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RESEARCH ARTICLE

# Inhibition of human carbonic anhydrase isozymes I, II and VI with a series of bisphenol, methoxy and bromophenol compounds

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## Abstract

Carbonic anhydrase inhibitors (CAI) are valuable molecules as they have several therapeutic applications, including anti-glaucoma activity. In this study, inhibition of three human carbonic anhydrase (hCA, EC 4.2.1.1) isozymes I, II and VI with a series of bisphenol and bromophenol derivatives was investigated. Molecular docking studies of a set of such inhibitors within CA I and II were also performed.  $K_i$  values of the molecules **2–9** were in the range of 10.025–892.109  $\mu\text{M}$  for hCA I, 1.437–59.107  $\mu\text{M}$  for hCA II and 11.143–919.182  $\mu\text{M}$  for hCA VI, respectively. Reported inhibitory activities of molecules **2–9** will assist in better understanding of structure-activity relationship studies of CAI.

**Keywords:** Carbonic anhydrase, molecular docking, bisphenol, bromophenol, hCA II, hCA VI

## Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc(II)-dependent enzyme that catalyses the reversible hydration of carbon dioxide to hydrogencarbonate and a proton. This enzyme plays an important role in physiological anion exchange processes<sup>1,2</sup>. Thus far, at least 16 CA isozymes have been described in mammals<sup>2–6</sup>. CA II is primarily found in red blood cells and also in many other secretory tissues of the gastrointestinal tract, kidney, lung, eye and so on<sup>2–4</sup>. CA VI is a secretory enzyme that was initially described in the ovine parotid gland, saliva and normal human serum<sup>4,5</sup>. Other CA isoforms are found in a variety of tissues where they participate in several important biological processes such as acid–base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and electrolyte secretion<sup>1–13</sup>.

CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited/activated for the treatment of a range of disorders such as oedema, glaucoma, obesity, cancer, epilepsy and osteoporosis<sup>8–10,13</sup>.

Recently, Our and Supuran's groups investigated the interaction of CA I and II isozymes with several types of phenols such as the simple phenol, hydroxy-/methoxy-substituted benzoic acids as well as di-/tri-methoxy benzenes, anti-oxidant bisphenols and several of its substituted derivatives, for example, salicylates and some of their derivatives<sup>11–13</sup>. In this study, we extended these earlier investigations to bromophenols, some of which are widely used as anti-oxidant food additives or drugs. Bromophenols, frequently isolated from red algae of the family Rhodomelaceae, have some important biological activities<sup>14–22</sup>. The bisphenols or related methoxy

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derivatives **3–6** were previously demonstrated to possess strong anti-oxidant properties<sup>16</sup>.

In this study, we have purified human CA I, II and VI (hCA I, hCA II and hCA VI) from human blood and examined the *in vitro* inhibition effects of some bisphenol, metoxy and bromophenol compounds on these enzymes.

## Materials and methods

CNBr-activated sepharose 4B, protein assay reagents, p-aminobenzene sulfonamide L-tyrosine, 4-nitrophenylacetate (NPA) and chemicals for electrophoresis were purchased from Sigma-Aldrich Co, Munich, Germany. All other chemicals were of analytical grade and obtained from either Sigma or Merck.

### CA purification assay

Purification of hCA I and hCA II were performed as previously described<sup>13</sup>. The methods proposed by Murakami and Sly and Kivela et al. were modified and used for purification of CA VI from human serum<sup>47</sup>. Serum was obtained from fresh human blood at the Blood Center of the Research Hospital at Atatürk University. The blood samples were centrifuged at 5000 rpm for 15 min, the precipitant was removed and serum was isolated. The pH was adjusted to 8.7 with solid Tris. Sepharose-4B-aniline-sulfanylamine affinity column was equilibrated with 25-mM Tris-HCl/0.1-M Na<sub>2</sub>SO<sub>4</sub> (pH 8.7). The affinity gel was washed with 25-mM Tris-HCl/22-mM Na<sub>2</sub>SO<sub>4</sub> (pH 8.7). The human carbonic anhydrase (hCA-VI) isozyme was eluted with 0.25 M H<sub>2</sub>NSO<sub>3</sub>H/25cmM Na<sub>2</sub>HPO<sub>4</sub> (pH = 6.7). All procedures were performed at 4°C<sup>4,5</sup>.

