (m, 5H, C_6H_5). ^{13}C-NMR

($CDCl_3$) ppm: 40.03 (NCH_3), 21.07 (p-CH_3), 22.51 (o-CH_3), 138.00 (C_1), 140.34, 140.54 (C_2), 128.05, 128.21 (C_3), 136.83, 137.19 (C_4), 149.37 ($C_{1'}$), 124.66 ($C_{2'}$), 127.72 ($C_{3'}$), 124.27 ($C_{4'}$). MS $C_{25}H_{30}BN$ (EI): $M^{+\bullet}$: 355 (13%); $M^{+\bullet}$ – MesH: 235 (100%).

Preparation of dimesityl para-toluenesulfonamidoborane, **6** To a solution of *p*-toluenesulfonamide (0.60 g; 3.51 mmol) in 5 ml THF at −78 °C was added with stirring *t*-BuLi (3.51 mmol; 1.7 M in pentane). After 2 h stirring at room temperature, Mes_2BF (0.94 g; 3.51 mmol) in 5 ml THF was added to the mixture. After 5 h further stirring at room temperature, LiF was centrifuged off. The white residual powder (0.97 g) obtained by evaporation of the solvent in vacuum was identified as $Mes_2BNHSO_2C_6H_4p$-CH_3. Yield: 67%. m.p.: 118–120 °C. IR ($CDCl_3$): 3267 cm^{-1} (NH). ^{1}H NMR ($CDCl_3$) ppm: 2.22 (s, 3H, p-CH_3), 2.29 (s, 3H, p-CH_3), 1.97 (s, 6H, o-CH_3), 2.18 (s, 6H, o-CH_3), 6.72 (s, 2H, C_6H_2), 6.76 (s, 2H, C_6H_2), 2.41 (s, 3H, p-$CH_3C_6H_4$), 7.15 (d, 2H, $J_{bc} = 9$ Hz, H_b), 7.36 (d, 2H, $J_{bc} = 9$ Hz, H_c). ^{13}C-NMR ($CDCl_3$) ppm: 21.10, 21.32 (p-CH_3), 22.77, 22.89 (o-CH_3), 137.78 (C_1), 139.81, 140.72 (C_2), 129.27 (C_3), 138.46 (C_4), 141.80 (C_a), 127.53 (C_b), 127.69 (C_c), 144.03 (C_d), 21.64 (CH_{3a}). ^{11}B-NMR ($CDCl_3$) ppm: 48.40. MS $C_{25}H_{30}BNO_2S$ (EI): $M^{+\bullet}$: 419 (15%); $M^{+\bullet}$ – Mes: 300 (80%).

Preparation of dimesitylsulfonamidoborane, **7** Following the procedure used for $Mes_2BNHSO_2C_6H_4p$-CH_3: To $LiNHSO_2C_6H_4NH_2$ prepared from sulfonamide (0.70 g; 4.04 mmol) in 10 ml THF and *t*-BuLi (4.07 mmol; 1.7 M in pentane) was added Mes_2BF (1.09 g; 4.07 mmol) to give 0.45 g of $Mes_2BNHSO_2C_6H_4p$-NH_2 as a white powder after washing twice with pentane. Yield: 27%. m.p.: 80–82 °C. IR (DMSO): 3389 and 3478 cm^{-1} (NH_2), 3255 cm^{-1} (NH). ^{1}H-NMR (DMSO) ppm: 2.17 (s, 6H, p-CH_3), 2.04 (s, 12H, o-CH_3), 6.67 (s, 4H, C_6H_2), 6.52 (d, 2H, $^3J_{bc} = 7.2$ Hz, H_b), 7.21 (d, 2H, $^3J_{bc} = 7.2$ Hz, H_c), 5.92 (s, 1H, NH), 6.74 (s, 2H, NH_2). ^{13}C-NMR (DMSO) ppm: 20.87 (p-CH_3), 21.95 (o-CH_3), 140.07 (C_2), 127.69 (C_3), 137.90 (C_4), 152.79 (C_a), 112.10 (C_b), 128.53 (C_c). MS $C_{24}H_{29}BN_2O_2S$ (EI): $M^{+\bullet}$: 420 (26%); $M^{+\bullet}$ – Mes: 301 (100%).

