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SHORT COMMUNICATION

Analysis of saponins and phenolic compounds as inhibitors of α-carbonic anhydrase isoenzymes

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Abstract

A series of phenolic and saponin type natural products such as quercetin, rutin, catechin, epicatechin, silymarin, trojanoside H, astragaloside IV, astragaloside VIII and astrasieversianin X, were investigated for their inhibitory effects against the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). We here report inhibitory effects of these compounds against five α-CA isozymes (hCA I, hCA II, bCA III, hCA IV and hCA VI). Most of the phenolic and saponin type compounds inhibited the isoenzymes quite effectively at low micromolar K_I-s ranging between 0.1 and 4 µM, whereas a few derivatives were ineffective (K_I-s > 100 µM). The results were remarkable which might lead to design of novel CAIs with a diverse inhibition mechanism compared to sulfonamide/sulfamate inhibitors.

Keywords: Carbonic anhydrase, inhibition, natural phenolics, saponins

Introduction

Carbonic anhydrase (EC 4.2.1.1., CA) is a pH regulatory/metabolic enzyme in all life forms. It catalyzes the hydration of carbon dioxide to bicarbonate and the corresponding dehydration of bicarbonate in acidic medium with regeneration of CO_2. Sixteen CA isozymes have been described up to now in mammals, of which the most active catalysts are known as CA II and CA IX. The first one is found primarily in red blood cells but also in many other secretory tissues of the gastrointestinal tract, kidneys, lungs, eye, CNS, etc., whereas the second one is a tumour-associated isoform[1–9]. Other CA isoforms are found in a variety of tissues where they participate in several important biological processes such as acid-base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and electrolyte secretion[1–3]. On this basis, CA isozymes are important therapeutic targets with the potential to be inhibited/activated for the treatment of a range of disorders such as edema, glaucoma, obesity, cancer, epilepsy and osteoporosis[1–7].

Our groups recently investigated the interaction of hCA I, II, VI isozymes with several types of natural and synthetic compounds, such as catechol, resorcinol, progallol, gallic acid, tannic acid and several of their substituted derivatives[10–13]. Here we extend our earlier investigations to a series of compounds (**1–13**), some of which are widely used as antioxidant food additives or as drugs. Among the various natural or synthetic phenolic compounds with antioxidant properties, these compounds are well known for their superior reactive oxygen species quenching abilities[11,12]. They have been reported

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to possess anticancer, antimutagenic, antibacterial, antiviral or anti-inflammatory activities[11–13]. Quercetin (**1**), rutin (**2**), catechin (**3**), epicatechin (**4**), apigenin (**5**), members of the flavonoid family are ubiquitously present in food, including vegetables, fruits, tea, and red wine. Several biological properties of these compounds 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 aging[13]. Silymarin (**6**), a plant derived flavolignan, was isolated from the fruits and seeds of the milk thistle (*Silybum marianum*[14]), being purportedly reported to be useful for the treatment of cancer, varicose veins, menstrual problems, depression, low breast milk production, liver disorders, cirrhosis and chronic hepatitis[14]. Compounds **7–10**, belonging to the saponin class, were purified from *Astragalus pycnocephalus* var. *pycnocephalus* FISCHER by using chromatographic methods. Their structures were determined by means of spectroscopic methods[15–17]. Phenol (**11**), catechol (**12**), and resorcinol (**13**) are widely used prodrugs or disinfectants[12].

In the present study, we have purified human CA I, II, IV, VI, bovine CA III, and investigated the *in vitro* inhibition effects of the compounds **1–13** by using esterase activity of the isoenzymes with 4-nitrophenyl acetate as substrate.

Materials and methods

Chemicals

Quercetin, rutin, catechin, epicatechin, apigenin, silymarin, phenol, resorcinol, catechol, Sepharose 4B, protein assay reagents, 4-nitrophenylacetate (NPA) were obtained from Sigma-Aldrich Co. All other chemicals were analytical grade and obtained from Merck.