### CA activity assay and kinetic studies

CA activity was assayed by following the change in absorbance at 348 nm of 4-NPA to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer (Shimadzu UV-VIS) according to the method described by Verpoorte et al.<sup>23</sup> The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4-mL 0.05-M Tris-SO<sub>4</sub> buffer (pH 7.4), 1-mL 3-mM 4-NPA, 0.5-mL H<sub>2</sub>O and 0.1-mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. The inhibitory effects of the phenolic compounds were examined. All compounds were tested in triplicate at each concentration used. Different concentrations of the compounds were used. Control cuvette activity in the absence of inhibitor was taken as 100%. For the compounds, an activity (%)–[inhibitor] graphs were drawn. In these experiments, 4-NPA was used as substrate at five different concentrations (0.15–0.75 mM). K<sub>1</sub>-s were obtained from IC<sub>50</sub> by the Cheng–Prusoff equation, and the Lineweaver–Burk curves were drawn<sup>24</sup>.

### Protein determination

Protein quantity was determined spectrophotometrically at 595 nm according to the Bradford method during the

purification steps, using bovine serum albumin as the standard<sup>25</sup>.

### SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the enzymes. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli procedure. A 20 µg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without the dye<sup>26</sup>.

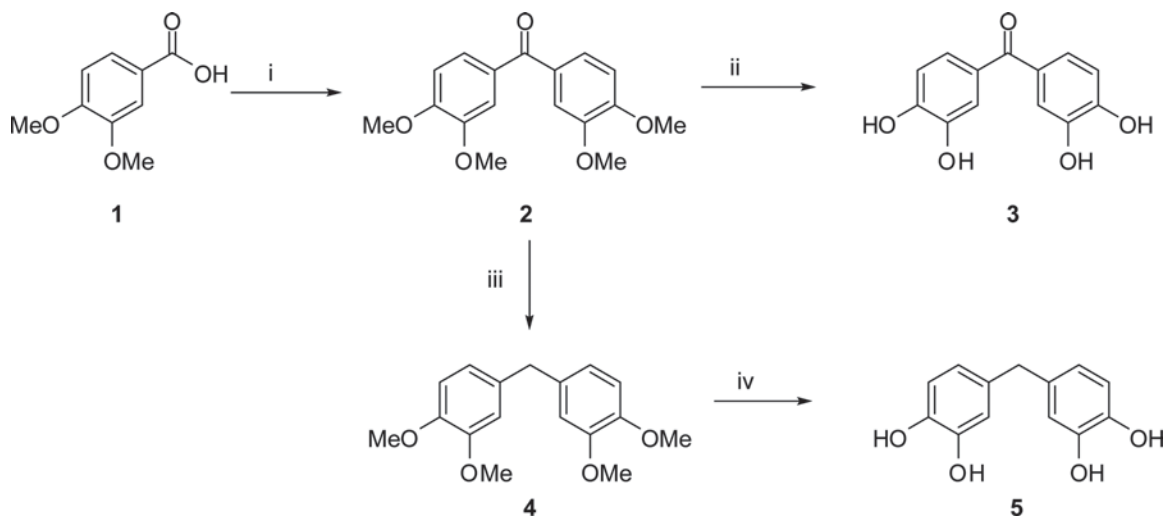
### Molecular docking

Glide-Induced Fit Docking (IFD) program of Schrodinger molecular modelling platform has been used for docking studies (Figures 2–6). Extra precision (XP) option is used<sup>26–28</sup>.

## Results and discussion

Since simple phenol (PhOH) has been shown to be the only competitive inhibitor with CO<sub>2</sub> as substrate for the main isoform of CA, that is, human CA II (hCA II)<sup>29</sup>, we investigated these phenolic derivatives. The X-ray crystal structure for the adduct of hCA II with phenol<sup>29</sup> showed that this compound binds to CA by anchoring its OH moiety to the zinc-bound water/hydroxide ion of the enzyme active site, through a hydrogen bond as well as through a second hydrogen bond to the NH amide of Thr199, an amino acid conserved in all α-CAs and critically important for the catalytic cycle of these enzymes<sup>3–13</sup>. The hydrophobic part of the hCA II active site contains the phenyl moiety of phenol, where CO<sub>2</sub> binds in the precatalytic complex, explaining the behaviour of phenol as a unique CO<sub>2</sub> competitive inhibitor. Supuran et al. has recently investigated the interactions of phenol and some of its substituted derivatives with all mammalian CA enzymes<sup>10–13</sup>. Their results demonstrated low micromolar/submicromolar inhibitory of molecules as well as the possibility to design isozyme-selective CA inhibitors (CAIs). The inhibition profile of various CA isozymes with this class of agents is very diverse, with inhibition constants ranging from the millimolar to the submicromolar range for many simple phenols<sup>10–13</sup>. Thus, it seemed reasonable to us to extend the previous studies<sup>10–13</sup>, including in this investigation phenols with clinical and anti-oxidant applications as food additives, such as compounds **3–6**<sup>16</sup>. Other structurally related derivatives such as **2**, **7** and **8** were also included in this study.

