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RESEARCH ARTICLE

Carbonic anhydrase inhibitors: *in vitro* inhibition of α isoforms (hCA I, hCA II, bCA III, hCA IV) by flavonoids

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Abstract

A series of flavonoids, such as quercetin, catechin, apigenin, luteolin, morin, were investigated for their inhibitory effects against the metalloenzyme carbonic anhydrase (CA). The compounds were tested against four α -CA isozymes purified from human and bovine (hCA I, hCA II, bCA III, hCA IV) tissues. The four isozymes showed quite diverse inhibition profiles with these compounds. The flavonoids inhibited hCA I with K_i s in the range of 2.2–12.8 μ M, hCA II with K_i s in the range of 0.74–6.2 μ M, bCA III with K_i s in the range of 2.2–21.3 μ M, and hCA IV with inhibition constants in the range of 4.4–15.7, with an esterase assay using 4-nitrophenyl acetate as substrate. Some simple phenols/sulfonamides were also investigated as standard inhibitors. The flavonoids incorporate phenol moieties which inhibit these CAs through a diverse, not yet determined inhibition mechanism, compared to classic inhibitors such as the sulfonamide/sulfamate ones.

Keywords: Carbonic anhydrase, flavonoid, inhibitor, phenol

Introduction

Flavonoids are polyphenols incorporating diphenylpropanes scaffolds. The four major classes are the 4-oxo-flavonoids (flavones, flavonols, etc.), anthocyanins, isoflavones, and the flavan-3-ol derivatives (catechins and tannins¹). About 6500 naturally occurring flavonoids have been identified up to date². They are important constituents of the human diet, and the daily intake of flavonoids is approximately 200–1000 mg.³ Major dietary sources of flavonoids include for example: beverages (cola, coffee, tea, fruit juices, red wine), fruits and vegetables, nuts^{4,5}. The proposed health optimizing activities of the flavonoids are due to the following actions: (i) antioxidant properties through the ability to scavenge reactive oxygen species (ROS) or through their influences on the redox status (ii) modulation of signal transduction pathways involved in cell proliferation and angiogenesis (iii)

influencing enzymes participating in estrogen biosynthesis (iv) modulation of enzymes necessary for metabolic activation of pro-carcinogens and detoxification of carcinogens^{5,6}.

The hydration of carbon dioxide to bicarbonate and protons, a simple, but essential reaction, is catalyzed by a superfamily of enzymes which have been investigated in detail⁷. These enzymes, known as carbonic anhydrases (CAs, EC 4.2.1.1) are pH regulatory/metabolic enzymes in all life kingdoms, being found in organisms all over the phylogenetic tree. Sixteen CA isoforms are presently known in mammals^{7,8}. Mammalian CAs were the first enzymes of this type to be isolated and studied in detail, and many of them are established therapeutic targets with the potential to be inhibited or activated to treat a wide range of disorders.^{7–11} There are currently known diuretics, antiglaucoma, antiepileptic, antiobesity, and

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anticancer drugs based on CAIs, and they target various mammalian α -CA isoforms⁷. CAs are inhibited by four main mechanisms: (i) coordination of the inhibitor to the Zn(II) ion by replacing the zinc-bound water/hydroxide ion and leading to a tetrahedral geometry of Zn(II) (e.g. the sulfonamides, Figure 1A^{7,12}). (ii) Addition of the inhibitor to the metal coordination sphere, when the Zn(II) ion is in a trigonal bipyramidal geometry (e.g. the thiocyanate adduct, Figure 1B^{7,12}). (iii) Anchoring of the inhibitor to the Zn(II)-bound solvent molecule, i.e. a water or hydroxide ion (e.g. phenol, Figure 1C¹³) (iv) Occlusion of the CA active site entrance, with coumarins^{9–11,14} lacosamide⁹ and fullerenes¹⁵ binding in this way, as shown schematically in Figure 1D for a hydrolyzed coumarin derivative¹⁰. All these binding modes have been demonstrated by means of X-ray crystallography of enzyme–inhibitor adducts^{7–15}.

It should be noted that passing from sulfonamides and their bioisosteres (sulfamates, sulfamides, etc.) to inhibitors occluding the entrance of the active site, the role of the Zn(II) ion is constantly diminishing in its interaction with the inhibitor molecule^{10–15}. This phenomenon 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, whereas the regions with the highest variation

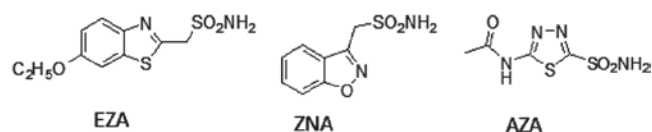


Figure 2. Structures of common used CA inhibitors.

