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Kinetic and docking studies of phenol-based inhibitors of carbonic anhydrase isoforms I, II, IX and XII evidence a new binding mode within the enzyme active site

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ABSTRACT

Carbonic anhydrases (CAs, EC 4.2.1.1) are inhibited by sulfonamides, inorganic anions, phenols, coumarins (acting as prodrugs) and polyamines. A novel class of CA inhibitors (CAIs), interacting with the CA isozymes I, II (cytosolic) and IX, XII (transmembrane, tumor-associated) in a different manner, is reported here. Kinetic measurements allowed us to identify hydroxy-/methoxy-substituted benzoic acids as well as di-/tri-methoxy benzenes as submicromolar–low micromolar inhibitors of the four CA isozymes. Molecular docking studies of a set of such inhibitors within CA I and II allowed us to understand the inhibition mechanism. This new class of inhibitors binds differently compared to all other classes of inhibitors known to date: they were found between the phenol-binding site and the coumarin-binding site, filling thus the middle of the enzyme cavity. They exploit different interactions with amino acid residues and water molecules from the CA active site compared to other classes of inhibitors, offering the possibility to design CAIs with an interesting inhibition profile compared to the clinically used sulfonamides/sulfamates.

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1. Introduction

An increasing number of different chemotypes than the sulfonamides[1–3] and their isosteres were reported in the last period to act as carbonic anhydrase (CAs, EC 4.2.1.1) inhibitors (CAIs).[1–13] Among them are the phenols[8,9] and thiols,[2,8] the coumarins (acting as prodrugs),[10a,b] the antiepileptic drug lacosamide,[10c] the polyamines[11] and some fullerenes derivatives.[13] Indeed, CAs are zinc proteins which catalyze the hydration of carbon dioxide to bicarbonate and protons,[1–3] and are present in high amounts in organisms all over the phylogenetic tree. The metal ion is critical for catalysis, as the apoenzyme is devoid of any catalytic activity.[1–5] In mammals, CAs are present in many tissues, under the form of 16 different isoforms.[1] The chemical species involved in the CA-catalyzed processes CO_2, bicarbonate, and protons, are essential molecules/ions in many physiologic processes in all organisms. Thus, CAs play crucial roles in processes connected with respiration and transport of CO_2/bicarbonate, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes thoroughly studied in vertebrates.[1–13]

The CAIs belong to four main classes: (i) sulfonamides (and their isosteres, such as sulfamates, sulfamides and similar derivatives) and metal complexing anions, which coordinate to the Zn(II) ion from the enzyme active site in tetrahedral or trigonal bipyramidal geometries of the metal ion (Fig. 1A and B),[1–4] (ii) phenols (such as the simple phenol C_6H_5OH),[8,9] which bind to the zinc-coordinated water molecule/hydroxide ion from the active site, through a network of two hydrogen bonds (Fig. 1C),[8] (iii) the polyamines,[11] such as spermine, spermidine and congeners, which bind rather similar but not identical to phenols, that is, by anchoring to the water molecule/hydroxide ion coordinated to Zn(II), Figure 1D and (iv) the recently reported class of effective CAIs, the coumarins

Abbreviations: CA, carbonic anhydrase; hCA, human CA; CAI, CA inhibitor; SAR, structure–activity relationship.

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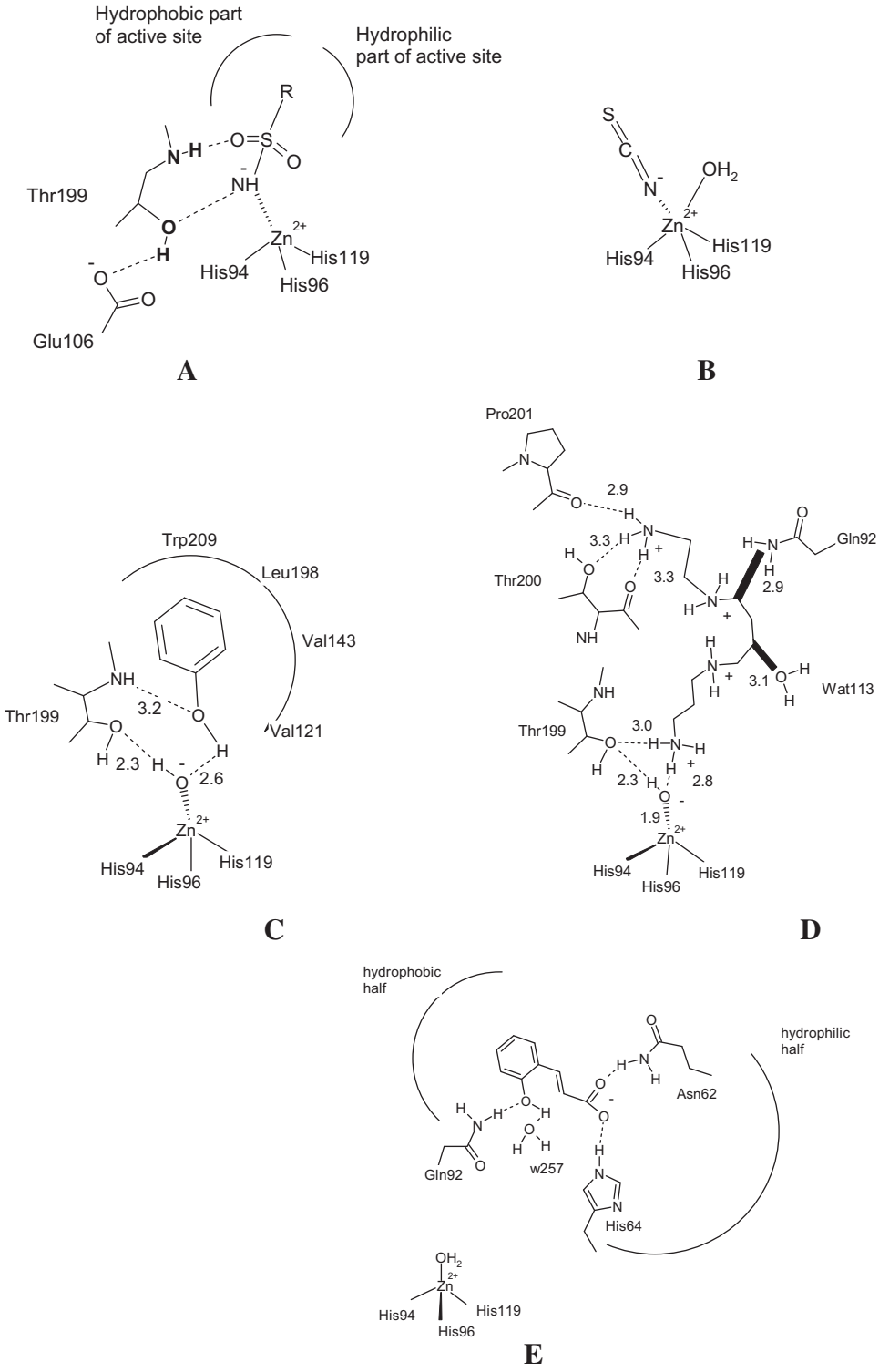