Bis(3,4-dimethoxyphenyl)methanone (**6**) was obtained from the reaction of 3,4-dimethoxybenzoic acid (**6**) with veratrole in polyphosphoric acid according to the literature method<sup>20,22</sup>. The Wolff–Kishner reduction of **3** with hydrazine hydrate and KOH at 190°C gave bis(3,4-dimethoxyphenyl)methane (**4**). The literature procedure<sup>14–20</sup> described for *o*-demethylation of aryl



Scheme 1.

methyl ethers with  $\text{BBr}_3$  was applied to compounds **1** and **3** to give corresponding phenols bis(2,3-dihydroxyphenyl)methanone (**3**) and 4,4'-methylenedibenzen-1,2-diol (**5**) (Scheme 1). Compounds **2–9** were synthesised according to our previously published procedures<sup>14–22</sup>.

The purification of the two CA isozymes was performed with a simple one-step method by a Sepharose-6B-aniline-sulfanilamide affinity column chromatography<sup>30–32</sup>. hCA I was purified, 102-fold with a specific activity of 865.7 EU/mg and overall yield of 48.4%, hCA II was purified, 625-fold with a specific activity of 5306 EU/mg and overall yield of 52.2% and hCA VI was purified, 75.3-fold with a specific activity of 213 EU/mg and overall yield of 17.8%<sup>22–25,29–35</sup>. SDS polyacrylamide gel electrophoresis was performed after the purification of these enzymes, and the electrophoretic pattern was photographed (Figure 1). Inhibitory effects of bisphenol, methoxy and bromophenol compounds on enzyme activities were tested under *in vitro* conditions;  $K_i$  values were calculated from Lineweaver–Burk graphs and are given in Table 1<sup>26</sup>.

We report here the first study on the inhibitory effects of bisphenol, methoxy and bromophenol compounds of type **2–9** on the esterase activity of hCA I, II and VI. Acetazolamide (AZA) **12** has been used as a negative control in our experiments and for comparison reasons<sup>13,30</sup>. The previous reports by Senturk et al.<sup>30–32</sup> investigated other anti-oxidant phenol derivatives (including salicylic acid and propofol) by using esterase assay. Data of Table 1 show the following regarding inhibition of hCA I, II and VI with phenols **2–9**, by an esterase assay<sup>23</sup>, with 4-NPA as substrate:

(i) Against the slow cytosolic isozyme hCA I, compounds **2–9** behave as weak inhibitors, with  $K_i$  values in the range of 18.086–892.109  $\mu\text{M}$ . 1,2,5-tribromo-3,4-dimethoxybenzene **9** was an ineffective hCA I inhibitor ( $K_i$  of 892.109  $\mu\text{M}$ ), similarly to the structurally related compound **4** ( $K_i$  537.311  $\mu\text{M}$ ). A second group of derivatives, including **6** and **7**, showed better inhibitory activity as compared with the previously mentioned methoxybenzenes, with  $K_i$  values of 25.341–32.145  $\mu\text{M}$ ,