Preparation of dimesitylboronic acid, **8**[28] To dimesitylfluoroborane (0.51 g; 1.90 mmol) in 2 ml THF was added with stirring at room temperature a large excess of NH_4OH (5 ml). Benzene was added after 3 h further stirring at 20 °C. The benzene phase was separated and dried on Na_2SO_4. After evaporation of the solvent, the white residue (0.32 g) was identified to Mes_2-BOH. Yield: 63%. m.p.: 141 °C. IR ($CDCl_3$): 3598 cm^{-1} (OH free), 3410 cm^{-1} (OH bonded). ^{1}H-NMR ($CDCl_3$) ppm: 2.30 (s, 18H, CH_3), 6.85 (s, 4H, C_6H_2), 5.94 (s, 1H, OH). ^{13}C-NMR ($CDCl_3$) ppm: 21.29 (p-CH_3), 22.52 (o-CH_3), 136.92 (C_1), 141.19 (C_2), 128.47 (C_3), 139.00 (C_4). ^{11}B-NMR ($CDCl_3$) ppm: 49.29. MS $C_{18}H_{23}BO$ (EI): $M^{+\bullet}$: 266 (4%); $M^{+\bullet}$ – MesH: 146 (2%).

Biochemistry

Human CA I and CA II cDNAs were expressed in Escherichia coli strain BL21 (DE3) from the plasmids pACA/HCA I and pACA/HCA II as previously describes[31,32] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by Lindskog's group,[31,32] and enzymes were purified by affinity chromatography according to the method of Khalifah *et al.*[33] Enzyme concentrations were determined spectrophotometrically at 280 nm, using a molar absorptivity of 49 $mM^{-1} \cdot cm^{-1}$ for CA I and 54 $mM^{-1} \cdot cm^{-1}$ for CA II, respectively, based on Mr = 28.85 kDa for CA I, and 29.3 kDa for CA II, respectively.[34,35]

Initial rates of 4-nitrophenyl acetate hydrolysis were monitored spectrophotometrically, at 400 nm and 25 °C, with a Cary 3 apparatus interfaced with an IBM compatible PC.[36] Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 10^{-2} and 10^{-6} M. A molar absorption coefficient ($\varepsilon = 18{,}400$ $M^{-1} \cdot cm^{-1}$) was used for the 4-nitro-

phenol formed by hydrolysis under the conditions of the experiments (pH 7.80), as reported by Pocker and Stone.[36] Ionic strength was maintained constant at 0.1 with Na_2SO_4. Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each inhibitor, and the values reported throughout the paper are the averages of such results. IC_{50} represents the molarity of inhibitor producing a 50% decrease in the enzyme catalyzed hydrolysis of 4-nitrophenyl acetate. Enzyme concentrations were: 10.7 nM for hCA I and 3.9 nM for hCA II. Co(II)-CA II has been prepared as described previously.[16]

Calculations and Docking

All the molecular mechanics and dynamics calculations were done with the program MOE (Molecular Operating Environment).[37] The starting point for the modelling of inhibitor binding within the CA active site was from the X-ray structure of the adduct of phenol with hCA II reported by Christianson's group.[38] This structure is not deposited in the Brookhaven Protein Database and was available directly from its authors. The second phenol molecule, bound distantly (15 Å) from the Zn(II) ion has been removed from the active site, whereas the phenol molecule bound within the hydrophobic pocket has been replaced by diphenylboronic acid. A number of 30 minimisation cycles have been performed with a tether constraint on the heavy atoms gradually lowered till complete relaxation.

RESULTS AND DISCUSSION

Dimesitylfluoroborane **1** was prepared by the Grignard reaction (Scheme 1) in a manner similar to that described in reference 28 and this compound was the starting material for obtaining all others boron derivatives (**2**) to (**8**) investigated here. Dimesitylboronic acid (**8**)[28] was obtained by ammoniacal hydrolysis of (**1**) as described in the literature.[39] The amino and sulfonamido derivatives were prepared by amino-metallation of (**1**) according to Scheme 1.