Figure 1. CA inhibition with: zinc binders such as sulfonamides (A) and inorganic anions (B); compounds anchoring to the zinc-bound water/hydroxide ion, such as phenol (C) and spermine (D), and compounds occluding the entrance to the CA active site cavity, exemplified by the hydrolyzed coumarin, *trans*-2-hydroxycinnamic acid (E). Figures represent distances (in Å), as determined by X-ray crystallographic techniques.[8–11] Hydrogen bonds are represented as dashed lines. All these binding modes have been proven by means of X-ray crystallography on enzyme-inhibitor adducts.[1,8–11]

and thiocoumarins,[10] which have an inhibition mechanism not dependent of Zn(II), and bind (in hydrolyzed form) in the same active site region as the activators, occluding the entrance to the active site (Fig. 1E).[10a,b] Fullerenes[13] and lacosamide[10c] bind similarly to the hydrolyzed coumarins[10] mentioned above, that is, by occluding the entrance to the enzyme active site cavity.

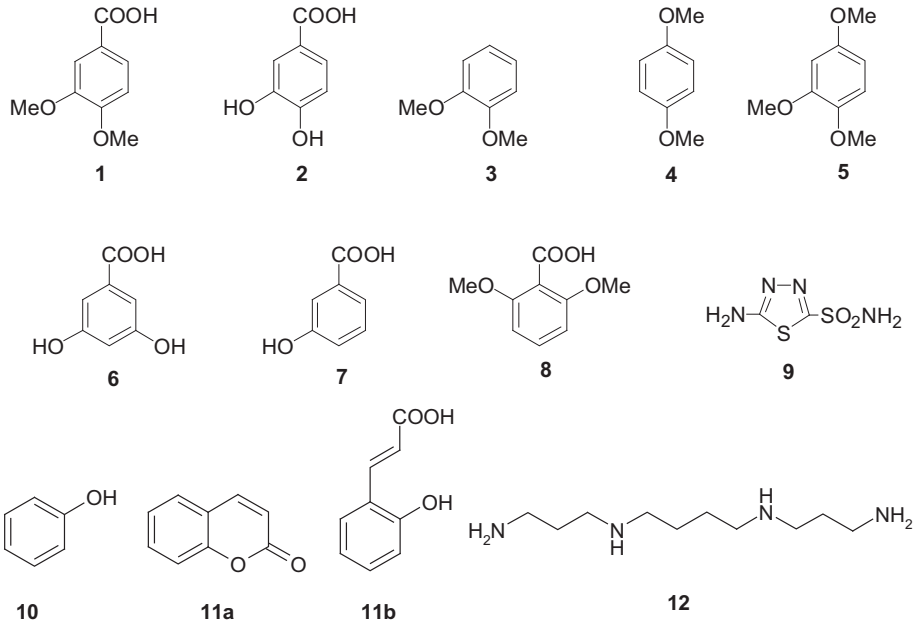
Here we report another class of CA modulators, discovered by considering phenols as leads. Our groups investigated ultimately[9,14–17] a rather large number of phenols, both simple such compounds[9] as well as natural product based derivatives,[14] possessing a large variety of structures. Although we have identified many interesting leads, with submicromolar–low nanomolar

activity against some physiologically relevant CA isoforms (e.g., CA VA and VB, mitochondrial isoforms involved in biosynthetic reactions)[14d] the main drawback of this class of CAIs is related to the very restricted knowledge of their precise binding mode to the enzyme, as only one such adduct has been characterized so far by means of X-ray crystallography, the CA II—C_6H_5OH adduct.[8] The main problem encountered with this class of CAIs is related either to the low solubility in aqueous media for the potent, structurally complex such compounds, or the low affinity to the enzyme for the simple, water soluble derivatives, which precluded us in obtaining good quality crystals of adducts of these inhibitors with various CA isoforms. Thus, here we investigated a number of such derivatives by kinetic measurements and in silico[18,19] techniques, allowing us to understand in some detail the inhibition mechanism of phenol-based compounds.

2. Results and discussion

2.1. Chemistry and CA inhibition studies

The phenol-based compounds **1**–**8** as well as the sulfonamide **9** have been included in the study. Sulfonamide **9** (5-amino-1,3,4-thiadiazole-2-sulfonamide) was used as a standard, as the sulfonamides constitute the best studied class of CAIs, and they also possess clinical applications.[1] In this particular case, the hCA II—**9** adduct has been thoroughly characterized by means of kinetic and X-ray crystallographic studies (PDB code 2HNC)[20] and this could be used as a standard for the enzyme inhibition studies with compounds **1**–**8**. Thus, sulfonamide **9**, similar to **1**–**8**, have been submitted to the in silico procedures reported subsequently in this paper.