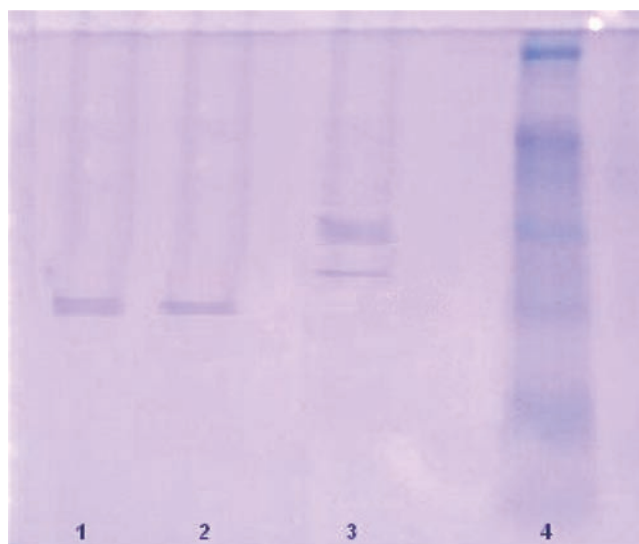
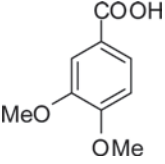
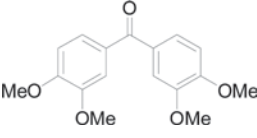
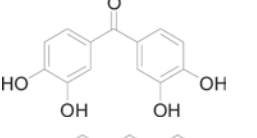
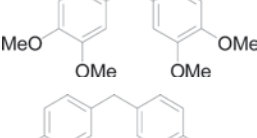
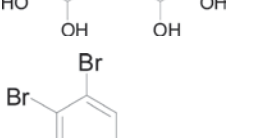
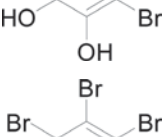
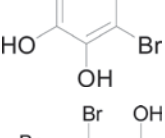
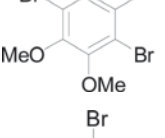
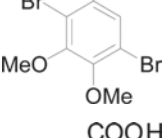
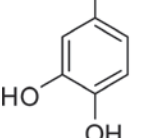
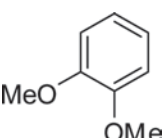


Figure 1. SDS polyacrylamide gel electrophoresis bands of carbonic anhydrase (CA) I, II, VI and standard proteins (Lane 1, CA I; Lane 2, CA II; Lane 3, CA VI; Lane 4, standards: rabbit phosphorylase B [97,400 Da], bovine albumin [66,000 Da], chicken ovalbumin [45,000 Da], bovine carbonic anhydrase [29,000 Da] and chicken egg white lysozyme [16,500 Da]).

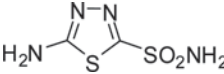
(Table 1). Therefore, the nature of the groups in *ortho*- and *ortho'*- to the phenolic OH moiety strongly influences hCA I inhibitory activity. It is also interesting to note that the biphenyl derivatives **3** and **5** were much better hCA I inhibitors as compared with the corresponding methoxy-bisphenols **2** and **4** from which they were prepared. AZA **12** is also a medium CA I with this assay and substrate against hCA I ( $K_i$  of 26.2  $\mu\text{M}$ )<sup>30</sup>. Kinetic investigations (Lineweaver–Burk plots, data not shown) indicate that similarly to sulfonamides and inorganic anions<sup>3–13,36–47</sup>, all the investigated compounds act as non-competitive inhibitors with 4-NPA as substrate, that is, they bind in different regions of the active site cavity as compared with the substrate. However, the binding site of 4-NPA itself is unknown,

Table 1. hCA I, II and VI inhibition data with compounds 1-11 and acetazolamide (12), by an esterase assay with 4-nitrophenylacetate as substrate<sup>23</sup>.

Structure	Compounds	K <sub>i</sub> (μM)		
		hCA I	hCA II	hCA VI
	<b>1</b>	6.83 <sup>a</sup>	6.18 <sup>a</sup>	—
	<b>2</b>	403.128	47.832	292.531
	<b>3</b>	18.025	1.437	11.423
	<b>4</b>	537.311	59.107	382.473
	<b>5</b>	18.356	5.636	13.692
	<b>6</b>	25.341	26.376	11.143
	<b>7</b>	32.145	29.820	29.138
	<b>8</b>	193.240	—	158.592
	<b>9</b>	892.109	—	919.182
	<b>10</b>	1.08 <sup>a</sup>	0.47 <sup>a</sup>	—
	<b>11</b>	10.4 <sup>a</sup>	0.50 <sup>a</sup>	—

(Continued)

Table 1. (Continued).

Structure	Compounds	K <sub>i</sub> (μM)		
		hCA I	hCA II	hCA VI
	<b>12</b>	26.2 <sup>a</sup>	0.37 <sup>a</sup>	0.34 <sup>b</sup>

Mean from at least three determinations. Errors in the range of 1%-3% of the reported value (data not shown).

hCA, human carbonic anhydrase.

