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Carbonic anhydrase inhibitors. Inhibition of mammalian isoforms I–XIV with a series of natural product polyphenols and phenolic acids

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ABSTRACT

A series of phenolic acids and phenol natural products, such as *p*-hydroxybenzoic acid, *p*-coumaric acid, caffeic acid, ferulic acid, gallic acid, syringic acid, quercetin, and ellagic acid, were investigated for their inhibitory effects against the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). All mammalian isozymes of human (h) or murine (m) origin hCA I–hCA XII, mCA XIII and hCA XIV were inhibited in the low micromolar or submicromolar range by these (poly)phenols (K_Is in the range of 0.87–7.79 μM). *p*-Hydroxybenzoic acid was the best inhibitor of all isozymes (K_Is of 0.87–35.4 μM) and the different isozymes showed very variable inhibition profiles with these derivatives. Phenols like the ones investigated here possess a CA inhibition mechanism distinct of that of the sulfonamides/sulfamates used clinically or the coumarins. Unlike the sulfonamides, which bind to the catalytic zinc ion, phenols are anchored at the Zn(II)-coordinated water molecule and bind more externally within the active site cavity, making contacts with various amino acid residues. As this is the region with the highest variability between the many CA isozymes found in mammals, this class of compounds may lead to isoform-selective inhibitors targeting just one or few of the medicinally relevant CAs.

1. Introduction

Phenols were recently investigated[1–4] in detail as inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1).[5] CAs, of which 16 isoforms are presently known in mammals,[5] are inhibited by three main mechanisms: (i) coordination of the inhibitor to the Zn(II) ion (critical for catalysis) from the enzyme active site by replacing the zinc-bound water/hydroxide ion and leading to a tetrahedral geometry of Zn(II) (Fig. 1A), or by addition of the inhibitor to the metal coordination sphere, when the Zn(II) ion is in a trigonal bipyramidal geometry (Fig. 1B);[5,6] (ii) by anchoring of the inhibitor to the Zn(II)-bound solvent molecule, that is, a water or hydroxide ion. Phenols[7] and polyamines[8] bind in this way, as shown schematically in Figure 1C for phenol; or (iii) by occlusion of the entrance to the active site cavity,[9] when the inhibitors bind in the activator binding region from the CA active site.[10–12] Coumarins[9] and lacosamide[13] bind in this way to, as shown schematically in Figure 1D. It should be noted that passing from the first type of inhibitors (the classical ones, that is, sulfonamides and their bioisosteres such as sulfamates, sulfamides, etc.) mentioned above to the last ones, the role of the Zn(II) ion is constantly diminishing in its interaction with the inhibitor. This has important consequences for the drug design of CA inhibitors (CAIs) because the bottom of the active site cavity is very much conserved in the 16 CA isozymes described so far in mammals,[5,6] whereas the regions with the highest variation in amino acid sequence and as a consequence with the highest degree of structural diversity are just those at the entrance of the active site. Indeed, phenols,[1–4] but also coumarins[9] and other types of non-zinc binder inhibitors[8] were recently shown to lead to isozyme-selective CAIs, a goal very difficultly achievable with the classical sulfonamide/sulfamate inhibitors.[5,6]

Exploration of novel chemotypes belonging to the phenol class is thus constantly being pursued in our laboratories,[1] in the search of interesting leads. Indeed, the CAIs have applications in the design of various pharmacological agents, such as diuretics,[5,14] antiglaucoma,[5,14–16] or anticonvulsant agents,[5,15–18] whereas more recent drug design studies evidenced some other classes of such derivatives as molecules of interest for developing novel therapies for obesity[18] and cancer[5,19–22] based on selective inhibition of CA isozymes involved in such pathologies. Continuing our earlier work in the field of phenols as possible CAIs, we report here an inhibition study of the 12 catalytically active mammalian isozymes CA I–XIV with a panel of phenols and polyphenols, some of which are natural products. Several low micromolar and submicromolar CAIs have been thus detected which represent interesting new leads for obtaining even tighter binding compounds.