There are several reasons why we concentrated on the phenol based derivatives **1**–**8**, which include 3,4-dimethoxybenzoic acid **1**, 3,4-dihydroxybenzoic acid **2**, 1,2- and 1,4-dimethoxybenzene (**3** and **4**), 1,2,4-trimethoxybenzene **5**, 3,5-dihydroxybenzoic acid **6**, 3-hydroxybenzoic acid **7** and 2,6-dimethoxybenzoic acid **8**, as possible CAIs:

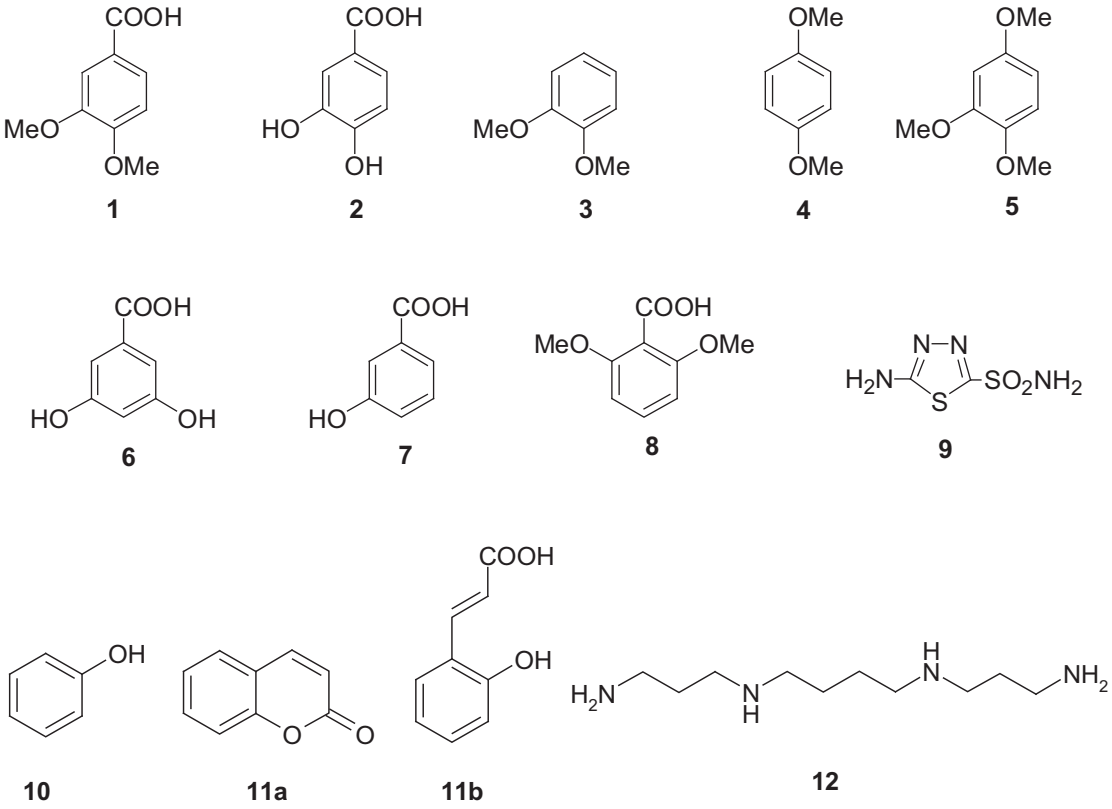
(i) Several phenols possessing a variety of structures, including natural product phenolic acids (4-hydroxy-benzoic acid; caffeic acid, *p*-coumaric acid; ferulic acid, gallic acid, and syringylic acid),[14b] showed promising inhibitory action against several isoforms, and selectivity for some of them. Compounds **1**–**8** investigated here bear structural resemblance with most of these derivatives, but in order to understand the contribution of the carboxyl, phenolic OH, and methoxy moieties to the CA inhibitory properties, the set investigated here also includes compounds without carboxylic acid moieties, such as the dimethoxy-benzenes **3** and **4**, as well as 1,2,4-trimethoxybenzene **5**, in addition to the mono-/di-hydroxy benzoic acids and their methoxy derivatives. It should be mentioned that ethers have never been investigated before as CAIs, since it is considered that they do not bear any moiety normally associated with CA inhibition in their molecule.

(ii) The simple phenol **10** binds in a rather unique mode to the enzyme,[8] as shown in Figure 1C, whereas the phenol with a more complex structure **11b**, formed by the enzyme active site mediated hydrolysis of coumarin **11a**, binds in an even more intriguing way,[10] occluding the entrance of the active site cavity, as shown in Figure 1E. However, between these two binding sites there are almost 5 Å of space in which no inhibitors have been detected so far, except for the very recently reported class of polyamines.[11] Indeed, spermine **12** was recently crystallized in adduct with hCA II, and its binding (shown schematically in Fig. 1D) revealed a completely new inhibition mechanism, which resemble that of phenol, but the scaffold of the polyamine extends towards the more external part of the active site (whereas phenol is buried rather deep in the active site). Thus, we have assayed compounds **1**–**12** as inhibitors of four physiologically relevant CA isoforms, that is, the cytosolic, widespread hCA I and II, and the transmembrane, tumor-associated CA IX and XII (Table 1).