<sup>a</sup>From ref. 30.

<sup>b</sup>From ref. 58.

but it is presumed to be in the same region as that of CO<sub>2</sub>, the physiological substrate of this enzyme<sup>29</sup>.

(ii) A better inhibitory activity has been observed with compounds **2-9** investigated in this study for the inhibition of the rapid cytosolic isozyme hCA II (Table 1). Thus, two derivatives, that is, **2** and **4**, showed moderate hCA II inhibitory activity with K<sub>i</sub>-s in the range of 47.832-59.107 μM (Table 1), whereas the remaining six derivatives were quite effective hCA II inhibitors, with K<sub>i</sub>-s in the range of 1.437-29.82 μM (Table 1). Structure-activity relationship is thus quite sharp for this small series of bisphenol, methoxy and bromophenol compounds: the 4,4'-dimethoxy-bis derivatives **2** and **4** are ineffective leads. This trend is maintained when different groups are present in the *para* or *meta* position to the phenol OH moiety, such as in compounds **6** and **7**. Without methoxy-group, same compounds **3** and **5** are also effective hCA II inhibitors. The best hCA II inhibitor in this series of derivatives was the bulky, bisphenol derivative **3**, which has a K<sub>i</sub> value of 1.437 μM. Derivative **3** showed better inhibitory profile compared with compounds **2**, **4-7**. It must be stressed that K<sub>i</sub>-s measured with the esterase method are always in the micromolar range because hCA I and II are weak esterases<sup>48-56</sup>.

(iii) 1,2,5-tribromo-3,4-dimethoxybenzene **9** and some of its congeners such as **2**, **4** and **8** are also weak inhibitors of the secreted isozyme hCA VI, with K<sub>i</sub>-s of 158.592-919.182 μM. However, again the 3,4,5,6-tetra-bromobenzene-1,2-diol **7** is medium potency inhibitors (K<sub>i</sub> of 29.138 μM), and derivatives **3**, **5** and **6** show a higher affinity for this isozyme, with inhibition constants in the range of 11.143-13.692 μM (Table 1).

In a recent study, it was reported that derivatives of salicylic acid<sup>13</sup>, a simple compound lacking the sulfonamide, sulfamate or related functional groups that are typically found in all known CA inhibitors, acts as a CA I inhibitor and could represent the starting point for a new class of inhibitors that may have advantages for patients with sulfonamide allergies (acetylsalicylic acid acts as a prodrug, being hydrolysed *in situ* with formation of a sodium salicylate derivative, which is the real enzyme inhibitor)<sup>51</sup>. However, Innocenti et al.<sup>52</sup> showed that compared with sulphonamides, thioxolone was inefficient for generating isozyme-selective inhibitors. Because it is found that except for hCA I, which was inhibited in the nanomolar range (K<sub>i</sub> of 91 nM), the remaining 12 mammalian CA isoforms (CA II-CA XV) were inhibited with

a very flat profile by this compound ( $K_i$ -s in the range of only 4.93–9.04  $\mu\text{M}$ ). In contrast to thioxolone, 3,5-dichloro-4-hydroxybenzenesulfonamide as well as the clinically used heterocyclic sulfonamide AZA showed  $K_i$ -s in the range of 58 nM–8.6  $\mu\text{M}$  and 2.5 nM–200  $\mu\text{M}$ , respectively, against the 13 investigated mammalian CAs. The sulfonamide zinc-binding group is thus superior to the thiol group (from the thioxolone hydrolysis product) for generating CA inhibitors with a diverse and sometimes isozyme-selective inhibition profile against the mammalian enzymes. However, it is critically important to explore further classes of potent CAIs to detect compounds with a different inhibition profile as compared with the sulfonamides and their bioisosteres and to find novel applications for the inhibitors of these widespread enzymes.

Bisphenol, methoxy and bromophenol compounds **2–9** influence the activity of CA isozymes due to the presence of the different functional groups (OH,  $\text{OCH}_3$  and Br) present in their aromatic scaffold. Thus, our findings indicate another class of possible CAIs of interest, in addition to the well-known sulfonamides/sulfamates/sulfamides, the phenols/biphenyl diphenols bearing bulky *ortho* moieties in their molecules. Some bisphenol, methoxy and bromophenol compounds investigated in this study showed effective hCA I and II inhibitory activity, in the low micromolar range, by the esterase method, which usually gives  $K_i$ -s an order of magnitude higher as compared with the  $\text{CO}_2$  hydrase assay<sup>51–58</sup>. These findings point out that substituted phenolic compounds may be used as leads for generating potent CAIs eventually targeting other isoforms, which

have not been assayed yet for their interactions with such agents.

