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Carbonic anhydrase inhibitors. Antioxidant polyphenols effectively inhibit mammalian isoforms I–XV

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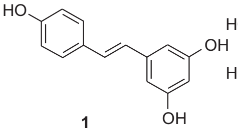
ABSTRACT

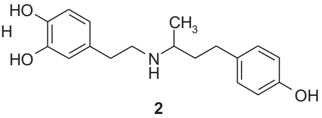
A series of polyphenolic derivatives, including resveratrol, dobutamine, curcumin, catechin and silymarine were investigated for the inhibition of all the catalytically active mammalian isozymes of the metalloprotein carbonic anhydrase (CA, EC 4.2.1.1), that is, CA I–CA XV. These polyphenols effectively inhibited CAs, with K_Is in the range of 380 nM–12.02 μM. The various isozymes showed quite diverse inhibition profiles with these compounds, which possess scaffolds not present in other investigated CA inhibitors (CAIs). These data may lead to drug design campaigns of effective CAIs possessing a diverse inhibition mechanism compared to sulfonamide/sulfamate inhibitors, based on such less investigated scaffolds.

Among the various natural products (NPs) with antioxidant properties, phenols and polyphenols are very active in quenching reactive oxygen species (ROS).[1] They are reported to possess anticancer, anti-carcinogenic, antimutagenic, antibacterial, antiviral or anti-inflammatory activities.[1–4] Compounds such as resveratrol **1**, dobutamine **2**, curcumin **3**, catechin **4**, and silymarin **5** are polyphenols possessing unrelated scaffolds but strong antioxidant properties.[2–4] Resveratrol **1** is found in red wine and is thought to induce protection against cardiovascular disease.[2a] Dobutamine **2** is a drug used to treat or to image acute heart failure, but also in cardiac surgery or in cases of septic or cardiogenic shock.[5] Curcumin (diferuloylmethane) **3**, the yellow pigment isolated from the root of *Curcuma longa*, has several biological effects, the most important being its anti-carcinogenic properties.[4a] Curcumin was demonstrated to inhibit several carcinogenic stimuli in mice.[4b] Catechin **4** is a polyphenolic antioxidant plant metabolite,[2] being also present in the human diet in tea, fruits, vegetables.[2] Silymarin **5**, a plant derived flavonoid belonging to the benzopyranone class, is isolated from fruits and seeds of the milk thistle (*Silymarin marianum*),[3] being purportedly reported to be useful for the treatment of cancer, varicose veins, menstrual problems, depression, low breast milk production, liver disorders, cirrhosis, chronic hepatitis.[3]



1

Resveratrol



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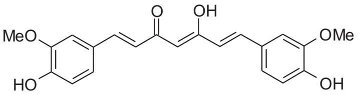
Dobutamine



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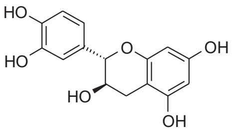
Curcumin



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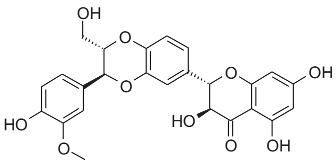
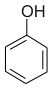
Catechin



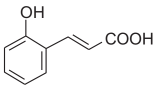
5

Silymarine



6

Phenol



7

2-Hydroxycinnamic acid

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The common denominator of all these compounds is the fact that they incorporate in their molecule the phenolic moieties, present in **6**, the simple phenol, which was also reported to act as an inhibitor of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1).[6–10] Indeed, phenol **6** binds to CA in a diverse manner compared to the classical inhibitors of the sulfonamide type, which coordinate to the Zn(II) ion from the enzyme active site by substituting the fourth, non-protein ligand, a water molecule or hydroxide ion.[6] X-ray crystal studies from Christianson's group showed phenol to be anchored by means of its OH moiety to the fourth zinc ligand (a water molecule but most probably a hydroxide ion, as the crystallization was performed at pH >8 and the pK_a of that water molecule is around 7) by means of a hydrogen bond.[7] A second hydrogen bond has been evidenced between the oxygen atom of phenol and the amide NH of Thr199, an amino acid residue conserved in all α-CAs (Fig. 1A).[7] Other phenols, such as those formed by the active site mediated hydrolysis of coumarins,[11,12] such as 2-hydroxycinnamic acid **7**, bind in a different way to CAs, occluding the entrance to the active site cavity (Fig. 1B). Coumarins,[11,12] lacosamide[13] and fullerenes[14] derivatives bind in this way to CA, as shown schematically in Figure 1B for a hydrolyzed coumarin derivative **7** and this binding-site coincides with the activator-binding site of these enzymes.[15]