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

(i) Against the slow and highly abundant isoform hCA I,[1,2] the phenol-based compounds **1**–**8** showed inhibition constants in the range of 0.55–10.4 μM, which are in the same range as those of the lead molecule, phenol **10** (K_I of 10.2 μM) or the hydrolyzed coumarin **11b** (K_I of 3.1 μM). Furthermore, the sulphonamide **9** showed an inhibition constant of 8.60 μM, in the same range as phenols **1**–**8** and **10**, whereas spermine **12** is a much weaker inhibitor of this isoform, with a K_I of 231 μM. One important observation of this study is that the 3,5-dihydroxy-benzoic acid **6** is a much more effective CAI compared to all other known classes of

Table 1
Inhibition data with compounds **1**–**9**, phenol **10**, the hydrolyzed coumarin **11a** (giving the *trans*-2-hydroxycinnamic acid **11b**) and spermine **12**, against CA isozymes I, II, IX and XII, by a stopped-flow technique monitoring the CO_2 hydration reaction



Compound	K_I* (μM)			
	hCA I	hCA II	hCA IX	hCA XII
1	6.83	6.18	8.25	6.70
2	1.08	0.47	4.45	4.09
3	10.4	0.50	8.63	8.36
4	8.21	3.35	4.15	4.02
5	5.96	1.43	4.06	7.80
6	0.55	0.51	4.41	3.67
7	2.37	0.60	4.53	3.53
8	9.67	1.02	9.07	8.23
9	8.60	0.060	0.041	0.033
10	10.2	5.50	8.80	9.20
11b	3.10	9.20	>1000	>1000
12	231	84	13.3	27.6

*Mean from 3 different assays. Errors were in the range of ± 5 % of the reported values.

inhibitors (K_I of 0.55 μM). Its position isomer **2** is the second best hCA I inhibitor in this series, whereas the compound with only one OH moiety, **7**, has an even lower inhibitory activity, but still in the low micromolar range (K_I of 1.08 μM for **2** and of 2.37 μM for **7**, respectively). The least effective inhibitor is 1,2-dimethoxy-benzene **3** (K_I of 10.4 μM) which is however rather similar with that of phenol **10**. Thus, a second conclusion is that phenolic ethers as those investigated here may act as CAIs (this class of compounds was not investigated earlier as CAIs). The position isomer of compounds **3** and **4**, had an increased affinity for the enzyme, which was further increased for the trimethoxy compound **5** (K_I of 5.96 μM). The remaining compounds incorporating both carboxyl, hydroxyl-/methoxy moieties, such as **1, 2**, and **8**, showed inhibition properties intermediate between those of the potent inhibitors (3,5-dihydroxy-benzoic acid **6**) and the weak inhibitors (dimethoxy-benzenes **3** and **4**) investigated here.

(ii) The physiologically dominant isoform hCA II[1] was also inhibited by the phenol-based compounds **1–8**, with inhibition constants in the range of 0.47–6.18 μM (Table 1). SAR is however completely different compared to the inhibition of hCA I discussed above. Thus, unexpectedly, the best hCA II inhibitors were compounds **2**, **3, 6** and **7**, which sowed K_I's in the range of 0.47–0.60 μM. Two other derivatives, **5** and **8**, had K_I of 1.02–1.43 μM, whereas the weakest inhibitors were **1** and **3** (K_I's of 3.35–6.18 μM). The two phenols for which the X-ray crystal structure is available (in complex with hCA II) possess the same range of inhibitory activity, with a K_I of 5.50 μM for **10**, and of 9.20 μM for **11b**. Spermine **12** is a very weak hCA II inhibitor (a K_I of 84 μM) whereas the sulfonamide **9** a very strong one (K_I of 60 nM). Thus, against this isoform it is possible to generate submicromolar, medium potency phenol-based inhibitors (e.g., **2, 3, 6–8**) but their efficacy is not as high as that of the sulfonamides. However the fact that the very simple compounds **1–8** show such a good, low micromolar or submicromolar level of inhibition is unexpected and remarkable, since they do not contain moieties normally associated with potent CA inhibitory properties.

(iii) The tumor-associated[3,12c] isoform hCA IX was also inhibited by compounds **1–8**, with K_I's in the range of 4.06–9.07 μM. Compared to the other two isoforms discussed above, this is more flat SAR, with less variation of the inhibitory power. Thus, except **1, 3** and **8**, which were the worst inhibitors (K_I's of 8.25–9.07 μM), the remaining derivatives had this parameter in the range of only 4.06–4.53 μM (Table 1). This is probably due to the fact that the hCA IX active site is considerably larger compared to that of hCA I and II, as recently reported by X-ray crystallography by one of our groups.[12c] Thus, hCA IX is moderately inhibited by these phenol-based compounds **1–8**, whereas sulfonamides (such as **9**) act as much more effective inhibitors (K_I of 41 nM). Phenol **10** and spermine **12** are weaker CAIs against hCA IX compared to **1–8**, with K_I's of 8.8–13.3 μM, whereas the coumarin **11a** (the prodrug of the phenolic compound **11b**) is not at all inhibitory (K_I >1000 μM). However, it should be mentioned that coumarins with a different substitution pattern were recently shown to act as low nanomolar inhibitors of CA IX, even if the lead used (just compound **11a** discussed here) was ineffective as an inhibitor of this isoform. That is one of the reasons why we appreciate that the phenolic compounds (e.g., ethers, hydroxyl acids, etc.) investigated here might be useful leads for generating potent inhibitors targeting this cancer-associated enzyme.[21] The second tumor-associated isoform, hCA XII; showed an inhibition profile with compounds **1–8** quite similar to that of hCA IX. Thus, these derivatives inhibited hCA XII wit inhibition constants in the range of 3.53–8.36 μM, with the same flat SAR noted above for hCA IX. Actually SAR for the two isoforms were rather similar, probably also due to the fact that active sites of the two transmembrane isoforms (CA IX and XII) are more similar with each other[12c] compared to those of the cytosolic ones CA I and II discussed above. Again sulfonamide **9** was the best hCA XII inhibitor (K_I of 33 nM), whereas phenol **10** and spermine **12** were less effective CAIs (K_I's of 9.2–27.6 μM). The coumarin **11a** was ineffective as an hCA XII inhibitor.