### In silico studies

In this study, to better understand the binding mechanisms of studied molecules, fully flexible docking methodology for both receptor residues and docked ligands was used. Docking studies are performed using Glide XP-IFD algorithm, which was implemented with the Prime module under Schrodinger molecular modelling package<sup>26–28,56,57</sup>. The compounds **2–9** were docked at the binding site of the targets (hCA I and hCA II). Glide/IFD docking scores of docked inhibitors at hCA I and II targets and corresponding binding interactions were tabulated in Table 2 (supporting information).

Table 2. Docking binding scores of the molecules.

Compounds	Docking Binding Score (kcal/mol)	
	hCA-I (kcal/mol)	hCA-II (kcal/mol)
1 <sup>a</sup>	–10.5	–6.00
2	–6.26	–4.90
3	–4.61	–6.52
4	–5.66	–7.09
5	–3.91	–4.68
6	–3.72	–4.51
7	–2.28	–4.21
8	–2.07	–5.00
9	–4.53	–4.48
10 <sup>a</sup>	–10.5	–7.20
11 <sup>a</sup>	–2.60	–5.30
12 <sup>a</sup>	–3.10	–4.10

<sup>a</sup>From ref. 26.

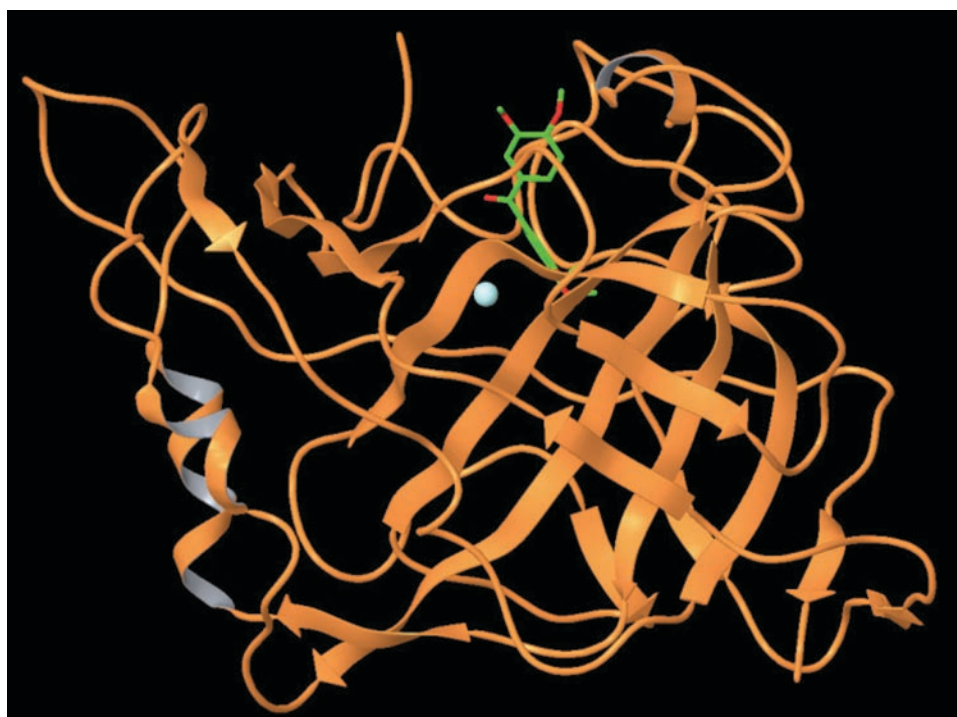


Figure 2. Docking of compound 2 within the hCA II active site.