Purification of CA isozymes by affinity chromatography

Purification of hCA I and hCA II were previously described[7]. Fresh citrated human whole blood obtained from the Blood Center of the Research Hospital at Atatürk University. Cells were washed three times by centrifugation at 1000*g* at 4 ± 6°C for 20 min in four volumes of 25 mM Na_2HPO_4 (pH = 7.4) buffer. Supernatant and fluffy coat were removed. The erythrocytes were lysed in 10 volumes of 5 mM Na_2HPO_4 (pH = 7.4) buffer, containing 1 mM EDTA. After 20 min, the haemolysate was centrifuged at 10000*g* for 60 min. The particulate fraction was washed four times in the same buffer. The membranes were centrifuged down at 15000*g* for 60 min. pH was adjusted to 8.3 with solid Tris. Sepharose-4B-aniline-sulfanilamide affinity column equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.3). The affinity gel was washed with 25 mM Tris-HCl/25 mM Na_2PO_4 (pH 8.3). Finally, human CA IV (hCA IV) isozyme was eluted with 25 mM Tris-HCl/0.5 M $NaClO_4$ (pH 7.4). Fresh non-citrated human whole blood obtained from the Blood Center of the Research Hospital at Atatürk University. The blood samples were centrifuged at 5000 rpm for 15 min and precipitant was removed. The serum was isolated. The pH was adjusted to 8.7 with solid Tris. Sepharose-4B-aniline-sulfanilamide affinity column equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25mM Tris-HCl/22 mM Na_2SO_4 (pH 8.7). The human CA (hCA VI) isozyme was eluted with 0.25 M H_2NSO_3H/25 mM Na_2HPO_4 (pH = 6.7)[5]. All procedures were performed at 4°C. Bovine CA III was obtained from flank steak and purified using a modification of the method of Tu et al. (1986)[18]. Hundred grams of cubed flank steak was homogenized in small batches in a blender with a total of 150 mL of buffer A (10 mM Tris/H_2S0_4 buffer, pH 8.0, containing 1 mM mercaptoethanol). The resulting product was centrifuged at 4000*g* for 30 min, and the supernatant pooled. This crude enzyme solution was filtered through Whatman No. 1 filter paper. The clear protein solution was passed through a 2 × 50-cm gel exclusion column using the buffer A. The active fractions were determined and concentrated to lyophilizer. This solution was applied to 2.5 × 50-cm ion exchange column and eluted with the buffer A. The active fractions comprising the major protein peak were pooled and concentrated in 1 mM mercaptoethanol.

Esterase activity assay

CA activity was assayed by following the change in absorbance at 348 nm of 4-NPA to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer according to the method described by Verpoorte et al.[19]. The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05 M Tris-SO_4 buffer (pH 7.4), 1 mL 3 mM 4-NPA, 0.5 mL H_2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution.

In vitro *inhibition study*

The inhibitory effects of compound **1–13**, and acetazolamide (**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-NPA was used as substrate at five different concentrations (0.15–0.75 mM). The Lineweaver–Burk curves were drawn[20].

Protein determination

Protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method using bovine serum albumin as a standard[21].

Results and discussion

Phenol **11** binds to CA in a different manner from the classical inhibitors of the sulfonamide type, for example **AZA**, 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[10–12,22]. The X-ray crystal structure of the adduct of hCA II with phenol[22] showed that this compound binds to CA by anchoring its OH moiety to the zinc-bound water/hydroxide ion of the active site through a hydrogen bond, as well as through another 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[6–12,22].

We recently investigated the interactions of phenol and some of its substituted derivatives with all mammalian CAs[10–12], 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 was very variable with inhibition constants ranging from the millimolar to the submicromolar levels for many simple phenols[10–12]. Thus, it seemed reasonable to extend the previous studies[10–12] including, in this investigation, phenolic compounds with clinical and antioxidant applications as food additives, such as compounds **1–6**, **12** and **13**[10–12,15–23]. Other structurally related derivatives such as **7–10**, were also included in our study (Figure 1).

The purification of the CA isozymes was performed with a simple one step method by a Sepharose-4B-aniline-sulfanilamide affinity column chromatoghrapy[24].