Thus, by binding in a non-classical way to CAs, phenols and polyphenols as well as their derivatives constitute interesting leads for identifying novel types of CA inhibitors (CAIs). Indeed, in this class of pharmacological agents, the sulfonamide are in clinical use for more than 50 years as diuretics and systemically acting antiglaucoma drugs.[7,16–20] However, in addition to the established diuretic/antiglaucoma effects of these CAIs, it has recently emerged that they have potential as anticonvulsant, antiobesity, anticancer, antipain and antiinfective drugs.[16–20] But critical barriers to the design of CAIs as therapeutic agents are related to the high number of isoforms in mammals (i.e., 16 CAs, of which 13 have catalytic activity), their rather diffuse localization in many tissues/organs, and the lack of isozyme selectivity of the presently available inhibitors of the sulfonamide/sulfamate type.[7,16–20] In fact, among the sulfonamide derivatives in clinical use, there are no compounds which selectively inhibit only CA isoforms with therapeutic value, although their inhibition profiles against the 13 mammalian isozymes are highly variable and can be used for the structure-based drug design of novel generation, isoform-selective inhibitors (inhibition data against all human (h) CA isoforms with clinically used sulfonamides/sulfamates are provided in Ref. 7a).

In the search of CAIs with interesting inhibition profiles, and considering the published work on phenols as inhibitors of these enzymes,[6,11,21] we report here an inhibition study of the 13 catalytically active mammalian isoforms CA I–XV with polyphenols 1–**5**, some of which are NP derivatives. They incorporate between 2 and 5 phenolic moieties in their molecules and scaffolds which are quite variable, representing thus an interesting starting point for different chemotypes belonging to the CAIs (up to now only mono- or biphenols have been investigated).[6,11,21] In fact, in an earlier study[21] we have reported nanomolar inhibitors of the mitochondrial isoforms CA VA and VB in a library of fungal metabolite phenols/polyphenols belonging to the NP class of derivatives.

Data of Table 1, in which the inhibition of these CA isoforms with polyphenols **1–5** as well as phenol **6** and 2-hydroxycinnamic acid **7**, reported earlier,[8a,11] (included for comparison reasons), allow us to evidence the following structure–activity relation (SAR) features for these CAIs:[22]

(i) Resveratrol **1** showed effective inhibition of all 13 investigated CA isoforms, with inhibition constants in the range of 0.81–9.36 μM. The isoforms which showed the strongest inhibition with this polyphenol were hCA IX and XII (tumor-associated) as well as hCA XIV (transmembrane isoform, like the other two mentioned above). It is interesting to note that these isoforms are the most similar with each other among the 16 CAs known to date in mammals, as show by phylogenetic analysis and examination of their 3D structures as obtained by X-ray crystallography.[19b] The second remarkable feature is that resveratrol is a submicromlar inhibitor of these CAs, some which are known to be involved in tumorigenesis and cancer progression.[18,19b,20] Several other investigated CAs, such as hCA I, hCA II, hCA IV, hCA VA, hCA VB, hCA VII and hCA XIII were less effectively inhibited by **1**, with inhibition constants in the range of 2.21–4.75 μM (Table 1). Except CA IV which is membrane-bound, the other isoforms in this subgroup are cytosolic or mitochondrial (CA VB and VA). The least susceptible isozymes to be inhibited by **1** were hCA III (cytosolic), hCA VI (secreted) and mCA XV (membrane-associated), with K_Is in the range of 8.07–9.36 μM. It is thus evident that resveratrol **1** is a much more efficient inhibitor compared to the lead molecule **6** (except for CA III, for which phenol **6** is slightly better an inhibitor compared to **1**), with the efficiency increase sometimes quite