2.2. In silico studies

Although compounds **1–8** are low molecular inhibitors, we were unsuccessful in crystallizing them in complex with any CA isoforms (we have tried to obtain adducts with hCA I, II and IX, proteins rather easily crystalizable).[2,12c,20–22] This is probably due to the fact that some of these compounds are highly lipophilic (e.g., **3, 4** and **5**) and completely insoluble in the aqueous buffer at pH 7.5. For this reason, in this study, fully flexible docking methodology for both receptor residues and docked ligands was used by Glide XP (extra precision)–Induced Fit Docking (IFD) algorithm which was implemented with the Prime module under Schrodinger molecular modeling package.[18,19] The compounds **1–9** 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 hCA-II targets and corresponding binding interactions were tabulated at Tables 2 and 3, respectively.

We have performed docking studies of compounds **1–9** within the hCA I and II active sites, by using as templates hCA I and II adducts for which the X-ray crystal structures have been reported in complexes with activators (e.g., hCA I—L-His, PDB code 2FW4[17]) or inhibitors (the hCA II—phenol adduct;[8] the hCA II—**9** adduct, PDB code 3HNC) and the hCA II—**11b** adducts (PDB code 3F8E).[10b] Sulfonamide **9** together with compounds **1–8** have been docked to the active sites of hCA I and II. The results of the docking experiments are presented in Table 2 for hCA I, and Table 3 for hCA II, respectively, and are shown in some details in Figures 2–5.

As seen from data of Tables 2 and 3, there are many amino acid residues/water molecules participating in hydrogen bonds and close van der Waals, <4 Å contacts with the inhibitors **1–9** when bound to the enzyme active sites, for both hCA I and II. Most of these amino acids include Thr199 (one of the gate keeper residues of this enzyme)[1,2] as well as the residues lining the CO_2 binding pocket, that is, Val121, Val143, Leu198, Val207 and Trp209.[23] None

Table 2
Molecular docking binding scores and binding interactions of compounds **1–9** within the hCA I active site. Residues/water molecules participating in hydrogen bonds and close van der Waals contacts (<4 Å) with the inhibitors are shown

Compound number	Docking Score (kcal/mol)	H-bonds	Close van der Waals contacts
1	−10.5	Thr199	Leu198, Val143, Val207, Leu141, Ala121, Phe91, Gln92, His94, H_2O 336, Trp209
2	−10.5	H_2O 336, Thr199	Phe91, Leu141, H_2O 336, Leu198, Ala121, His94, H_2O 32, Trp209, His200, His119
3	−2.6	H_2O 32	His200, H_2O 336, H_2O 215, Val143, Val207, Leu198, Trp209, Leu198, Thr199,
4	−4.2	—	His94, H_2O 336, His119, H_2O 346, Phe91, Leu141, Val143, Val207, Leu198, Trp209, His200, Thr199
5	−3.8	Thr199	Phe91, Leu141, Ala121, H_2O 336, Leu198, H_2O 32, His200, Val143, Trp209,
6	−7.7	Thr199, Gln92	Phe91, Leu198, Ala121, His119, His94
7	−9.2	Thr199, His119	Trp209, Leu198, Ala121, H_2O 336, His94, His119, Gln92
8	−8.3	H_2O 336	Val 207, H_2O 336, Leu198, Leu141, Val143, Trp209, Phe191, Gln 92, His94, H_2O 215, H_2O 32, His200, His119
9	−3.1	Thr199	Leu141, Phe91, Ala121, Val143, Val207, Trp209, Leu198, H_2O 32, His200, His94, His119

Table 3
Molecular docking binding scores and binding interactions of compounds **1–9** within the hCA II active site. Residues/water molecules participating in hydrogen bonds and close van der Waals contacts (<4 Å) with the inhibitors are shown

Compound number	Docking score (kcal/mol)	H-Bonds	Close van der Waals Contacts
1	−6.0	H_2O 423, H_2O 454, H_2O 453, H_2O 438	H_2O 423, H_2O 454, H_2O 453, H_2O 438, Val121, Leu198, Leu141, Ile91, Trp123, Phe131
2	−7.2	H_2O 454, H_2O 438, Thr 199, Gln 92	H_2O 454, H_2O 438, Thr 199, Gln 92, Ile 91, Leu 141, His 122, Leu 198, Val 121, H_2O 423
3	−5.3	H_2O 423, H_2O 438	H_2O 423, H_2O 438, H_2O 454, Trp209, Thr199, Val143, Val207, Leu141, Ser197, Ala142, Val121, Leu198
4	−4.3	—	H_2O 453, Pro207, Tyr7, His64, Gly6, Gly63, Phe131, Trp5
5	−5.0	Thr199, H_2O 438	H_2O 438, Thr199, Ala142, His122, Ile91, H_2O 454, H_2O 423, Val207, Val143, Trp209, Val121, Leu198, Leu141
6	−6.4	H_2O 424, H_2O 453, Thr200	H_2O 424, H_2O 453, Thr200, Tyr7, Trp16, Asn244, His64, Trp5
7	−8.5	H_2O 423	H_2O 423, Leu198, H_2O 438, H_2O 454, Val121, Phe 131, Leu141, Phe131, Val135
8	−8.4	H_2O 453, Asn62, Thr200, Trp5	Trp5, Thr200, H_2O 453, Asn62, H_2O 423, H_2O 424, H_2O454, Leu203, His64
9	−4.1	Thr199, Thr200, H_2O 438, H_2O 423	H_2O 438, H_2O 423, H_2O 454, Leu198, Gln92, Phe131