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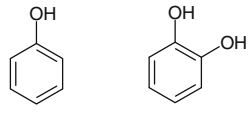
2. Results and discussion

2.1. Chemistry

We used phenol **1** and pyrocatechol **2** as lead compounds, as they have been investigated earlier[2] for their interaction with isoforms CA I–XIV and provided noteworthy results. As stressed in the introduction, these compounds possess a mechanism of enzyme inhibition quite distinct from that of sulfonamides,[5] clinically used CAIs, such as acetazolamide **3**, which has been included in our study for comparison reasons.



1

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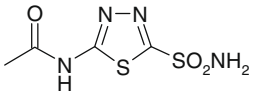

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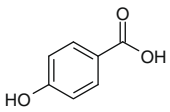

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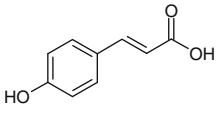

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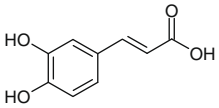

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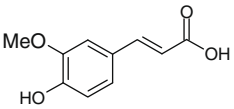

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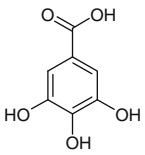
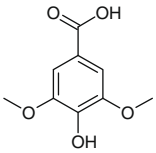
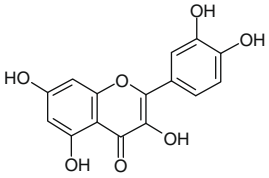


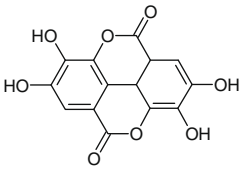
8: Gallic Acid



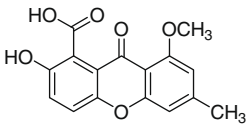
9: Syringic Acid



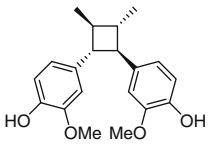
10: Quercetin



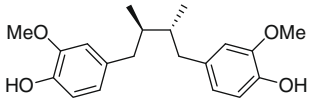
11: Ellagic Acid



12: xanthone



13: endiandrin A



14: (-)-dihydroguaiaretic acid

We decided to investigate mono- and polyphenols which possess various side chains in their molecules, such as the simple *p*-hydroxybenzoic acid **4** but also the natural products *p*-coumaric acid (one of the predominant phenolic acids acylating the cell walls of grasses)[23] **5**, caffeic acid[24] **6** and ferulic acid **7**,[24] which possess one or two phenolic OH groups and the ethenyl-carboxy moiety found in 2-hydroxycinnamic acid, a CAI recently discovered by our group[9] which has been shown by means of X-ray crystallography to bind in a completely new manner to the enzyme active site,[9] as represented schematically in Figure 1D.

Gallic acid **8** and its bis-methylated derivative syringic acid **9**, naturally occurring polyphenols widespread in plants,[25] were also included in this study together with quercetin **10** and ellagic acid **11**, compounds with a more complicated scaffold which are bioactive polyphenols that were reported to possess antioxidant and anti-inflammatory activities.[26] These last two derivatives share some structural similarity with the xanthone **12**, endiandrin A **13** and (−)-dihydroguaiaretic acid **14**, natural compounds also possessing phenolic moieties grafted on a more complex organic scaffold, and significant CA inhibitory activity, as recently reported by Davis et al.[1]

Thus, all these compounds have been included in this study for at least two reasons: (i) the simple scaffolds **4**–**9** have not been investigated earlier for their interaction with CA isozymes, even if they possess structural elements found in potent and selective CAIs (such as the 2-hydroxycinnamic acids),[9] whereas the natural product phenols derivatives **10** and **11** possessing more complicated scaffolds might mimic in a way the investigated derivatives **12**–**14**[1] which showed nanomolar and selective inhibition of the mitochondrial isozymes CA VA and CA VB;[1] and (ii) many of the natural products phenols/polyphenols **5**–**11** possess significant biological activity, some of their derivatives being in clinical evaluation as antioxidant, antitumor or antibacterial agents, even if their targets are largely unknown at the moment.[23b–26]

2.2. Carbonic anhydrase inhibition

Inhibition data against all the catalytically active mammalian α-CA isozymes, that is, CA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII and XIV with compounds **1**–**11** are presented in Table 1, and they were obtained at pH 7.4 in 10 mM Hepes buffer, at 25 °C, by a stopped-flow assay monitoring the CO_2 hydration reaction.[27] The sulfonamide **3** (and previously investigated phenols **1** and **2**)[2] inhibition data are also shown for comparison reasons in Table 1.

