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Flavones and structurally related 4-chromenones inhibit carbonic anhydrases by a different mechanism of action compared to coumarins

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ABSTRACT

An inhibition study of several carbonic anhydrase (CA, EC 4.2.1.1) isoforms with flavones and aminoflavones, compounds possessing a rather similar scaffold with the coumarins, recently discovered inhibitors of this enzyme, is reported. The natural product flavone and some of its hydroxylated derivatives did not show time-dependent inhibition of the CAs, sign that they are not hydrolyzed within the enzyme active site as the (thio)coumarins and lactones. These compounds were low micromolar inhibitors of hCA I, II, IX and XII, with K_Is in the range of 1.88–9.07 μM. A series of substituted 2-amino-3-phenyl-4*H*-chromen-4-ones, incorporating chloro- and methoxy substituents in various positions of the heterocycle, were then prepared and assayed as hCA I and II inhibitors, showing activity in the micromolar range. Some of these derivatives, as well as *cis* + *trans* resveratrol, were then assayed for the inhibition of all catalytically active mammalian CA isoforms, hCA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV and mCA XV (h = human, m = murine enzyme). These derivatives inhibited these CAs in the submicromolar—low micromolar range. Flavones, although not as active as the coumarins, may be considered as interesting leads for the design of non-sulfonamide CA inhibitors.

Coumarins (2-chromenones) were recently discovered to act as prodrug-type inhibitors[1–3] of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1).[4] These compounds initially bind (probably as benzopyrylium phenol tautomers) to the enzyme active site, and undergo an in situ hydrolytic process of the lactone ring through the esterase activity of the CAs. The 2-hydroxycinnamic acids thus formed thereafter act as the real CA inhibitors (CAIs).[1–4] As substituted 2-hydroxycinnamic acids are rather bulky, due to steric hindrance they cannot bind near the zinc ion from the enzyme active site, where most other classes of inhibitors bind.[4] Thus, the coumarin binding site is unique among al CAIs investigated so far, being at the entrance of the cavity, far away from the catalytically crucial Zn(II) ion, where only CA activators were observed to bind earlier.[5–9] As this is the active site region where the amino acid residues are the most variable among the 16 mammalian CA isoforms known to date, many coumarins showed a high degree of selectivity for inhibiting CA isoforms with pharmacologic relevance for obtaining antiglaucoma (CA IV, CA XII),[10] antiepileptic (CA VII, CA XIV),[11] antiobesity (CA VA, CA VB)[12] or antitumor (CA IX, CA XII)[4,13] agents, over offtarget CA isoforms CA I and II.[1–4] Furthermore, thiocoumarins,[2] 2-thioxo-coumarins,[7] 5- and 6-ring membered lactones/thiolactones[8] and coumarin/δ-lactone oximes,[9] possesssing scaffolds resembling the coumarin one, were recently shown to act as CAIs with a similar mechanism of action as the coumarins, that is, they are prodrugs undergoing active site hydrolysis through the CA esterase activity, with generation of the final inhibitor, which does not interact with the Zn(II) ion from the enzyme active site.

Flavones, 4-chromenones, are isomers of coumarins (2-chromenones), but this class of derivatives was scarcely investigated as CAIs. Only one such paper reported the inhibition of four mammalian CAs, i.e., hCA I, II, bCA III and hCA IV (h = human, b = bovine isoform) with several natural product flavones, such as quercetin, catechin, apigenin, luteolin and morin.[14] In thats paper, an esterase method to assay CA inhibition has been used, with 4-nitrophenyl acetate as substrate.[14] As many CAs are weak esterases, it is not surprising that only micromolar inhibition has been observed with these naturl product flavones in the study by Ekinci et al.[14]

In the present study we investigated in more detail the inhibition mechanism of CAs with flavones on one hand, and on the other one we report the synthesis of a series of 2-amino-4-chromenones,