The amino groups linked to the boron atom were chosen either from a sterically non-hindered series, in order to obtain small amino-boron substituents (**2**, **3**) or on the contrary, from an aromatic series (**4**, **5**) in order to compare the arylsulfonamide-boron derivatives (**6**, **7**) with the corresponding unsubstituted sulfonamides, known to act as efficient CA inhibitors.[4,8] B-N bonds are known to be very stable because of the return of the nitrogen electron free pair in the vacant orbital of the boron atom.[40,41] However, an electron-attracting substituent on nitrogen enhances the reactivity towards water, yielding the corresponding boron-hydroxy compound.[39] We chose to work with mesityl groups on the boron because of their stabilization effects towards hydrolysis demonstrated for other such organo-metallic derivatives.[42]

Inhibition data for compounds **1–8** towards isozymes hCA I and hCA II, with 4-nitrophenyl acetate as substrate, are shown in Table I.

As seen from the above data, the dimesitylboronic acid (**8**), the corresponding amine (**2**) and the hydrazine (**3**) behave as efficient

$$2\,BF_3.Me_2O + 2\,MesMgBr \longrightarrow \underset{\mathbf{1}}{Mes_2BF} + MgBr_2 + MgF_2 \quad \text{(eq. 1)}$$

$$\underset{\mathbf{1}}{Mes_2BF} + R(R')NM \longrightarrow Mes_2BNR(R') + MF \quad \text{(eq. 2)}$$

M = Li, Na

R(R')N = H_2N (**2**), $H_2N(H)N$ (**3**), Ph_2N (**4**), Ph(Me)N (**5**),
$CH_3PhSO_2(H)N$ (**6**), $H_2NPhSO_2(H)N$ (**7**).

SCHEME 1

TABLE I Inhibition of isozymes hCA I and II with inhibitors of type **1–8**. Sulfanilamide and 4-toluenesulfonamide were included as standards, being well known sulfonamide inhibitors

Inhibitor	IC_{50} (μM)*	
	hCA I**	hCA II**
1	36 (5)	30 (2)
2	9 (0.5)	2.2 (0.3)
3	10 (1)	3.6 (0.4)
4	> 3000	> 3000
5	> 3000	> 3000
6	54 (4)	43 (2)
7	59 (3)	44 (1)
8	12 (1)	3.4 (0.2)
4-Toluenesulfonamide	22 (2)	2.5 (0.1)
Sulfanilamide	28 (3)	3.0 (0.2)

*Mean (standard error from 3 determinations). **Enzyme concentrations were: 10.7 nM for hCA I and 3.9 nM for hCA II.

inhibitors of both CA isozymes, with potencies comparable to those of the unsubstituted aromatic sulfonamides, the classical inhibitors of these zinc enzymes.

Disubstitution of the nitrogen atom with aryl or aryl/alkyl moieties, such as in compounds (**4**) and (**5**), leads to completely inactive compounds as CA inhibitors. The *N*-monosubstitution by a *p*-toluenesulfonamide or sulfanilamide group (compounds **6** and **7**, respectively) has as a consequence the maintaining of activity, but it is drastically reduced as compared to that of the unsubstituted parent compound (**2**) (and the unsubstituted sulfonamides). The fact that the measured activities are quite different from those obtained in the presence of the hydrolysis products of compounds (**6**) and (**7**) (i.e., dimesitylboronic acid and unsubstituted sulfonamides), confirm that these activities are due indeed to compounds (**6**) and (**7**) themselves. It also seems of interest to have NH_2 or OH group next to boron in these new CA inhibitors, due to the possibility the interaction of such moieties with active site residues important for catalysis (i.e., the metal ion, or the metal ion bound water molecule/hydroxide ion). But, regarding the activities of compounds (**2**) and (**3**), the distance between the NH_2 group and boron does not have any effect on the biological activity. If may also be noted that there are no changes in activity when the terminal CH_3 in compound (**6**) is replaced by a NH_2 (compound **7**) moiety. The derivatives (**1**), (**2**), (**3**), (**6**), (**7**) and (**8**) are more efficient hCA II than hCA I inhibitors.