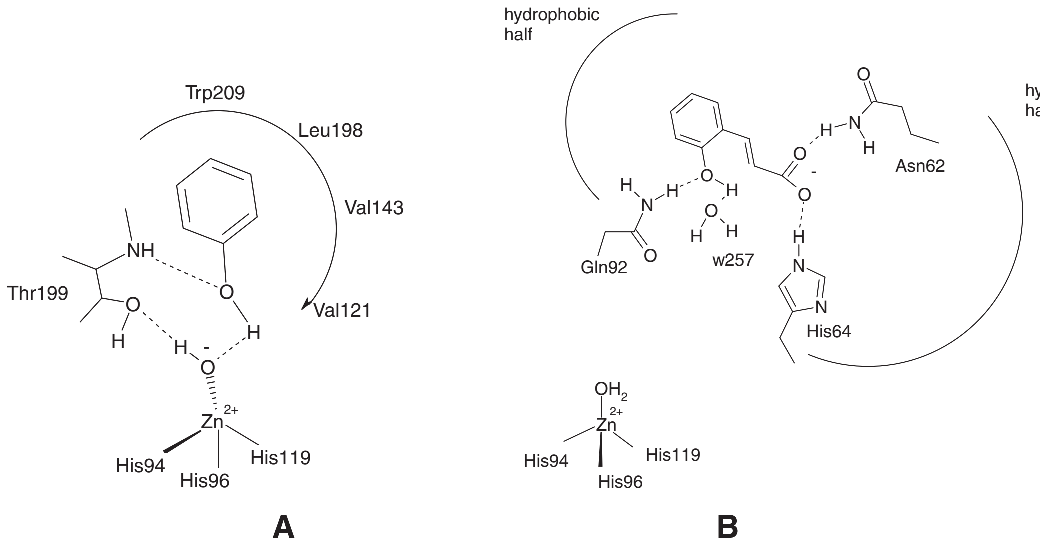

Figure 1. Binding of phenols and their derivatives to CAs. (A) Simple phenol **6** binds by anchoring to the Zn(II) coordinated water/hydroxide ion.[6] (B) 2-Hydroxycinnamic acid 7 (in situ hydrolysis product of coumarin)[11,12] binds at the entrance of the active site cavity, in the activator-binding site.[11,12,15]

Table 1
Inhibition of CA isoforms I–XV with compounds **1**–**7** by a stopped-flow, CO_2 hydrase assay[22]

Isoform[*]	K_I[**] (μM)						
	1	**2**	**3**	**4**	**5**	**6**[a]	**7**[b]
hCA I	2.21	1.92	2.41	2.42	1.49	10.2	3.1
hCA II	2.77	0.48	0.38	1.84	2.51	5.5	9.2
hCA III	9.09	7.40	11.30	3.58	6.43	2.7	>1000
hCA IV	4.47	8.98	4.97	4.90	8.96	9.5	62.3
hCA VA	4.75	0.73	10.25	4.21	4.08	218	>1000
hCA VB	4.64	0.89	9.46	4.02	4.56	543	578
hCA VI	8.07	9.47	9.94	4.91	9.70	208	>1000
hCA VII	4.35	4.30	9.30	0.45	4.71	710	>1000
hCA IX	0.81	9.82	4.05	5.03	10.15	8.8	>1000
hCA XII	0.95	4.35	3.48	4.72	9.05	9.2	>1000
hCA XIII	4.09	9.53	6.85	10.51	4.82	697	>1000
hCA XIV	0.83	12.02	11.73	11.55	11.64	11.5	>1000
mCA XV	9.36	0.39	5.09	7.68	0.65	10.5	>1000

[*] h = human, m = murine isoform.

[**] Errors in the range of ±10% of the reported value (from three different assays).

[a,b] From Refs. 8a,11.

dramatic between the two compounds (e.g., against hCA VII, **1** is 163-times more effective as CAI compared to **6**). 2-Hydroxycinnamic acid **7** (the hydrolysis product of the 'prodrug' coumarin inhibitor) was also much less effective compared to **1** as a CAI against most isoforms (Table 1). It is thus obvious that the presence of the 3,5-dihydroxy-styrenyl moiety in **1** leads to an important increase of CA inhibitory properties for **1** compared to the lead **6**.