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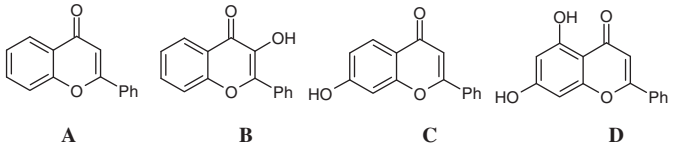
Table 1
CA inhibition data of isoforms hCA I, II, IX and XII with flavones **A–D**, by a stopped-flow CO_2 hydrase assays[15]



A **B** **C** **D**

Compd	K_I[a] (μM)							
	hCA I		hCA II		hCA IX		hCA XII	
	15 min	12 h	15 min	12 h	15 min	12 h	15 min	12 h
A	1.89	1.88	8.71	8.80	4.13	4.22	7.22	7.33
B	5.21	5.10	9.30	9.20	5.54	5.63	6.59	6.65
C	3.37	3.43	2.24	2.32	4.92	4.85	8.40	8.51
D	5.49	5.42	4.38	4.31	2.65	2.71	8.96	9.07

Enzyme and inhibitor were incubated for 15 min (standard assay and for 12 h, respectively).[15]
[a] Errors in the range of ±10% of the reported values, from three different assays (data not shown).

a chemotype not investigated earlier for CA inhibition, and the enzyme inhibitory properties of such compounds against a rather large range of CA isoforms.

Initially, we investigated whether the simple, natural product flavone **A**, 3-hydroxyflavone **B**, 7-hydroxy-flavone **C** and 5,7-dihydroxyflavone **D**, act as CAIs against the physiologically relevant isoforms hCA I, II (cytosolic), IX and XII (transmembrane, tumor-associated enzymes)—Table 1.[15] As seen from data of Table 1, both the standard 15 min incubation between enzyme and inhibitors has been used,[15] as well as the 12 h incubation period, which may afford for an eventual in situ hydrolytic process of the chromenone, as for the coumarins, or as for carbonyl compounds, which have been reported by Pocker's group to be substrates and subsequently inhibitors for CA II.[16,17] Indeed, both aliphatic and aromatic aldehydes, as well as α-ketoesters were reported to be hydrated by CA II, with formation of aldehyde/ketone diols which subsequently inhibited the enzyme activity.[16,17]

Data of Table 1 show that the flavone **A** and its hydroxylated derivatives **B–D** act as low micromolar inhibitors against isoforms hCA I, II, IX and XII, and that the incubation period (of 15 min–12 h) between enzyme and inhibitor has no consequences on the inhibitory power of these compounds, which showed K_Is in the range of 1.88–8.80 μM against hCA I, of 5.10–9.30 μM against hCA II, of 2.24–8.51 μM against hCA IX and of 2.65–9.07 μM against hCA XII, respectively (Table 1). This is a clear sign that the flavones **A–D** do not undergo any hydrolytic process catalyzed by the CAs, and that they inhibit the enzyme in a similar manner with the phenols.[18–20] Simple phenol, PhOH, as well as many of its substituted derivatives, are known to effectively inhibit all CA isoforms, usually in the micromolar range.[18–20] Furthermore, the adduct of hCA II with PhOH has been characterized by means of X-ray crystallography by Christianson's group,[18] who showed this compound to possess a particular inhibition mechanism compared to other classes of CAIs. PhOH was found anchored by means of its OH moiety to the zinc-coordinated water molecule/hydroxide ion, whereas a second hydrogen bond between its OH moiety and the OH of Thr199, further stabilized the adduct.[18]

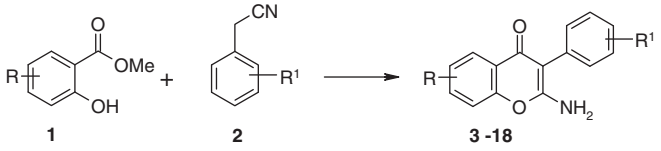
Considering the interesting CA inhibitory data obtained with the simple natural product flavones **A–D**, and using the **A** scaffold as lead molecule, we report the synthesis of a series of aminoflavones and structurally related derivatives, of types **3–23** as well as a coumarin (compound **24**) structurally related to these scaffolds (Schemes 1–3) and their CA inhibitory properties (Tables 2 and 3).