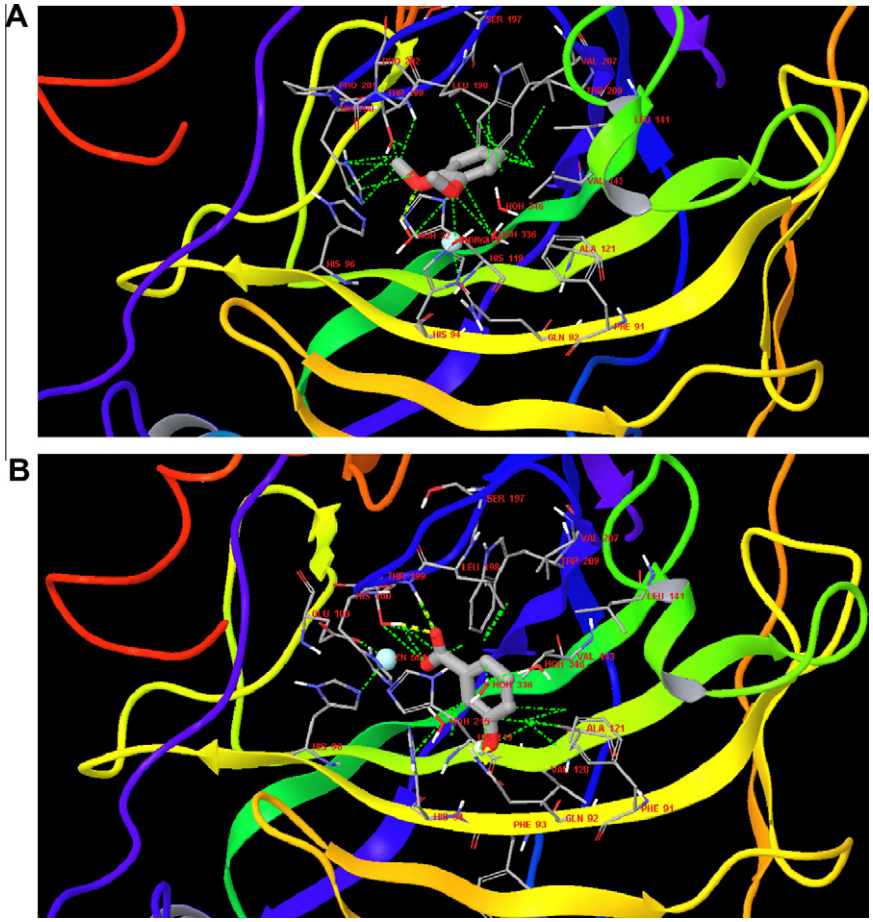


Figure 2. Docking of compounds **3** (A) and **7** (B) within the hCA I active site. Amino acid residues and water molecules with which the inhibitors interact are evidenced. The Zn(II) ion (bluish sphere) and protein backbone (ribbon representations) are also shown. Hydrogen bonds and Van der Waals interactions were represented with yellow and green colored dashed lines. For details of the interaction see also Table 2.

Figure 3. Superposition of the adducts of compounds **3**, **7** and **11b** (hydrolyzed coumarin) within the hCA I active site.

of these inhibitors except the sulfonamide **9** was found coordinated to the Zn(II) ion from the two CA active sites. This is a very important result which points to the fact that the phenol-based derivatives **1–8** may share the same inhibition mechanism as the simple phenol **10** or the hydrolyzed coumarins, that is, the phenol derivative **11b**, both of which do not interact with the metal ion from the enzyme active site and bind either within the hydrophobic, CO_2 binding pocket (phenol **10**, see also Fig. 1C) or towards the exit of the active site, occluding it (phenol **11b**, see Fig. 1E). We thus concentrated on two rather different derivatives, the highly lipophilic dimethoxy-benzene **3** and the very hydrophilic 3-hydroxy-benzoic acid **7**, for which we generated detailed binding modes, based on the docking results (Figs. 2–5).

Indeed, as seen from Figures 2 and 3, the binding of the two derivatives within the hCA I active site is done without interaction with the zinc ion, by means of an extended network of hydrogen bonds and van der Waals interactions involving His200, water 336, water 215, Val143, Val207, Leu198, Trp209, Leu198, Thr199 (for the lipophilic compound **3**) and Trp209, Leu198, Ala121, water336, His94, His119, Gln92 (for **7**), respectively. It may be observed (Fig. 3) that the more compact, phenol-like veratrole **3** binds deeper within the active site compared to 3-hydroxy-benzoic acid **7**, which binds more towards the coumarin-binding site. Thus, the new inhibitors reported here exploit binding pockets which are found between the phenol-binding site and the coumarin binding sites in the hCA I cavity, a binding mode never evidenced earlier for other classes of the CAIs.

The binding to the hCA II active site of the two compounds **3** and **7**, is rather similar to what discussed above for hCA I (Figs. 4 and 5). Thus, **3** binds deep within the hCA II active site, but does not interact with the metal ion, similar to phenol **10**[8] (Figs. 4A and 5A). Its two methoxy groups are anchored within the active site by means of two hydrogen bonds with two water molecules (water 423, and water 438) and further stabilized by van der Waals interaction with water 454, Trp209, Thr199, Val143, Val207, Leu141, Ser197, Ala142, Val121, Leu198 (basically all the amino