The following should be noted regarding the inhibition of these mammalian α-CA isozymes with phenols **4**–**11**:

(i) Against isozyme hCA I phenols **4**–**11** exhibited excellent inhibitory activity, with inhibition constants in the range of 0.92–4.15 μM, being more effective than the simple phenol **1** (K_I of 10.2 μM) or pyrocatechol **2** (which is a very ineffective hCA I inhibitor, with a K_i of 4 mM).[2] The best inhibitors were *p*-hydroxybenzoic acid **4** and *p*-coumaric acid **5**, whereas the least effective ones gallic acid **8** and syringic acid **9**. Thus, small structural changes in the molecules of these phenols have important consequences for their potency as hCA I inhibitors. For example, the incorporation of the *p*-carboxy moiety in phenol **1**, leads to **4** which is a 11 times more potent inhibitor compared to the lead **1**. Introduction of the carboxyethenyl moiety (again in *para*) leads to a 9.5 times increase of the inhibitory potency of **5** over **1**. However a second OH phenolic moiety in *meta* (as in **6**) or its methylation (as in **7**) lead to a decrease of the inhibitory power of these derivatives over the lead **5**. The more complex polyphenols **10** (pentaphenol) and **11** (tetraphenol) were on the other hand effective hCA I inhibitors, with K_Is of 2.32–2.68 μM. Only acetazolamide 3 was slightly a better hCA I inhibitor compared to the investigated phenols (K_I of 0.25 μM).