Substituted methyl salicylates **1** and benzylcyanides **2** were the starting materials for obtaining the 2-aminoflavones **3–18**,[21] by a classical cyclization reaction leading to this ring system (Scheme 1).[22] In an analogous manner were then obtained the iminoflavone derivatives **20** and **21**, by using *ortho*-amino methyl benzoates instead of salicylates (Scheme 2). Coumarin **24** was on the other hand obtained by condensation of 5-chloro-salicyclic aldehyde with 4-methoxybenzyl cyanide, as shown in Scheme 3. The moieties present in compounds **3–24** as substituents of the 3-phenyl ring and of the 4-chromenone benzo-moiety were of the halogeno (chlorine) and methoxy type, in order to explore structure–activity relationship (SAR) for such substitution patterns.

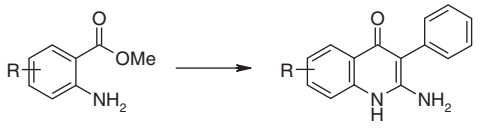
Compounds **3–24** were initially assayed for the inhibition of the major cytosolic isoforms hCA I and II (Table 2). It may be observed that both isoforms were inhibited with IC_{50}-s in the micromolar range, i.e., IC_{50} of 4.61–95.7 μM against hCA I, and of 3.62–36.7 μM against hCA II. The SAR for flavones/coumarins **3–24** is in fact rather straightforward. Thus, for hCA I, three compounds were weakly active, that is, **4, 14** and **16**, possessing IC_{50}-s in the range of 46.2–95.7 μM. The remaining derivatives were around one order of magnitude more effective as hCA I inhibitors, with IC_{50}-s in the range of 4.61–9.85 μM (Table 1). Small structural changes in these compounds led to important differences of inhibitory power. For example compounds **6** and **7**, regiomers differing only by the position of the chlorine atom in position 6 or 7 of the heterocyclic ring, differed by a factor of 5.1 in inhibiting this isoform. Compounds **4** and **20** differ only by the substitution of the endocyclic O atom by an NH group, and again their activity against hCA I differed by a factor of 4.75. It is interesting to note that coumarin **24** was also weakly active (low micromolar inhibitor) against these two isoforms, possessing the same activity as most of the flavones investigated here. In fact, we showed earlier[1b,2,3] that a bulky substituent in position 3 of the coumarin ring, interferes with the hydrolysis of the lactone moiety from the coumarin prodrug inhibitor. As a consequence, 3-substituted coumarins (possessing bulky, aromatic 3-substituents) do not show time-dependent CA inhibition (as other coumarins do)[1b,2,3] but inhibit these enzymes similarly to the phenols, that is, probably a benzopyrylium phenol is the real CAI. This observation[1b] has been reconfirmed here by the inhibition data of compound **24**.

hCA II was also effectively inhibited by compounds **3, 5–13, 15, 17–24**, with IC_{50}-s in the range of 3.62–9.43 μM. Several compounds, among which **4, 14**, and **16**, showed weaker inhibition, with IC_{50}-s in the range of 13.7–36.7 μM (Table 2). SAR was more or less similar with what discussed above for hCA I inhibition, with the nature and position of the substituents on the heterocyclic ring being the main factors influencing enzyme inhibitory activity.

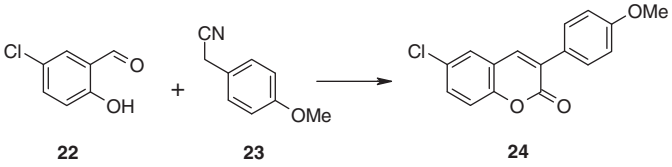
Two of the most active compounds against hCA II, that is, compounds **7** and **10**, as well as another important natural product phenol, resveratrol (as a mixture of 50% cis + 50% trans isomers, **RVT**) were investigated in detail for the inhibition of all catalytically active human isoforms, that is, hCA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV and mCA XV (m = murine isoform, as primates, including humans, do not express CA XV).[23] As seen from data of Table 3, where the inhibition constants (K_I) of these compounds are presented, the three derivatives were low micromolar or submicromolar CAIs. Amino-flavone **7** showed effective inhibition of hCA I and II (K_Is of 0.91–1.25 μM) and was slightly less effective



Scheme 1. Reagents and conditions: THF, NaH, 60 °C, 24 h, then 2 N HCl.