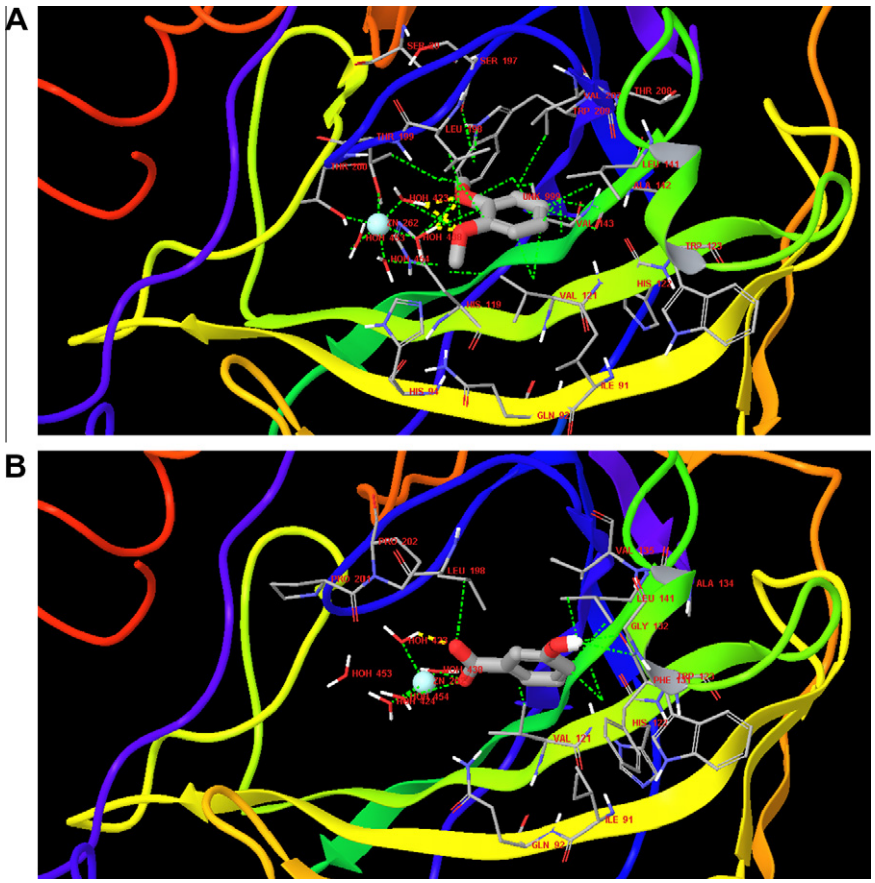


Figure 4. Docking of compounds **3** (A) and **7** (B) within the hCA II active site. Amino acid residues and water molecules with which the inhibitors interact are evidenced. The Zn(II) ion (bluish sphere in A and orange sphere in B) and protein backbone (ribbon representations) are also shown. Hydrogen bonds and Van der Waals interactions were represented with yellow and green colored dashed lines. For details of the interaction see also Table 3.

acids aligning the CO_2 binding site).[23] In the case of **7**, the binding site is situated towards the exit of the cavity, in the same region where hydrolyzed coumarins bind (Figs. 4B and 5B), as observed clearly when the adduct was superposed on the hCA II—11b adduct crystallographic structre.[10b] Compound **7** is also anchored within the active site by means of one hydrogen bond with a water molecule (water 423), and makes favourable van der Waals interactions with Leu198, water 438, water 454, Val121, Phe 131, Leu141, Phe131, Val135. It is interesting to note that even if they make so diverse contacts when bound to the hCA II active site (and they are found in very diverse regions of the cavity), the two inhibitors **3** and **7** possess quite similar affinity for this isoforms (K_I's of 0.50–0.60 μM, Table 1). As for hCA I, also in the case of hCA II these derivatives bind between the phenol-[8] and coumarin-[10] binding sites, exploiting thus a region of the cavity where no other CAIs have been found to date. Such data may be thus important for designing compounds with enhanced affinity and eventually more selectivity for various CA isoforms, since exactly the regions towards the exit of the cavity are those with the highest variability in amino acid residues.[7,22]

3. Conclusions

A novel class of efficient CAIs, interacting with the CA isozymes I, II (cytosolic) and IX, XII (transmembrane, tumor-associated) in a different manner compared to sulfonamides, sulfamates and other classes of inhibitors, is reported in this paper. Kinetic measurements allowed us to identify hydroxy-/methoxy-substituted benzoic acids as well as di-/tri-methoxy benzenes as submicromolar–low micromolar inhibitors of the four CA isozymes. Docking studies of a set of such inhibitors within CA I and II allowed us to understand the inhibition mechanism. This new class of inhibitors binds differently of all other CAIs known to date, being found between the phenol-binding site and the coumarin-binding site within the enzyme cavity. They exploit different interactions with amino acid residues and water molecules from the CA active site compared to other classes of inhibitors, offering the possibility to design compounds with a better inhibition profile compared to the clinically used sulfonamides/sulfamates.

4. Experimental protocols

4.1. Chemistry

Compounds **1–12** investigated here are commercially available derivatives from Sigma-Aldrich, Milan (Italy) and were used without further purification.

4.2. CA inhibition

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 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining a constant ionic strength), following the initial rates of 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