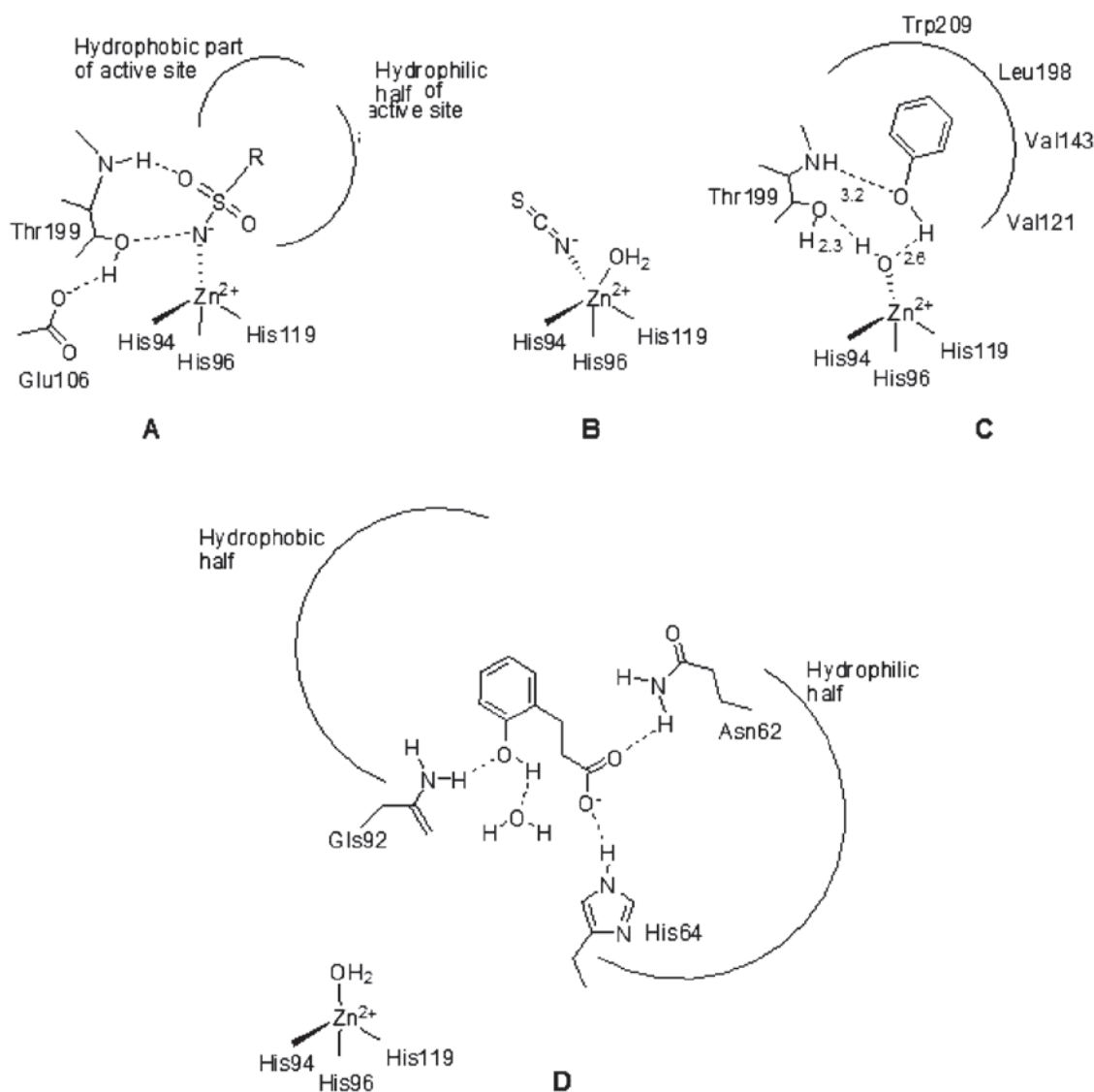


Figure 1. Schematic representation for the three main CA inhibition mechanisms: (A) Sulfonamides (and their isosteres, sulfamate, and sulfamide) substitute the fourth zinc ligand and bind in tetrahedral geometry of the metal ion¹²; (B) Inorganic anion inhibitors (thiocyanate as an example) add to the metal ion coordination sphere leading to trigonal bipyramidal adducts¹²; (C) Phenols anchor to the Zn(II) coordinated water molecule/hydroxide ion¹³; (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^{10,11}.