Detailed information regarding the binding of these inhibitors within the enzyme active site could not be obtained by means of X-ray crystallographic studies of enzyme-inhibitor adducts, due to the low solubility of compounds (**1–8**) in aqueous solution, as well as due to their unknown stability in such systems. Furthermore, spectroscopic studies of adducts of Co(II)-CA II with these new inhibitors were not at all informative since the spectra of the adducts were similar with those of the uncomplexed enzyme (data not shown). The only other compounds that interact with CA but without changing the electronic spectrum of Co(II)-CA are phenol,[43] the only competitive inhibitor (with CO_2 as substrate) of this enzyme,[38] and histamine, a powerful CA activator.[19,44] The X-ray crystallographic structures of both these complexes with hCA II have been reported recently.[19,38] It appeared thus of interest to try to dock one of the inhibitors described here within the CA active site, starting from the crystallographic structure of one of the adducts mentioned above, and phenol was chosen since being more hydrophobic than histamine it is more structurally related to the inhibitors described in this paper. A simple, structurally related compound with the boronic acid inhibitor (**8**) was chosen for the molecular modeling experiment i.e., diphenylboronic acid, Ph_2BOH. Compound (**8**) (and presumably its congener Ph_2BOH) is a moderately-strong hCA II inhibitor, with an IC_{50} value of 3.4 μM (Table I), being much more effective than phenol, which possesses an inhibition constant of 10 mM against the same isozyme.[43] Phenol binds to the enzyme in a very specific manner. It forms a strong hydrogen bond with the zinc-bound water molecule from the hCA II active site, being at a distance of about 4 Å from Zn(II) (i.e., the

distance Zn-O (from phenol) is precisely 3.9 Å).[38] Phenol is bound within the hydrophobic half of the CA active site, being in Van der Waals contacts with the residues Val 121, Val 143, Leu 198, Thr 199 and Trp 209, as shown by the valuable X-ray crystallographic study of Nair *et al.*[38] After the calculation performed in the present study by docking Ph_2BOH within the hCA II active site, we observed that the boronic acid inhibitor was placed exactly within the hydrophobic site of the enzyme cavity, but more distant than phenol. More precisely, the oxygen atom of Ph_2BOH is situated at 7.9 Å from zinc (Figure 1), being in Van der Waals contacts with the following amino acid residues: Val 121, Phe 130, Val 135, Leu 141, Val 143, Val 207 and Pro 201 (all of which line the hydrophobic half of the CA active site – Figure 2).[14,18] The similarity between the binding of this hydrophobic inhibitor (Ph_2BOH) and that of histamine to hCA II[18,44] is of great interest. Both these compounds bind at the entrance of the active site, but unlike histamine which prefers the hydrophilic half (being in close contact with residues His 64 and Gln 92), Ph_2BOH prefers the hydrophobic half of the active site (Figures 1 and 2). Histamine, and other activators of the same type, participate in proton shuttling processes between the active site and the reaction medium, by means of their proton accepting moieties. Their binding is thus achieved in the same region of the active site where the "natural" proton shuttle of the enzyme is located, i.e., His 64.[15,18,44] On the contrary, phenol and the hydrophobic inhibitor Ph_2BOH bind at the opposite site of the cavity, within the hydrophobic half of it : phenol near the Zn(II) ion, within the postulated CO_2-binding site (which could not be precisely shown up to now) whereas Ph_2BOH binds at a more distant site, obturating in this way the access of