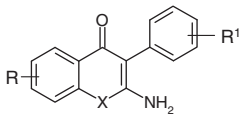


Scheme 2. Reagents and conditions: benzyl cyanide, THF, NaH, 60 °C, 24 h.



Scheme 3. Reagents and conditions: MeOH, 50% aq NaOH, rt, 24 h, then N HCl.

Table 2
Inhibitory activity (IC_{50}, μM)[a] of chromenone derivatives and *cis+trans*-Resveratrol (*c+t*-**RVT**) against hCA I and II



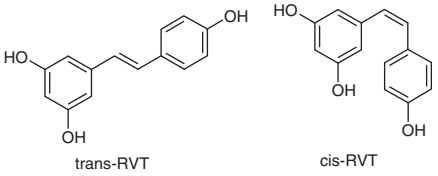
Compd	R	R^1	X	hCA I	hCA II
3	H	H	O	4.87	9.43
4	6-Cl	H	O	46.2	36.2
5	7-Cl	H	O	9.07	6.79
6	H	4-Cl	O	7.66	8.66
7	6-Cl	4-Cl	O	4.61	3.62
8	7-Cl	4-Cl	O	8.12	8.25
9	H	4-MeO	O	8.06	6.51
10	6-Cl	4-MeO	O	9.56	3.66
11	7-Cl	4-MeO	O	7.10	8.07
12	H	3,4-$(MeO)_2$	O	8.37	8.28
13	6-Cl	3,4-$(MeO)_2$	O	7.68	7.59
14	7-Cl	3,4-$(MeO)_2$	O	54.1	13.7
15	7-MeO	H	O	9.85	7.87
16	7-MeO	4-Cl	O	95.7	36.7
17	7-MeO	4-MeO	O	5.85	8.74
18	7-MeO	3,4-$(MeO)_2$	O	8.30	8.35
20	6-Cl	H	N	9.72	9.02
21	7-Cl	H	N	8.51	6.44
24	See Scheme 3			4.81	9.33

[a] Mean from 3 different assays, by a stopped-flow CO_2 hydrase assay.[15]

as inhibitor of hCA IV, VA, VB, hCA IX, hCA XIV and mCA XV (K_Is of 2.27–3.75 μM). hCA III was inhibited with a K_I of 5.90 μM, whereas the remaining isoforms (hCA VI, VII, XII and XIII) were the least inhibited ones, with K_Is of 7.95–9.67 μM. Compound **10**, in which one of the chlorine atoms of 7 was substituted by a methoxy group, also showed interesting inhibitory properties against these isoforms. Thus, hCA I, II, VA, VB, IX, XIV and mCA XV were inhibited with K_Is in the range of 1.65–2.83 μM (Table 3). hCA III and IV showed K_Is in the range of 3.60–4.58 μM, whereas hCA VI, VII, XII and XIII had K_Is in the range of 6.49–8.95 μM with this compound. The mixture of 50% cis + 50% trans isomers of **RVT** also inhibited all CAs in the low micromolar range, with K_Is of 1.75–9.53 μM. The best inhibited isoform was hCA II and the least inhibited one hCA IX.