(ii) Dobutamine **2** also showed interesting CA inhibitory properties. Thus, hCA II, VA, VB and mCA XV were inhibited in the submicromolar range by this compounds, with K_Is in the range of 0.39–0.89 μM. mCA XV was the most susceptible isoform to inhibition by **2** (K_I of 390 nM). A second group of enzymes, comprising hCA I, hCA VII and hCA XII showed weaker inhibition, with K_Is in the range of 1.92–4.35 μM, whereas hCA III, IV, VI, IX, XIII and XIV were the least sensitive to dobutamine, with inhibition constants in the range of 7.40–12.02 μM (Table 1).

(iii) An interesting inhibition profile against isoforms CA I–XV was observed for the diphenol curcumin **3** (Table 1). This compound was a submicromolar inhibitor of only hCA II (K_I of 380 nM), and was a moderate inhibitor of hCA I, hCA IV, hCA IX, hCA XII and mCA XV (K_Is in the range of 2.41–5.09 μM). The remaining isozymes, that is, hCA III, hCA VA, hCA VB, hCA VI, hCA VII, hCA XIII, and hCA XIV, were inhibited by curcumin with K_Is in the range of 6.85–11.73 μM. As for the preceding two compounds, curcumin has enhanced CA inhibitory properties compared to the leads **6** and **7** to which it has some structural resemblance.

(iv) Catechin **4**, possessing an even more complicated scaffold compared to **1**–**3** discussed above, showed effective CA inhibitory properties against all investigated isoforms (Table 1). Thus, hCA VII was inhibited in the submicromolar range (K_I of 450 nM) by this compound, whereas hCA XIII, hCA XIV and mCA XV were the least sensitive isoforms to this tetraphenol, with K_Is in the range of 7.68–11.55 μM. The remaining isoforms showed K_Is in the range of 1.84–5.03 μM. Catechin is thus a much more effective CAI compared to **6** and **7**, proving that the substitution pattern present in its scaffold (which is prone to many types of derivatization) may lead to even better CAIs belonging to the polyphenol class.

(v) The triphenol silymarin **5**, also showed good CA inhibitory properties, with inhibition constants in the range of 0.65–11.64 μM. Silymarin was a strong mCA XV inhibitor (K_I of 650 nM), whereas it showed less potent activity against hCA I, II, VA, VB, VII and XIII, with K_Is in the range of 1.49–4.82 μM. Isoforms which were the least inhibited by this compound were hCA III, IV, VI, IX, XII and XIV (K_Is in the range of 6.43–11.64 μM).

(v) The 13 CA isozymes showed quite different susceptibility to inhibition with this small group of polyphenols, although overall potency varied only between 380 nM and 12.02 μM. This may be explained considering that presumably these polyphenols anchor to the zinc-bound water/hydroxide ion, similar to the simple phenol **6** and then their scaffold participates in additional interaction with the enzyme active site. Hopefully, X-ray crystal data for CA—phenol adducts should be available soon and this may lead to a better understanding of this less investigated class of CAIs. Indeed, for example the coumarins, another recently described family of CAIs, led to low nanomolar, isozyme selective inhibitors (even if starting from the poorly active lead **7**).[11,12] They were obtained by simple derivatizations of the coumarin ring, but this feature of isoform selectivity is generally not shared by other classes of CAIs investigated so far, such as the sulfonamides or sulfamates, and this illustrates how important is to explore diverse chemotypes for finding good biological activity.

In conclusion, we investigated here the inhibition of all the catalytically active mammalian α-CA isozymes hCA I–mCA XV with a group of polyphenolic derivatives, including resveratrol, dobutamine, curcumin, catechin and silymarine. All these polyphenols effectively inhibited these enzymes, with K_Is in the range of 380 nM–12.02 μM. The various isozymes showed diverse inhibition profiles. These data may lead to drug design campaigns of more effective CAIs based on such less investigated scaffolds.

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22. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity. 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 for the α-CAs) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the 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 kinetic parameters and inhibition constants. 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 (10 mM) were prepared in distilled–deionized water and dilutions up to 0.01 μM were done thereafter with distilled–deionized water. 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 non-linear least-squares methods using PRISM 3, as reported earlier,[8–12] and represent the mean from at least three different determinations. Human/murine CA isozymes I–XV were prepared in recombinant form as reported earlier by our group.[8–12].