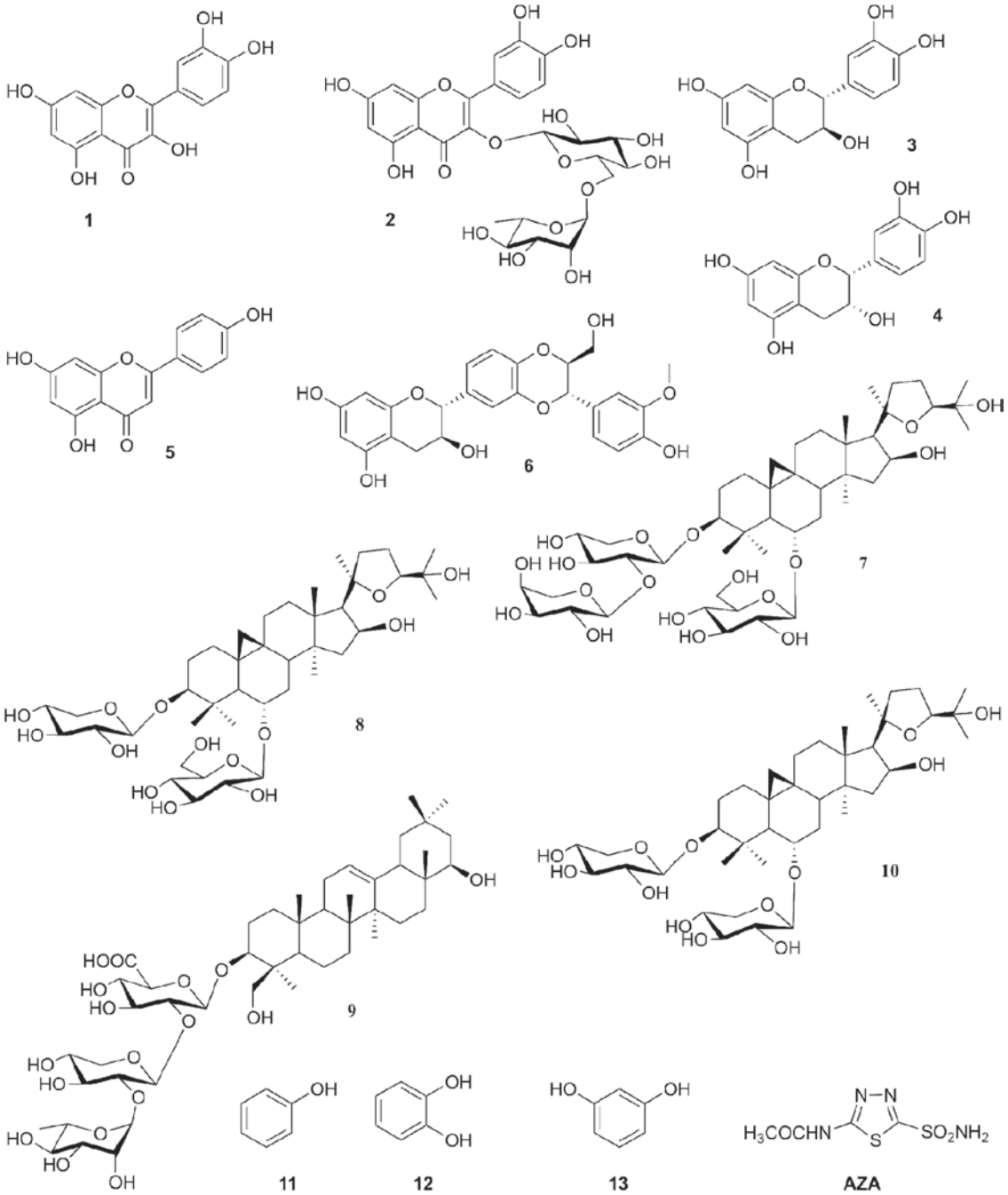

Figure 1. Structures of the compounds **1–13** and **AZA**.

Table 1. K_i values (μM) for compound **1–13** and **AZA** of some α-carbonic anhydrase isoforms in human (h) and bovine (b).

Compound	hCA I	hCA II	bCA III	hCA IV	hCA VI
1	2.68[a]	2.54[a]	3.73	7.89[a]	6.17[a]
2	1.73	0.83	4.77	3.76	7.82
3	2.42[a]	1.84[a]	9.71	4.90[a]	4.91[a]
4	2.32	1.24	8.93	3.98	4.36
5	0.97	0.113	2.21	1.12	1.49
6	1.49[b]	2.51[b]	5.68	8.96[b]	9.70[b]
7	0.94	0.47	1.38	2.41	4.71
8	1.47	0.97	1.93	2.75	6.96
9	1.98	1.13	2.18	3.94	7.37
10	2.21	1.28	2.49	4.36	9.78
11	10.2[c]	5.5[c]	4.63	9.5[c]	208[c]
12	4003[c]	9.9[c]	5.79	10.9[c]	606[c]
13	795[c]	7.7[c]	196	570[c]	550[c]
AZA	36.2	0.37	263	0.578	0.34

[a]Ref 47.
[b]Ref 48.
[c]Ref 49.

hCA I was purified, 111.2-fold with a specific activity of 945.51 EUmg^{-1} and overall yield of 64.3%. hCA II was purified, 792.6-fold with a specific activity of 7186 EUmg^{-1} and overall yield of 72.84%. hCA IV was purifed, 85.15-fold with a specific activity of 734.12 EUmg^{-1} and overall yield of 26.3%. hCA VI was purifed, 71.3-fold with a specific activity of 414.1 EUmg^{-1} and overall yield of 16.8%. Similarly, bCA III was purifed, 49.2-fold with a specific activity of 462.1 EUmg^{-1} and overall yield of 48.2%[3,19–27]. Inhibitory effects of compounds **1–13** on enzyme activities were tested under *in vitro* conditions; K_I values were calculated from Lineweaver–Burk graphs and are given in Table 1[19,20].