In conclusion, we present here a detailed ihibition study of several CA isoforms with flavones and aminoflavones, compounds possessing a rather similar scaffold with the coumarins, recently discovered CAIs. The natural product flavone and some of its

Table 3
CA I-XV inhibition data (K_I, μM)[a] with chromenones **7** and **10**, and *cis+trans*-**RVT**



	7	**10**	*c+t*-**RVT**
hCA I	1.25	2.26	2.18
hCA II	0.91	2.01	1.75
hCA III	5.90	4.58	6.75
hCA IV	3.91	3.60	4.01
hCA VA	2.27	2.58	2.86
hCA VB	2.48	2.82	3.24
hCA VI	8.58	8.95	8.63
hCA VII	7.95	6.75	8.98
hCA IX	3.40	2.41	9.53
hCA XII	9.31	6.49	8.13
hCA XIII	9.67	7.85	8.25
hCA XIV	3.28	2.83	1.95
mCA XV	3.75	1.65	2.57

[a] Mean from 3 different assays, by a stopped-flow CO_2 hydrase assay.[15]

hydroxylated derivatives did not show time-dependent inhibition of the CAs, sign that they are not hydrolyzed within the enzyme active site as the coumarins and lactones. These compounds were low micromolar inhibitors of hCA I, II, IX and XII, with K_Is in the range of 1.88–9.07 μM. A series of substituted 2-amino-3-phenyl-4*H*-chromen-4-ones, incorporating chloro- and methoxy substituents in various positions of the heterocycle, were then prepared and assayed as hCA I and II inhibitors, showing activity in the micromolar range. Some of these derivatives, as well as *cis+trans* resveratrol, were then assayed for the inhibition of all catalytically active mammalian CA isoforms, hCA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV and mCA XV. These derivatives inhibited these enzymes in the submicromolar—low micromolar range. Flavones, although not as active as the coumarins, may be considered as interesting leads for the design of non-sulfonamide CAIs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.03.071.

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15. 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) as buffer, and 20 mM $NaClO_4$ (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 (0.1 mM) were prepared in distilled–deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min–12 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 show the inhibition after 12 h incubation, which led to the eventual completion of the in situ hydrolysis of the heterocyclic ring, as reported earlier for couamrins.[1b,2] The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[1b,2] and represent the mean from at least three different determinations. CA isofoms were recombinant ones obtained in house as reported earlier.[1,2]
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21. *General procedure for 3-aryl-4H-chromen-4-ones* **3**–**18**. To a suspension of 80% sodium hydride (40 mmol) in THF (30 mL) was added a methyl salicylate **1** (11 mmol) and a benzyl cyanide **2** (10 mmol). The mixture was stirred at 60 °C for 24 h. After cooling, 2 N hydrochloric acid (20 mL) was added, and the formed precipitate filtered off, air dried, and crystallized with EtOH.
*2-Amino-3-phenyl-4H-chromen-4-one (**3**).* Yield 46%, mp 226–228 °C. ^{1}H NMR: δ 7.24 (s, 2H), 7.44–7.52 (m, 7H), 7.77–8.08 (m, 2H); IR (Nujol) 3484, 3230, 3133, 1636, 1604 cm^{-1}. Anal. Calcd for $C_{15}H_{11}NO_2$: C, 75.94; H, 4.67; N, 5.90. Found: C, 75.90; H, 4.66; N, 5.87.
*General Procedure for 2-Amino-arylquinolin-4(1H)-ones **20**, **21*** To a suspension of 80% sodium hydride (30 mmol) in THF (30 mL) was added benzyl cyanide (1.30 g, 11 mmol) and methyl 4- or 5-chloroanthranilate **19** (1,86 g, 10 mmol). The mixture was stirred at 60 °C for 24 h. The MeOH was then removed by rotary evaporation. Water (30 mL) was added, and the formed precipitate was filtered off, air dried, and crystallized with EtOH.
*Amino-6-chloro-3-phenylquinolin-4(1H)-one (**20**).* Yield 78%, mp 246–248 °C. ^{1}H NMR: δ 5.84 (s, 2H), 7.41–8.03 (m, 8H), 11.30 (s, 1H); IR (Nujol) 3493, 3263, 1640, 1628 cm^{-1}. Anal. Calcd for $C_{15}H_{11}ClN_2O$: C, 66.55; H, 4.10; N, 10.35. Found: C, 66.49; H, 4.12; N, 10.33.
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