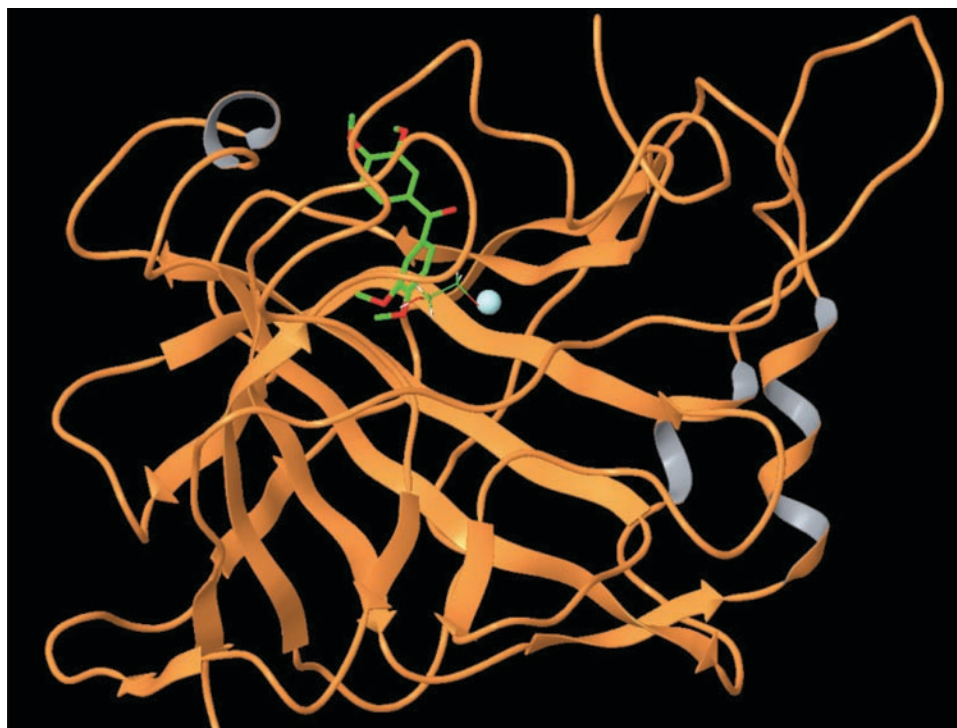


Figure 3. Binding mode of adduct and compound 2 at hCA-I.

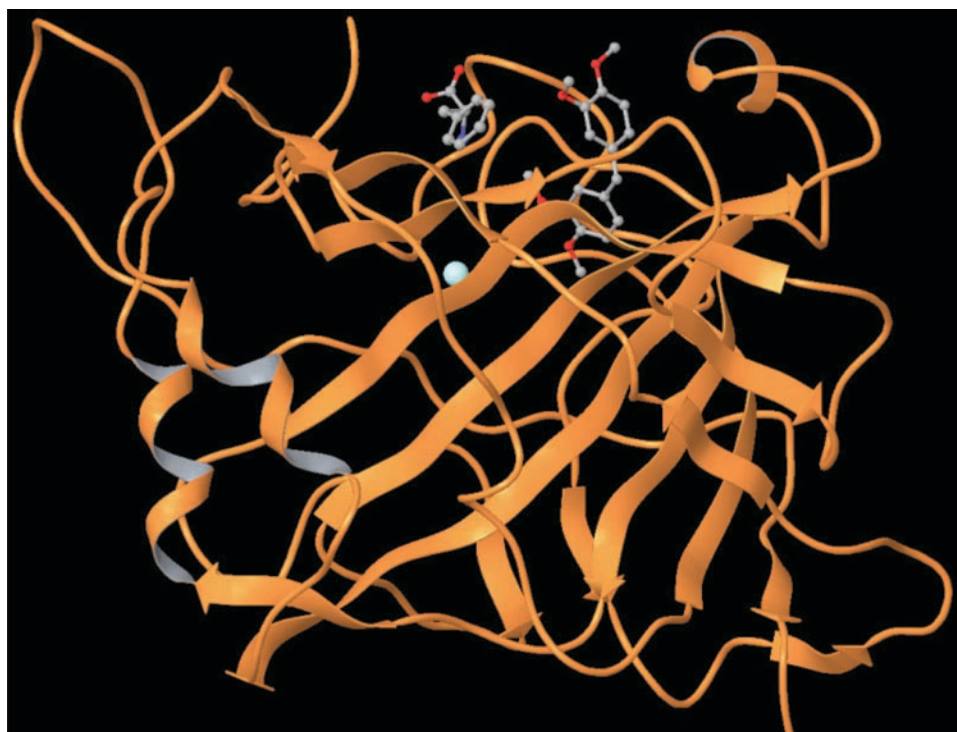


Figure 4. Docking of compound 2 within the hCA I active site.