For the first time, the inhibitory effects of a group of polyphenolic compounds, quercetin (**1**), rutin (**2**), catechin (**3**), epicatechin (**4**), apigenin (**5**), silymarin (**6**), phenol (**11**), catechol (**12**), resorcinol (**13**), and saponins, trojanoside H (**7**), astragaloside IV (**8**), astragaloside VIII (**9**), astrasieversianin X (**10**), on the esterase activity of hCA I, II, IV, VI and bCA III were investigated. The sulfonamide CAI–**AZA**[1–3] was used as negative control. By using esterase assay, the previous reports by Senturk et al.[11,12] investigated other phenol derivatives including salicylic acid and gallic acid. Based on the results given in Table 1, the following remarks were concluded regarding the inhibition of hCA I, II, IV, VI and bCA III by compounds **1–13** and AZA.

1. Against the slow cytosolic isozyme hCA I, compound **13** behaved as weak inhibitor, with K_I value of 795 μM[10,12]. Catechol (**12**) was ineffective with K_I of 4003 μM, whereas compound **11** (phenol) and **AZA** showed better inhibitory activity with K_I values of 10.2 and 36.2 μM, respectively (Table 1). It was also interesting to note that the polyphenolic compounds **1–6** were stronger hCA I inhibitors as compared to the simple phenolics **11, 12** and **13**, with K_I-s values in the range of 0.97–2.68 μM. The saponins (**7–10**) were found to be strong inhibitors for hCA I with K_I-s values ranging between 0.94 and 2.21 μM. Kinetic investigations (Lineweaver–Burk plots, data not shown) indicated that all the investigated compounds act as noncompetitive inhibitors with 4-NPA as substrate, similar to sulfonamides, metal ions and inorganic anions[2–7,28–38]. It means that 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 CO_2, the physiological substrate of this enzyme[23].
2. Significant inhibitory activities were observed with compounds **5**, **7** for the rapid cytosolic isozyme hCA II (Table 1). Six derivatives, i.e. **1**, **3**, **4**, **6**, **9–13**, showed moderate hCA II inhibitory activity with K_I-s in the range of 1.13–9.9 μM, whereas the remaining two derivatives **2** and **8** were quite effective hCA II inhibitors with K_I-s of 0.83 and 0.97 μM, respectively. The best hCA II inhibitor was found to be **5** with a K_I of 0.113 μM, better inhibitor than **AZA**, a clinically used sulfonamide. 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[34–38].
3. Compound **13** was a weak inhibitor of bCA III with K_I of 196 μM. While compounds **3** and **4** had medium potency (K_I of 8.93 and 9.71 μM, respectively), the phenolic compounds **6**, **11** and **12** showed higher affinity for this isozyme with inhibition constants in the range of 4.63–5.79 mM. **AZA** had K_I of 263 μM, whereas the remaining seven derivatives **1, 2, 5, 7–10** were quite effective bCA III inhibitors with K_I-s in the range of 1.38–3.73 μM (Table 1).
4. The membrane-anchored isoform hCA IV was poorly inhibited by five of the investigated phenols, (**1**, **6** and **11–13**, K_i-s = 7.89–570 μM). Compounds **2–4** and **7–10** were more effective hCA IV inhibitors, acting similarly to the lead **5** with inhibition constants in the range of 1.12–9.5 μM. These compounds were anyhow weaker inhibitors as compared to the **AZA** (K_I of 0.578 μM against hCA IV, Table 1).
5. Phenol (**11**) and some of its congeners such as **12** and **13** were also weak inhibitors of the secreted isozyme hCA VI, with K_I-s of 208–606 μM. Additionally, compounds **1–4** and **6–10** were moderate inhibitors once more (K_I of 4.36–9.78 μM), while the derivative **5** showed higher affinity for this isozyme with inhibition constant of 1.49 μM (Table 1).

In a recent study, it has been reported that the derivatives of salicylic acid act as a CA I inhibitor, and could represent the starting point for a new class of inhibitors that may have advantages for patients with sulfonamide allergies[10,38]. The influences of various substances on different proteins and enzymes have been investigated

by our group[39–46]. However, further investigations are needed in this area for understanding the behaviour of both enzymes and interacting compounds especially for medicinal chemists. CA inhibitors are of particular interest due to their potency to be used as drugs or prodrugs.

Phenolic compounds assayed in this study **1–6** influence the activity of CA enzyme due to the presence of different functional groups present in their aromatic scaffold. Saponins **7–10** are also quite effective against the isoenzymes. The present data point toward another class of possible CAIs of interest, in addition to the well-known sulfonamides/sulfamates/sulfamides, the phenols/biphenyl diphenols bearing bulky *ortho* moieties in their molecules. Compound **5** was the most remarkable inhibitor in the esterase method which usually gives K_I-s an order of magnitude higher as compared to the CO_2 hydratase assay[47–49].

Declaration of interest

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