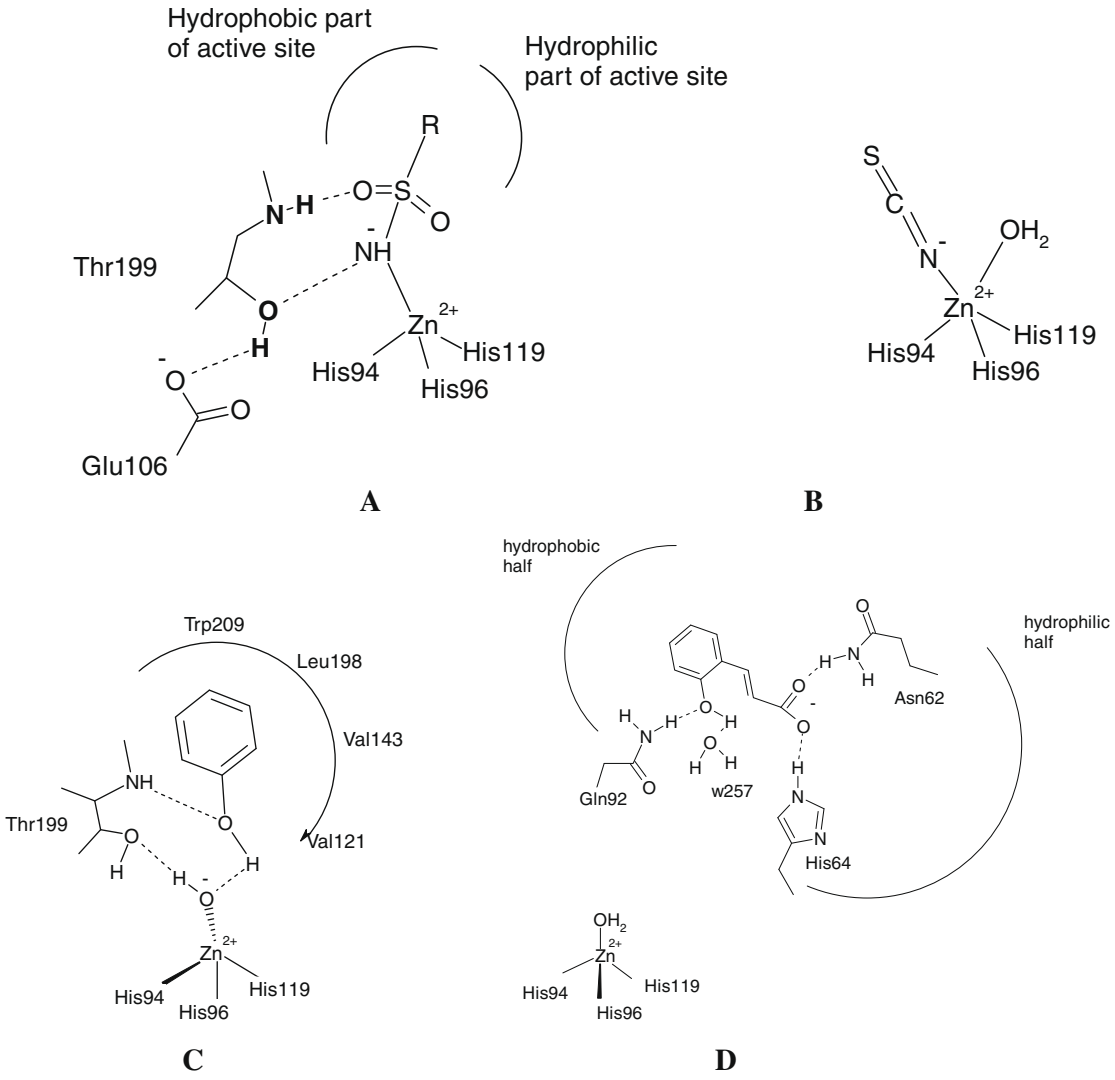


Figure 1. Schematic representation for the three main CA inhibition mechanisms: (A) Sulfonamides (and their isosteres, sulfamate and sulfamide) substitute the fourth zinc ligand and bind in tetragedral geometry of the metal ion;[5,6] (B) Inorganic anion inhibitors (thiocyanate as an example) add to the metal ion coordination sphere leading to trigonal bipyramidal adducts;[5,6] (C) Phenols anchor to the Zn(II)-coordinated water molecule/hydroxide ion;[7] (D) Coumarins (hydrolyzed in situ to 2-hydroxycinnamic acids) occlude the entrance of the active site cavity, interacting both with hydrophilic and hydrophobic amino acid residues. The inhibitor does not interact at all with the catalytically crucial Zn(II) ion which is coordinated by three His residues and a water molecule.[9]

Table 1
Inhibition of CA isozymes I–XV (of human = h, and murine = m origin) with phenols **1**, **2** and **4**–**11** and acetazolamide **3** as standard inhibitor[27]

Compound	K_I (μM) [b,c]											
	hCA I	hCA II	hCA III	hCA IV	hCA VA	hCA VB	hCA VI	hCA VII	hCA IX[d]	hCA XII[d]	mCA XIII	hCA XIV
1[a]	10.2[a]	5.5[a]	2.7[a]	9.5[a]	218[a]	543[a]	208[a]	710[a]	8.8[a]	9.2[a]	697[a]	11.5[a]
2[a]	4003	9.9	13.0	10.9	55.1	4.2	606	714	115	8.9	12.2	48.9
3[a]	0.25	0.012	200	0.074	0.063	0.054	0.011	0.0025	0.025	0.0057	0.017	0.041
4	0.92	0.87	6.61	7.78	3.67	7.13	5.70	4.08	3.73	6.27	7.79	6.16
5	1.07	0.98	7.57	9.60	5.96	7.76	6.72	5.23	5.33	8.01	10.1	6.68
6	2.38	1.61	10.0	10.1	6.49	9.08	7.33	6.42	7.87	9.06	10.9	8.71
7	2.89	2.40	11.1	10.8	7.04	10.5	8.45	7.41	9.87	9.78	12.2	9.43
8	3.20	2.25	7.49	9.80	4.08	9.97	6.13	6.07	6.99	7.78	9.86	7.03
9	4.15	3.19	8.58	10.6	6.34	35.4	7.55	7.81	8.20	9.01	11.0	9.14
10	2.68	2.54	8.10	7.89	6.81	11.9	6.17	4.84	7.00	9.39	9.03	5.41
11	2.32	2.18	10.5	9.08	7.59	12.7	7.06	6.32	9.37	10.1	10.3	8.91