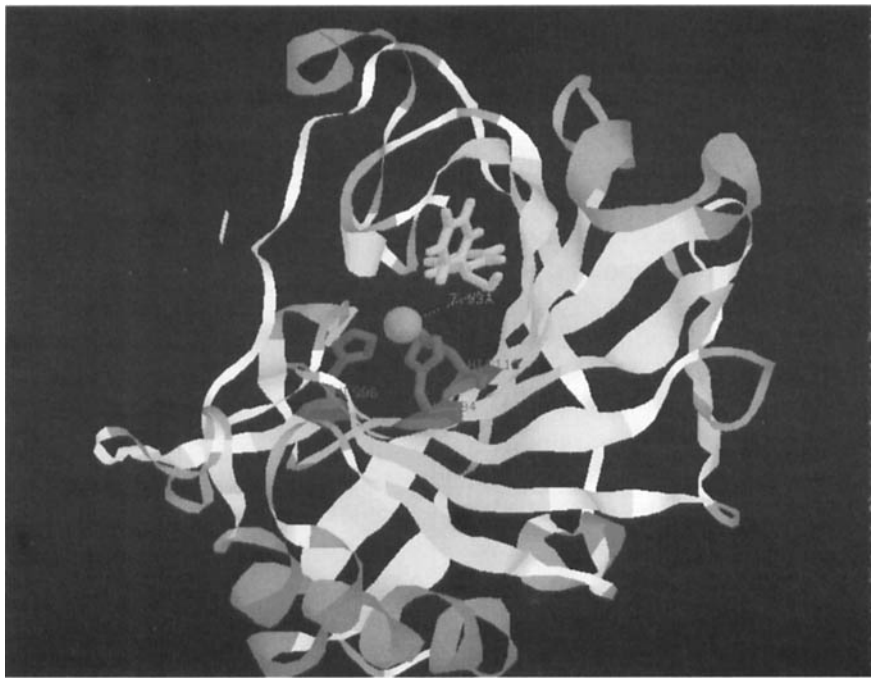


FIGURE 1 Diphenylboronic acid (in pink) docked within the active site of hCA II. The three zinc (red sphere) ligands, His 94, His 96 and His 119 (in blue) are also shown. The inhibitor is bound on the hydrophobic half, at the entrance of the active site cavity, with the OH moiety at 7.93 Å from zinc. (See Color Plate I).

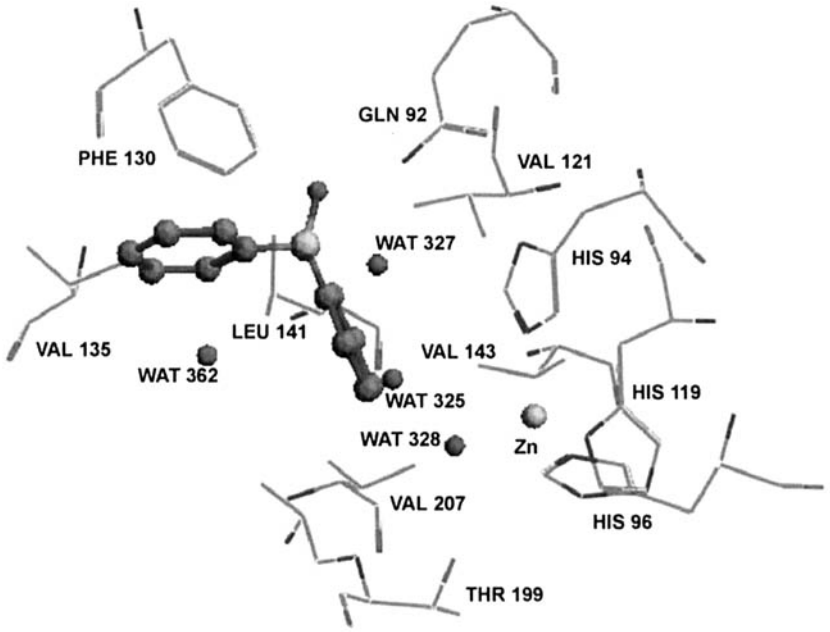


FIGURE 2 Interactions between Ph_2BOH and the hCA II active. The following moieties are shown: the Zn(II) ion and its three histidine ligands (His 94, His 96, His 119); the hydrophobic residues in close contact with the inhibitor molecule (Val 121, Phe 130, Val 135, Leu 141, Val 143 and Val 207), Gln 92 (situated towards the hydrophilic half of the active site) and Thr 199 (which orients the zinc bound hydroxide ion for catalysis). Four water molecules (Wat 325, 327, 328 and 362) are also present in the neighbourhood of the bound inhibitor. (See Color Plate II).

substrates to the nucleophilic ZnOH species. This may explain the increased efficiency of around 10 000 times of Ph_2BOH over PhOH in inhibiting hCA II. It must also be noted that the OH moiety of Ph_2BOH points towards the hydrophilic part of the active site (Figure 2) and that at least three water molecules are present in that region too.