## Conclusions

Together with *in vitro* studies, we also performed molecular docking studies of compounds 2–9 within the hCA I and II active sites. The hCA I and II adducts for

which the X-ray crystal structures have been reported in complexes with activators or inhibitors were used in the docking. AZA 12 together with compounds 2–9 have been docked to the active sites of hCA I and II.

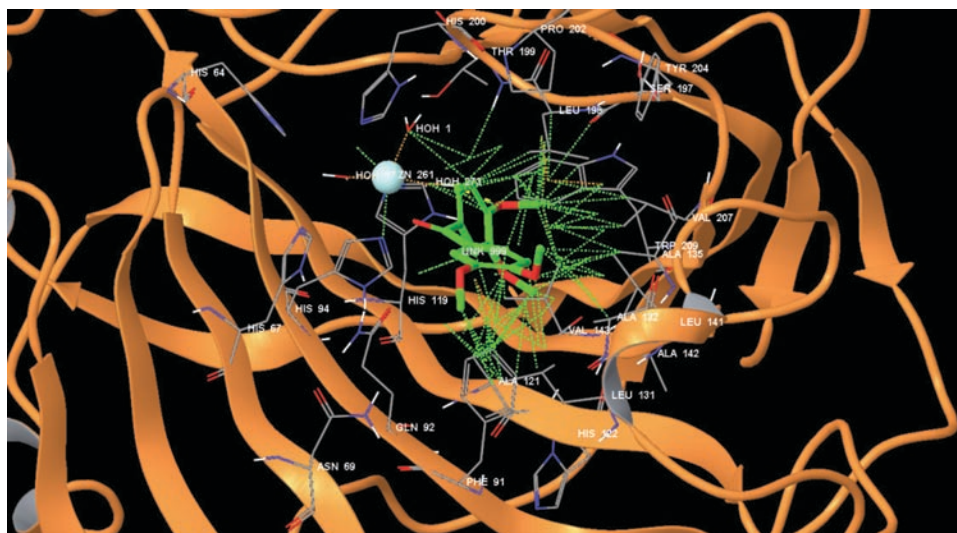


Figure 5. Docking of compound 4 within the hCA II active site.

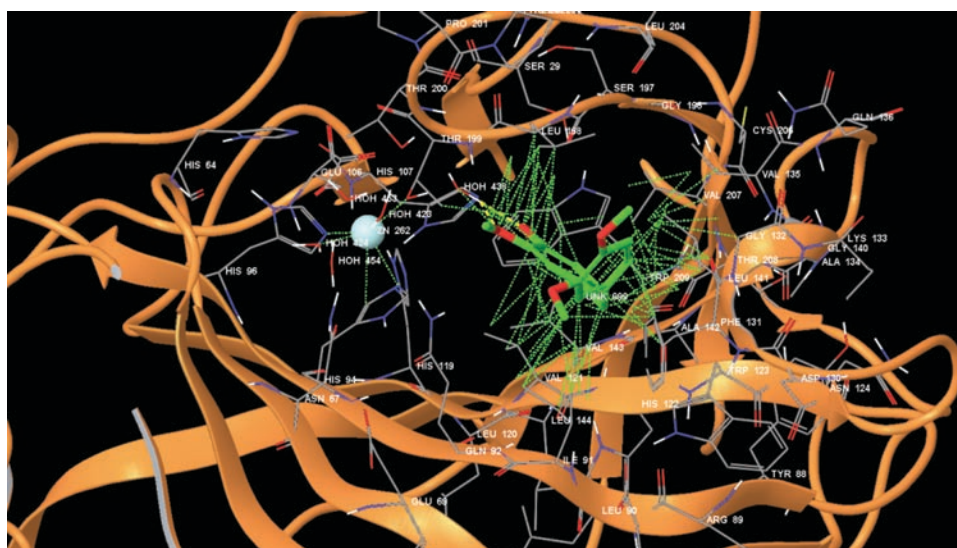


Figure 6. Binding mode of adduct and compound 4 at hCA-II.

The results of the docking experiments are presented in Table 2 (supporting information) for hCA I and II and are shown in some details in Figures 2–4. Such data may be important for designing compounds with enhanced affinity and eventually more selectivity for various CA isoforms.

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### Declaration of interest

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