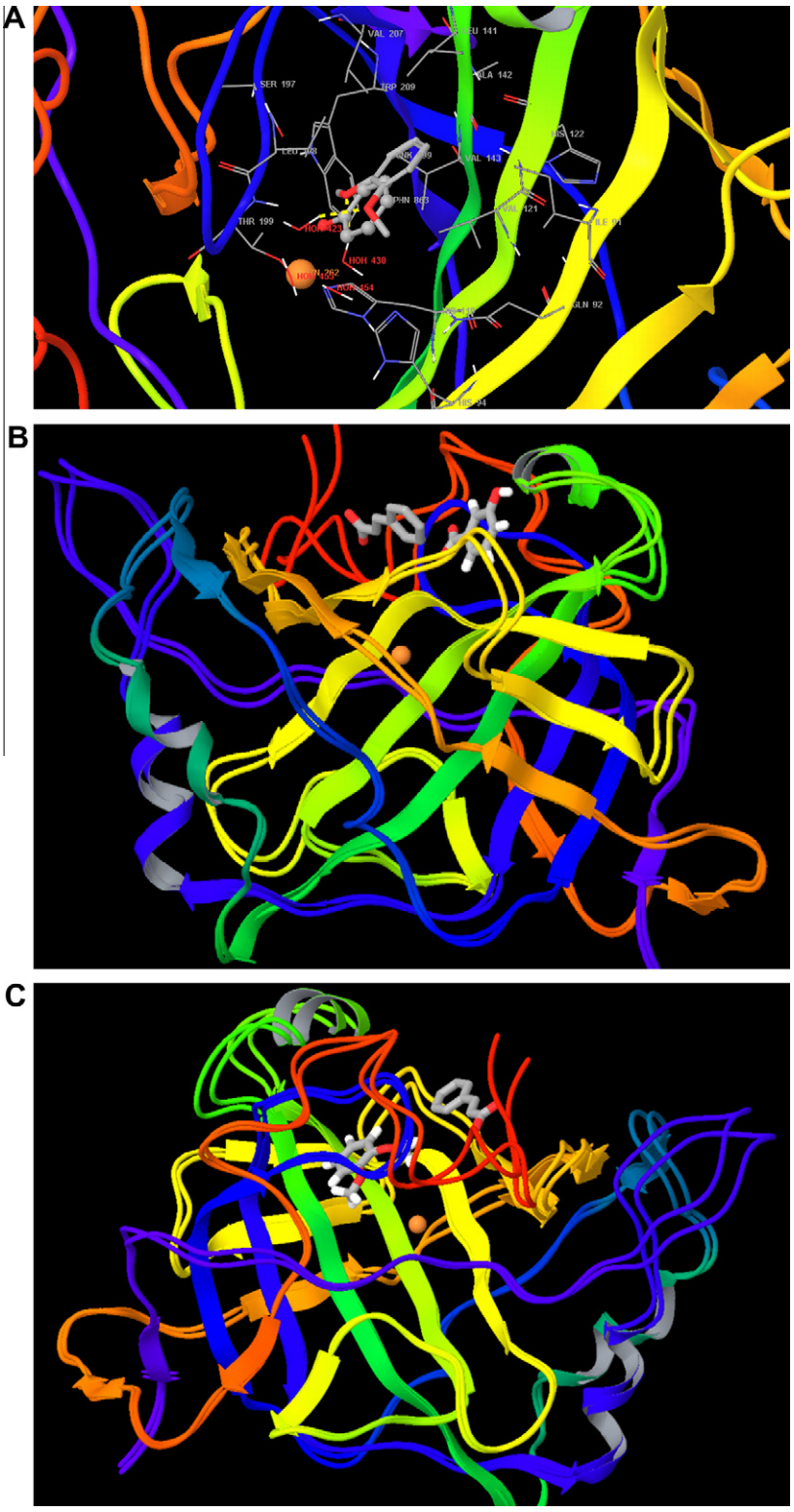


Figure 5. Superposition of the (A) hCA II—phenol (X-ray crystal structure)[8] with hCA II—**3** adducts; (B) hCA II—**11b** adduct (PDB file 3F8E[10b]) with the docked hCA II—**7** adducts; (C) hCA II—**11b** adduct (PDB file 3F8E[10b]) with the docked hCA II—**3** adducts. The Zn(II) ion is the central orange sphere whereas the protein backbone is represented as ribbon and the various inhibitors molecules as CPK stick models.

kinetic parameters and inhibition constants (5 different substrate concentrations have been used). 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 (0.1 mM) were prepared in distilled–deionized water and dilutions up to 0.01 nM were done thereafter with distilled–deionized water. Experiments were done using six different inhibitor concentrations, varying from 100 μM to 0.1 nM. Inhibitor and enzyme solutions were preincubated

together for 15 min to 24 h at room temperature (15 min) or 4 °C (all other incubation times) prior to assay, in order to allow for the formation of the E-I complex or for the eventual active site mediated hydrolysis of the inhibitor. Data reported in Table 1 shows the inhibition after 15 min incubation, as there were no differences of inhibitory power when the enzyme and inhibitors were kept for longer periods in incubation.[10] The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[10,14] and represent the mean from at least three different determinations. The four CA isozymes used in the experiments were recombinant ones, obtained as reported earlier.[12c,16]

4.3. In silico docking studies

Crystal structures of CAs in complex with inhibitors/activators: phenol **10**—CA II adduct, not available in PDB, file available through courtesy of Prof. D.W. Christianson, Univ of Philadelphia, and pdb files 3F8E[10b] (hCA II in complex with the hydrolyzed coumarin **11b**)[10b] and 2FW4[17] (CA I in complex with L-His) were used for the molecular docking calculations of compounds **1–9** within the active sites of the two CA isoforms (hCA I and II). Explicit water molecules and metal ions from the X-ray structures were kept for all the calculations. Before the docking simulations, the complexes were submitted to the protein preparation module of Schrodinger.[18] Compounds **1–9** were constructed using the Schrodinger's Maestro module and then geometry optimization was performed for these ligands using Polak-Ribiere conjugate gradient (PRCG) minimization (0.0001 kJ $Å^{-1}$ mol^{-1}, convergence criteria). Protonation states of ligands and residues were tested using LigPrep and Protein Preparation modules under Schrodinger package[18] at neutral pH (experimentally the compounds have been tested at pH of 7.4). The Glide-XP (extra precision) (v5.0)[19] combined with Induced Fit Docking (IFD) have been used for the docking calculations. IFD uses the Glide docking program to account the ligand flexibility and the refinement module, and the Prime algorithm to account for flexibility of the receptor.[19] Schrodinger's IFD protocol model uses the following steps (the description below is from the IFD user manual): (i) Constrained minimization of the receptor with an RMSD cutoff of 0.18 Å. (ii) Initial Glide docking of each ligand using a soft potentials (0.8 van der Waals radii scaling of non-polar atoms of ligands and receptor using partial charge cutoff of 0.15). (iii) Refinement of the docking poses was done using the Prime module of Schrodinger. Residues within 5.0 Å of ligand poses were minimized in order to form suitable conformations of poses at the active site of the receptor. (iv) Glide redocking of each protein–ligand complexes.

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