These findings may be of importance for the design of novel types of CA inhibitors: a compound possessing a strong zinc-binding function (for example of the sulfonamide type) but also structural elements that allow its interaction with this new hydrophobic binding site shown here for the first time, might show an increased efficiency towards the different CA isozymes, and also raises the possibility of designing isozyme-specific inhibitors. These results are interesting from an aspect of developing new drugs possessing less undesired side effects, because of their selectivity for diverse CAs.

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References

[1] F.J.W. Roughton and A.M. Clark (1951) In *The Enzymes*, Sumner, J.B. and Myrback, K. (Eds.), vol. **1**, pp. 1250–1266. Academic Press: New York.
[2] D. Hewett-Emmett and R.E. Tashian (1996) *Mol. Phylogenet Evol.*, **5**, 50–77.
[3] S. Lindskog and A. Liljas (1993) *Curr. Opin. Struct. Biol.*, **3**, 915–920.
[4] S. Lindskog and A. Liljas (1994) *Roum. Chem. Q. Rev.*, **2**, 243–258.

[5] T.H. Maren (1967) *Physiol. Rev.*, **47**, 595–782.
[6] C.T. Supuran (1994) In *Carbonic Anhydrase and Modulation of Physiologic and Pathologic Processes in the Organism*, Puscas, I. (Ed.), pp. 29–116. Helicon, Timisoara: Romania.
[7] K.S. Smith, C. Jakubzick, T.S. Whittam and J.G. Ferry (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 15184–15189.
[8] C.T. Supuran and A. Scozzafava (2000) *Expert. Opin. Ther. Patents*, **10**, 575–600.
[9] J. Bellingham, K. Gregory-Evans and C. Gregory-Evans (1998) *Biochem. Biophys. Res. Commun.*, **253**, 364–367.
[10] K.K. Kannan, M. Ramanadham and T.A. Jones (1984) In *Biology and Chemistry of the Carbonic Anhydrases*, Tashian, R. and Hewett-Emmett, D. (Eds.), pp. 49–60. New York Academy Science: New York.
[11] K. Hakansson, M. Carlsson, A. Svensson and A. Liljas (1992) *J. Mol. Biol.*, **227**, 1192–1204.
[12] A.E. Eriksson and A. Liljas (1993) *Proteins Struct. Funct. Genet.*, **16**, 29–42.
[13] P.A. Boriack-Sjodin, R.W. Heck, P.J. Laipis, D.N. Silverman and D.W. Christianson (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 10949–10953.
[14] A. Liljas, K. Hakansson, B.H. Jonson and Y. Xue (1994) *Eur. J. Biochem.*, **219**, 1–10.
[15] D.N. Silverman and S. Lindskog (1988) *Acc. Chem. Res.*, **21**, 30–36.
[16] F. Briganti, R. Pieratelli, A. Scozzafava and C.T. Supuran (1996) *Eur. J. Med. Chem.*, **31**, 1001–1010.
[17] I. Bertini and C. Luchinat (1983) *Acc. Chem. Res.*, **16**, 272–279.
[18] I. Bertini, C. Luchinat and A. Scozzafava (1982) *Struct. Bonding*, **48**, 45–92.
[19] F. Briganti, S. Mangani, P. Orioli, A. Scozzafava, G. Vernaglione and C.T. Supuran (1997) *Biochemistry*, **36**, 10384–10392.
[20] S. Mangani and K. Hakanson (1992) *Eur. J. Biochem.*, **210**, 867–871.
[21] A. Guerri, F. Briganti, A. Scozzafava, C.T. Supuran and S. Mangani (2000) *Biochemistry*, **39**, 12391–12397.
[22] F. Briganti, S. Mangani, A. Scozzafava, G. Vernaglione and C.T. Supuran (1999) *J. Biol. Inorg. Chem.*, **4**, 528–536.