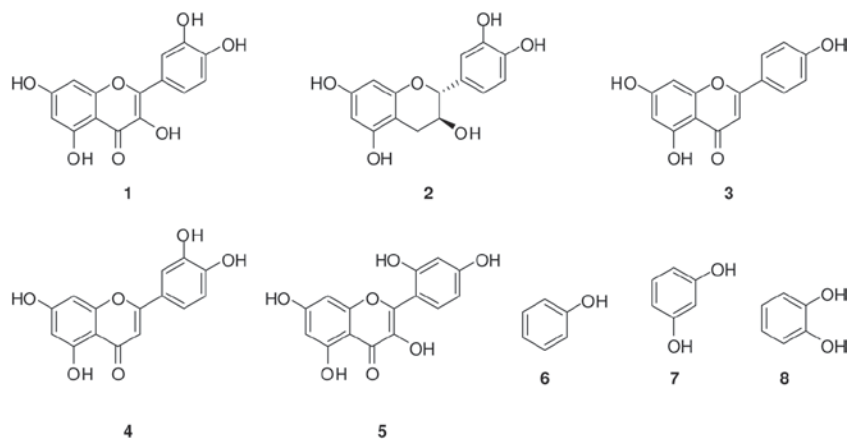


Figure 3. Structures of tested compounds 1–8.

in amino acid sequence, therefore the highest degree of structural diversity, are just those at the entrance of the active site^{7,8,12}. Indeed, phenols^{15–20} but also coumarins^{9,10} and other types of non-zinc binder inhibitors¹¹ were recently shown to lead to isozyme-selective CAIs, a goal difficult to achieve with the classical sulfonamide/sulfamate inhibitors^{7,22}.

Our groups recently investigated the interaction of hCA I, II, VI isozymes with several types of natural and synthetic compounds, such as the simple phenol, catechol, resorcinol, pyrogallol, gallic acid, tannic acid and several of its substituted derivatives, e.g. salicylates and some of their derivatives^{19,20}. Here we extend these earlier investigations to series of compounds 1–8, some of which are widely used as antioxidant food additives. Among the various natural or synthetic phenolic compounds with antioxidant properties, these compounds are very active in quenching ROS¹⁹. They are reported to possess anti-cancer, anti-carcinogenic, antimutagenic, antibacterial, antiviral or anti-inflammatory activities^{4–6}. Quercetin, catechin, apigenin, luteolin and morin, members of the flavonoids family, are ubiquitously present in food, including vegetables, fruits, tea, and red wine. Several biologic properties of these series have been reported to be beneficial to human health, including protection against various diseases, such as osteoporosis, certain forms of cancer, pulmonary and cardiovascular diseases, and aging⁶. Phenol, resorcinol, and catechol are widely used prodrugs or disinfectants^{3,6}.

In the present study, we have purified hCA I, hCA II, bCA III, and hCA IV and examined the *in vitro* inhibition effects of the compounds 1–8, using the esterase activity of these isoenzymes, with 4-nitrophenyl acetate as substrate.

Materials and methods

Chemicals

Quercetin, catechin, apigenin, luteolin, morin, phenol, resorcinol, catechol, Sepharose 4B, protein assay reagents, 4-nitrophenylacetate were obtained from Sigma-Aldrich Co. All other chemicals were analytical grade and obtained from Merck.

Purification of carbonic anhydrase isozymes by affinity chromatography

Purification of hCA I and hCA II were previously described^{17–21}. hCA IV was purified following the protocol described by Zhu and Sly²². Bovine CA III was obtained from flank steak and purified using a modification of the method of Tu et al. (1986²³). The active fractions comprising the major protein peak were pooled and concentrated in 1 mM mercaptoethanol.

Esterase activity assay

Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer according to the method described by Verpoorte et al.²⁴. The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL of 0.05 M Tris-SO₄ buffer (pH 7.4), 1 mL of 3 mM 4-nitrophenylacetate, 0.5 mL of H₂O and 0.1 mL of enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. Enzyme concentrations in the assay system were 9 nM for hCA I, 7 nM for hCA II, 7 nM for IV and 12 nM for bCA III.