[a] From Ref. 2.
[b] Errors in the range of ±5% of the reported data from three different assays.
[c] h = human; m = murine isozyme.
[d] Catalytic domain.

(ii) The physiologically dominant[5] isozyme hCA II was also effectively inhibited by compounds **4**–**11**, with K_Is in the range of 0.87–3.19 μM. The structure–activity relationship (SAR) is rather similar to what outlined above for hCA I (also because the two enzymes have a high sequence homology of amino acid present within the active site).[5] Thus, *p*-hydroxybenzoic acid **4** and *p*-coumaric acid **5** were the best inhibitors in this series of investigated compounds (K_Is of 0.87–0.98 μM), being 6.3–11.4 times more effective hCA II inhibitors compared to the leads **1** and **2**. The activity was then slightly diminishing for derivatives **6**–**11** (K_Is of 1.61–3.19 μM) as for the activity of the same derivatives against hCA I, but overall hCA II was more sensitive to these derivatives compared to hCA I (although the difference in activity is not so high). Acetazolamide **3** was a very effective, nanomolar inhibitor of hCA II (K_I of 12 nM).

(iii) Isoform hCA III, which is not inhibited by sulfonamides[28] (**3** has a K_I of 200 μM) is sensitive to phenol inhibition. Indeed, phenol **1** and pyrocatechol **2** are much better hCA III inhibitors than acetazolamide (K_Is of 2.7–13.0 μM). The phenols **4**–**11** investigated here also inhibited hCA III (K_Is in the range of 6.61–11.1 μM), being thus less effective compared to **1** and having activities better or in the same range as pyrocatechol **2**. The best hCA III inhibitors were *p*-hydroxybenzoic acid **4** and gallic acid **8** (K_Is of 6.61–7.49 μM). Compounds **5**, **9** and **10** also showed K_Is <10 μM, whereas the least effective hCA III inhibitors were caffeic acid **6**, ferrulic acid **7** and ellagic acid **11** (K_Is of 10.0–11.1 μM). Again small structural changes influence significantly the inhibitory activity (compare for example **4** and **7** which differ by a MeO and CH=CH moieties, whereas their inhibitory activity differs by a factor of 1.7).

(iv) The membrane-associated isoform hCA IV was moderately inhibited by all phenols **4**–**11**, with inhibition constants in the range of 7.78–10.8 μM, in the same range as the leads **1** and **2** (K_Is of 9.5–10.9 μM). Thus, SAR is rather flat in the case of this isoform, as all these derivatives show a limited variation of their potency in inhibiting hCA IV (a situation quite different from that of hCA I and II).

(v) The first mitochondrial isoform, hCA VA, was quite poorly inhibited by the simple phenols **1** and **2** (K_Is of 55.1–218 μM)[2] but quite effectively inhibited by the (poly) phenols **4**–**11** investigated here, with K_Is in the range of 3.67–7.59 μM (Table 1). The best inhibitors were again *p*-hydroxybenzoic acid **4** and gallic acid **8** (K_Is of 3.67–4.08 μM). Thus, there is an increase of inhibitory potency of 53.4–59.4 times of these two derivatives over the simple phenol **1**, which is indeed a very significant finding. SAR is also here rather flat, with all these derivatives showing K_Is <7.6 μM. Thus, polyphenols, unlike simple phenols are indeed good leads to obtain potent hCA VA inhibitors. Indeed, recently Davis et al.[1] identified phenols **12**–**14** as potent hCA VA inhibitors, with K_Is <100 nM.