[23] T.H. Maren (1991) The links among biochemistry, physiology and pharmacology in carbonic anhydrase mediated systems. In *Carbonic anhydrase – From Biochemistry and Genetics to Physiology and Clinical Medecine*, Botré, F., Gros, G. and Storey, B.T. (Eds.), pp. 186–207. VCH: Weinheim.
[24] I. Puscas and C.T. Supuran (1996) In *Aparelho Digestivo*, Coelho, J. (Ed.), pp. 1704–1734. MEDSI: Rio de Janeiro.
[25] P.J. Wistrand and A. Lindqvist (1991) Design of carbonic anhydrase inhibitors and the relationship between the pharmacodynamics and pharmacokinetics of acetazolamide. In *Carbonic anhydrase – From Biochemistry and Genetics to Physiology and Clinical Medicine*, Botré, F., Gros, G. and Storey, B.T. (Eds.), pp. 352–378. VCH: Weinheim.
[26] (a) M. Rivière-Baudet and C.T. Supuran (1996) *Main Group Met. Chem.*, **19**, 579–584; (b) M. Rivière-Baudet, C.T. Supuran, A. Scozzafava, F. Briganti, F. El Baz, Z. Ben Maarouf and P. Rivière (1997) *Main Group Met. Chem.*, **20**, 641–648.
[27] I. Fenesan, R. Popescu, A. Scozzafava, V. Crucin, E. Mateiciuc, R. Bauer, M.A. Ilies and C.T. Supuran (2000) *J. Enz. Inhib.*, **15**, 297–310.
[28] H.C. Brown and V.H. Dodson (1957) *J. Amer. Chem. Soc.*, **79**, 2302–2306.
[29] G.E. Coates and J.G. Livingstone (1961) *J. Chem. Soc.*, 4909–4913.
[30] M.E. Glogowski, P.J. Grisdale, J.L.R. Williams and T.H. Regan (1973) *J. Organomet. Chem.*, **54**, 51–62.
[31] C. Forsman, G. Behravan, A. Osterman and B.H. Jonsson (1988) *Acta Chem. Scand.*, **B42**, 314–318.
[32] G. Behravan, P. Jonasson, B.H. Jonsson and S. Lindskog (1991) *Eur. J. Biochem.*, **198**, 589–592.
[33] R.G. Khalifah, D.J. Strader, S.H. Bryant and S.M. Gibson (1977) *Biochemistry*, **16**, 2241–2247.
[34] L.E. Henderson, D. Henriksson and P.O. Nyman (1976) *J. Biol. Chem.*, **251**, 5457–5463.
[35] P.O. Nyman and S. Lindskog (1964) *Biochim. Biophys. Acta*, **85**, 141–151.
[36] Y. Pocker and J.T. Stone (1967) *Biochemistry*, **6**, 668–678.
[37] MOE, available from Chemical Computing Groups Inc., 1255 University Street, Suite 1600, Montreal, Quebec, Canada h3B 3X3.
[38] S.K. Nair, P.A. Ludwig and D.W. Christianson (1994) *J. Am. Chem. Soc.*, **116**, 3659–3660.
[39] Z. Ben Maarouf, C. Chazalette, M. Rivière-Baudet and P. Rivière (1999) *Main Group Met. Chem.*, **22**, 409–416.
[40] M. Vaultier and B. Carbone (1994) "Boron". In *Comprehensive Organometallic Chemistry*, volume II, Chap 11.
[41] M.F. Lappert, P.P. Power, A.R. Sanger and R.C. Srivastawa (1980) *Metal and Metalloids Amides*. Ellis Horwood: Chichester.
[42] P. Rivière, M. Rivière-Baudet and J. Satgé (1982) "Germanium". In: *Comprehensive Organometallic Chemistry I*, vol 2, Chap 10; *Comprehensive Organometallic Chemistry II*, vol. 12, Chap 5, (1995).
[43] I. Simonsson, B.H. Jonsson and S. Lindskog (1982) *Biochem. Biophys. Res. Commun.*, **108**, 1406–1412.
[44] C.T. Supuran and A. Scozzafava (2000) "Activation of carbonic anhydrase isozymes". In *The Carbonic Anhydrases – New Horizons*, Chegwidden, W.R., Carter, N. and Edwards, Y. (Eds.), pp. 197–219. Birkhauser Verlag: Basel, Switzerland.