In vitro inhibition study

The inhibitory effects of compound 1–8, **EZA**, **ZNA** and **AZA** were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used, starting from 1 nM to 1 mM. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor, Activity (%)–[Inhibitor] graphs were drawn. To determine K_i values, three different inhibitor concentrations were tested. In these experiments, 4-nitrophenylacetate was used as substrate at five different concentrations (0.15–0.75 mM). The Lineweaver-Burk curves were drawn²⁵.

Protein determination

Protein concentration during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as a standard²⁶.

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 procedure²⁷.

Results and discussion

Phenol binds to CA in a different manner from the classical inhibitors of the sulfonamide type, for example etazolamide (**EZA**), 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^{13,16}. The simple phenol has been shown to be the only competitive inhibitor with CO₂ as substrate for the main isoform of CA, i.e. human CA II (hCA II¹³) which is the reason for investigation of these phenolic compounds. The X-ray crystal structure for the adduct of hCA II with phenol¹³, 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^{13–20}. Our group has recently investigated the interactions of phenol and some of its substituted derivatives with all mammalian CAs^{18–20}, demonstrating some low micromolar/submicromolar inhibitors as well as the possibility to design isozyme selective CAIs. The inhibition profile of various CA isozymes with this class of agents is very variable, with inhibition constants ranging from the millimolar to the submicromolar range for many simple phenols^{13,20,28}. Thus, it seemed reasonable to us to extend the previous studies^{20,28}, including in this investigation phenolic compounds and flavonoids with clinical and antioxidant applications as food additives, such as compounds **1–8**^{20,28,29}.

The purification of the CA isozymes was performed with a simple one step method by a Sepharose-4B-aniline-sulfanilamide affinity column chromatography¹⁸. hCA I was purified, 104.7-fold with a specific activity of 917.16 EUmg^{–1} and overall yield of 54.2 %. hCA II was purified, 732.4-fold with a specific activity of 7045 EUmg^{–1} and overall yield of 59.36 %. hCA IV was purified, 89.18-fold with a specific activity of 748.86 EUmg^{–1} and overall yield of 27.8%. Similarly, bCA III was purified, 48.8-fold with a specific activity of 456.4 EUmg^{–1} and overall yield of 46.4%^{16–20,22,23}. Inhibitory effects of compounds **1–8** on enzyme activities were tested under *in vitro* conditions; K_i values were calculated from Lineweaver-Burk graphs and are given in Table 1²⁵.

We investigated the first study on the inhibitory effects of flavonoids, including quercetin, catechin, apigenin, luteolin, morin, phenol, resorcinol, and catechol on the esterase activity of hCA I, II, IV and bCA III. The sulfonamide CAIs **EZA**, **ZNA** and **AZA** have been used as a negative controls in our experiments, and for comparison reasons. The previous reports by Senturk et al.^{20,29}

Table 1. K_i values (μ M) for compound **1–8**, **EZA**, **ZNA** and **AZA** of some α -carbonic anhydrase isoforms in human (h) and bovine (b). ^aRef 29.

Compound	hCA I	hCA II	bCA III	hCA IV
Quercetin (1)	3.6	2.4	9.1	13.8
Catechin (2)	6.8	6.2 ^a	2.2	5.6
Apigenin (3)	4.1	2.7	11.6	9.1
Luteolin (4)	2.2	0.74	5.4	4.4
Morin (5)	12.8	4.4	21.3	15.7
Phenol (6)	17.3	7.4	4.6	14.3
Resorcinol (7)	828	9.4	219	634
Catechol (8)	4014	11.4	6.5	11.9
EZA	3.7	0.32	9.4	0.84
ZNA	14.8	1.1	8.5	38.4
AZA	36.2	0.37	263	0.58

investigated other antioxidant phenol derivatives (including salicylic acid and gallic acid) by using esterase assay. Data of Table 1 show the following regarding inhibition of hCA I, II, IV and bCA III with compounds **1–8**, **EZA**, **ZNA**, and **AZA**, by an esterase assay²⁴, with 4-nitrophenylacetate (4-NPA) as substrate:

1. Against hCA I, compound **7** behave as weak inhibitor, with K_i value in the range of 828.2 μ M. Catechol (**8**) was also an ineffective hCA I inhibitor (K_i of 4014 μ M). A second group of derivatives, compounds **1–6**, **EZA**, **ZNA** and **AZA**, showed better inhibitory activity with K_i values of between 2.2 to 36.2 μ M, (Table 1). Therefore, the nature of the groups in *ortho*-, *para*- and *meta*- to the phenolic OH moiety strongly influences hCA I inhibitory activity. It is also interesting to note that the polyphenolic compounds **1–5** were much better hCA I inhibitors as compared to the corresponding other phenolic compounds **7** and **8** from which they were prepared, with K_i-s in the range of 2.2–12.8 μ M. Acetazolamide **AZA** is also a medium CA I with this assay and substrate against hCA I (K_i of 36.2 μ M). Kinetic investigations (Lineweaver-Burk plots, data not shown) indicate that similarly to sulfonamides, metal ions and inorganic anions^{28–32}, all the investigated compounds act as noncompetitive inhibitors with 4-NPA as substrate, i.e. they bind in different regions of the active site cavity as compared to the substrate. However the binding site of 4-NPA itself is unknown, but it is presumed to be in the same region as that of CO₂, the physiological substrate of this enzyme^{13,29–32}.
2. A better inhibitory activity has been observed with compounds **1** and **3** investigated here for the inhibition of the rapid cytosolic isozyme hCA II (Table 1). Five derivatives, i.e. **2**, **5–8**, showed moderate hCA II inhibitory activity with K_i-s in the range of 2.4–11.4 μ M, Table 1), whereas the remaining two derivatives **4**, **EZA**, **ZNA** and **AZA** were quite effective hCA II inhibitors, with K_i-s in the range of 0.32–1.1 μ M, (Table 1). Structure-activity relationship (SAR) is thus quite sharp for this small series of mono, di,

tri-hydroxy compounds and flavonoids (Figures 2 and 3). The best hCA II inhibitor in this series of derivatives was **4**, which with a K_i of 0.74 μ M. It must be stressed that K_i -s measured with the esterase method are always in the micromolar range because hCA I and II are weak esterases^{28–33}.

- Compound **7** and **AZA** were weak inhibitors of bCA III, with K_i -s of 219 and 263 μ M. However, again compound **5** was medium potency inhibitor (K_i of 21.3 μ M), and compounds **1**, **3**, **EZA** and **ZNA**, show a higher affinity for this isozyme, with inhibition constants in the range of 8.5–11.6 M, whereas the remaining four derivatives **2**, **4**, **6**, **8** were quite effective bCA III inhibitors, with K_i -s in the range of 2.2–6.5 μ M, (Table 1).
- The membrane-anchored isoform hCA IV was poorly inhibited by five of the investigated compounds, that is, compounds **1**, **3**, **5–8** and **ZNA** which showed K_i -s in the range of 9.1–634 μ M. These derivatives incorporate either a 3-substituent in meta to the phenol OH moiety (such as resorcinol **7**). However, compounds **2** and **4** were more effective hCA IV inhibitors, inhibition constants in the range of 4.4 and 5.6 μ M. These compounds are anyhow much weaker inhibitors as compared to the **EZA** and **AZA** (K_i -s of 0.84 and 0.58 μ M against hCA IV, Table 1).

In a recent study it was reported that derivatives of salicylic acid, some natural phenolic compounds, as well as some organic nitrates^{16–20} acts as a CAIs. These simple compounds are lacking the sulfonamide, sulfamate, or related functional groups that are typically found in classical CAIs. It is critically important to explore further classes of potent CAIs in order to detect compounds with a different inhibition profile as compared to the sulfonamides and their bioisosteres and to find novel applications for the inhibitors of these widespread enzymes.

Conclusions

Phenols **6–8** and similar molecules inhibit the activity of CAs due to the presence of the different functional groups present in their scaffold, first of which the phenolic one which probably is anchored to the non-protein zinc ligand²⁸. Flavonoids **1–5** investigated here were quite effective, micromolar inhibitors of several CA isozymes. Our findings indicate thus another category of CAIs of interest, in addition to the well-known sulfonamides/sulfamates/sulfamides, the phenols/ diphenols bearing bulky *ortho* or *para* moieties in their molecules, or the coumarins/polyamines³³. Compound **4** showed the most effective inhibitory activity among derivatives investigated here, with K_i s in the low micromolar range, by the esterase method y . These findings point out that substituted phenolic compounds and flavonoids may be used as leads for generating more potent CAIs eventually targeting other isoforms which have not been assayed yet for their interactions with such agents.

Declaration of interest

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