(vi) Also the second mitochondrial isoform, hCA VB, was inhibited by compounds **4**–**11** investigated here, but its inhibition profile was rather different from that of hCA VA (with which it shares a high degree of sequence homology).[29] Thus, phenols **4**–**8** and **10**, **11** were medium potency inhibitors, with K_Is in the range of 7.13–12.7 μM, whereas syringic acid **9** was much less effective (K_I of 35.4 μM). It is interesting to note the 3.5 times difference of inhibitory activity between **8** and **9**, which differ only by the bis-methylation of the *meta* diphenolic moieties of syringic acid. Thus, SAR is rather sensitive to small structural changes in the molecule of the inhibitor in the case of this isoform, which is very different from the situation discussed above for hCA VA.

(vii) Similarly to hCA VA discussed above, the secreted (in saliva and milk)[5] isoform hCA VI was effectively inhibited by all (poly)phenols **4**–**11** (K_Is in the range of 5.70–8.45 μM), although the simple phenols **1** and **2** were highly ineffective as inhibitors of hCA VI (K_Is of 208–606 μM). SAR was again quite flat, with the best inhibitor being *p*-hydroxybenzoic acid **4** (K_I of 5.70 μM) and the worst one ferulic acid **7** (K_I of 8.45 μM). The sulfonamide 3 was a much more effective hCA VI inhibitor (K_I of 11 nM) compared to all phenols investigated to date (Table 1).

(viii) As for hCA VA and hCA VI, the simple phenols **1** and **2** are ineffective inhibitors of the cytosolic isoform hCA VII (K_Is of 710–714 μM), but the phenolic carboxylic acids **4**–**9** and the natural products **10** and **11** effectively inhibited it, with K_Is in the range of 4.08–7.81 μM. The SAR is very flat also in this case, a situation we recently observed for at least two classes of CAIs: the natural products investigated by Davis et al.[1] for the inhibition of hCA I, II, VA and VB, and a class of sugar sulfonamides reported by Lopez et al.[30] In the last case, a detailed X-ray crystal structure study of several adducts of hCA II with some of the reported sulfonamides, explained why such a flat SAR has been observed. Indeed, all the investigated compounds of Lopez et al.[30] inhibited several CA isozymes in the range of 3.7–4.9 μM. The X-ray crystal structures showed four of the structurally very different *S*-glycosyl primary sulfonamides to bind within the CA II active site cavity, but with few favorable interactions between moieties present in the inhibitor molecule and amino acid residues from the active site being evidenced.[30]

(ix) The two transmembrane, tumor-associated isozymes hCA IX and XII were also effectively inhibited by (poly)phenols **4**–**11**, with K_Is in the range of 3.73–9.87 μM for hCA IX, and of 6.27–10.1 μM for hCA XII, respectively. Thus, for hCA IX the newly investigated phenols are more effective inhibitors than the leads **1** and **2**, whereas for hCA XII the inhibitory power is in the same range as for **1**, **2**, with only **4** and **8** being more effective hCA XII inhibitors compared to the leads **1**, **2** and the remaining investigated phenols. As these isozymes are overexpressed in hypoxic tumors where there are also reactive oxygen species damaging the tissue,[31] the inhibition of these isoforms with compounds such phenols **4**–**11** also possessing antioxidant activity might be of interest to be studied in more details.

(x) The cytosolic isoform mCA XIII showed an inhibition profile with phenols **4**–**11** rather similar to hCA III. Indeed, these compounds showed K_Is in the range of 7.79–12.2 μM, whereas the lead **1** was a very ineffective inhibitor (K_I of 697 μM). Thus, the presence of additional substituents on the aromatic ring, as in pyrocatechol **2** and derivatives **4**–**11**, strongly enhances the inhibitory activity of these phenols compared to **1**. For example, the incorporation of a *p*-COOH moiety in the molecule of phenol **1**, as in **4**, leads to a 89.5 times increase of mCA XIII inhibitory activity. However, the sulfonamide **3** was the most effective inhibitor among the investigated compounds, with a K_I of 17 nM (Table 1).

(xi) The last transmembrane isoform, hCA XIV (which shows a high degree of homology with CA IX and XII)[20c] exhibited an inhibition profile with compounds **4**–**11** rather similar to that of hCA IX discussed above. Thus, all the investigated (poly)phenols were effective inhibitors with K_Is in the range of 6.16–9.43 μM and thus a rather flat SAR. They were anyhow better inhibitors than the lead **1** (K_I of 11.5 μM) and especially pyrocatechol **2** (K_I of 48.9 μM). However, again the sulfonamide 3 was the most effective inhibitor among the investigated compound (K_I of 41 nM).

A last aspect that we investigated here is related to the inhibition mechanism of these derivatives. Thus, *p*-coumaric acid **5** is an isomer with 2-hydroxycinnamic acid that we showed recently[9] to possess a novel mechanism of inhibition of all CA isozymes (Fig. 1D). Indeed, as discussed above, this compound does not interact with the catalytic Zn(II) ion or the water coordinated to it (similarly to phenol), but binds at the entrance of the active site cavity, occluding it and forming several favorable interactions with Asn62, His64, Gln92 and a water molecule.9 Another interesting aspect of this compound is that it acts as a prodrug, since it is formed by active site hydrolysis of coumarin.[9] Furthermore, this inhibition is time-dependent, with the final inhibitor, that is, 2-hydroxycinnamic acid, being formed after 4–6 h after incubation of enzyme and coumarin. Thus, we have investigated whether the phenolic acids from this study might possess a time-dependent CA inhibitory activity. Thus, we incubated hCA II with compounds **4**–**11** for variable periods of time (15 min–24 h) and measured the inhibition constants. In all cases, no time-dependent inhibition has been observed, that is, the K_Is were the same after all incubation periods (within the limits of experimental errors, data not shown). Thus, we can rule out an inhibition mechanism of type (iii) discussed in the Introduction. More probably, the phenols investigated here, of type **4**–**11**, inhibit CAs by a mechanism of type (ii), of the phenol type, anchoring to the water molecule from the enzyme active site. Further studies are warranted in this field, as only one X-ray crystal structure of CAs with a phenols is available at this moment, the one of CA II with **1**. More such crystallographic data would be critical for better understanding the CA inhibition mechanism with various types of phenols.

3. Conclusions

A detailed inhibition study of 12 mammalian isoform of the metalloenzyme CA, that is, CA I–XIV, with a series of (poly)phenols was performed. Several phenolic acids and phenol natural products, such as *p*-hydroxybenzoic acid, *p*-coumaric acid, caffeic acid, ferulic acid, gallic acid, syringic acid, quercetin, and ellagic acid, were investigated for their inhibitory with these enzymes. All mammalian isozymes hCA I–hCA XII, mCA XIII and hCA XIV were inhibited in the low micromolar or submicromolar range by these (poly)phenols (K_Is in the range of 0.87–7.79 μM). *p*-Hydroxybenzoic acid was the best inhibitor of all isozymes (K_Is of 0.87–35.4 μM) and the different isozymes showed very variable inhibition profiles with these derivatives. Phenols like the ones investigated here possess a CA inhibition mechanism distinct of that of the sulfonamides/sulfamates used clinically or the coumarins. Unlike the sulfonamides, which bind to the catalytic zinc ion, phenols are anchored at the Zn(II)-coordinated water molecule and bind more externally within the active site cavity, making contacts with various amino acid residues. As this is the region with the highest variability between the many CA isozymes found in mammals, this class of compounds may lead to isoform-selective inhibitors targeting just one or few of the medicinally relevant CAs.

4. Experimental

4.1. Chemistry

Buffers, phenols **4**–**11** and acetazolamide **3** were from Sigma–Aldrich (Milan, Italy) of highest purity available, and were used without further purification. All CA isozymes were recombinant ones produced and purified in our laboratory as described earlier.[1–4]

4.2. CA catalytic/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.[27] 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),[1–4] 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 0.001 μM done with the assay buffer mentioned above. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, as reported earlier,[1–4,9] and represent the mean from at least three different determinations.

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