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Coumarins incorporating hydroxy- and chloro-moieties selectively inhibit the transmembrane, tumor-associated carbonic anhydrase isoforms IX and XII over the cytosolic ones I and II

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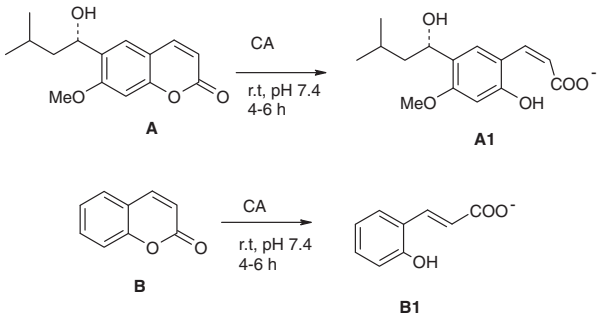
ABSTRACT

A series of coumarins incorporating hydroxy-, chloro- and/or chloromethyl-moieties in positions 3-, 4-, 6- and 7- of the heterocyclic ring were investigated for the inhibition of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1). These coumarins were very weak or ineffective as inhibitors of the house-keeping, offtarget isoforms CA I and II, but showed effective, submicromolar inhibition of the transmembrane, tumor-associated isoforms CA IX and XII. The nature and position of the groups substituting the coumarin ring greatly influenced CA inhibitory properties. 6-Hydroxycoumarin showed K_Is >100 μM against CA I and II, of 0.198 μM against CA IX and of 0.683 μM against CA XII, being thus a selective, efficient inhibitor for the tumor-associated over cytosolic isoforms. These compounds are also excellent leads for designing isoform-selective enzyme inhibitors.

Coumarins constitute a novel class of inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1), and their mechanism of action is different from that of all other known inhibitors.[1,2] Indeed most CA inhibitors (CAIs) investigated to date directly interact with the metal ion (which is Zn(II) in α-CAs) from the enzyme active site, directly coordinating to it (inorganic anions, sulfonamides and their isosteres, etc.)[3,4] or anchoring to the zinc-bound water molecule/hydroxide ion through a network of hydrogen bonds (phenols,[5] polyamines[6]) which stabilize the enzyme-inhibitor adduct. Whereas metal-complexing anions are weak CAIs, with affinities generally in the millimolar range,[7] sulfonamides and their isosteres (sulfamates, sulfamides, etc.) easily arrive to a low nanomolar inhibition potency.[3,4] Phenols and polyamines have an intermediate potency between the two extremes mentioned above (micromolar–nanomolar range, depending on the isoform and the substitution pattern of the inhibitor scaffold).[5,6] There are many X-ray crystal structures of adducts of all these classes of CAIs with several CA isoforms (of the 16 presently known in mammals),[2,3] which undoubtedly prove these different binding modes of the inhibitor to the enzyme.[7]

In contrast to CAIs which interact directly or indirectly with the metal ion, coumarins are mechanism based inhibitors, which may also be considered as prodrugs. Recently[1,2] we showed that the natural product coumarin **A** or the very simple non-substituted derivative **B** (and many of its congeners possessing various substitution patterns at the coumarin ring)[2] act as effective CAIs against many of the mammalian isoforms CA I–CA XV, but the real enzyme inhibitor is constituted by the hydrolyzed coumarins, such as compounds **A1** and **B1** (Scheme 1), formed from the original coumarins **A** and **B**, respectively. They have been evidenced by X-ray crystallography of enzyme-inhibitor adducts (Fig. 1) and investigated in detail also by kinetic methods.[1,2] The 2-hydroxy-cinnamic acids thus formed (**A1** and **B1**), bind in an unprecedented way to the



Scheme 1. Formation of 2-hydroxy-cinnamic acids **A1** and **B1** by the CA-mediated hydrolysis of coumarins **A** and **B**.

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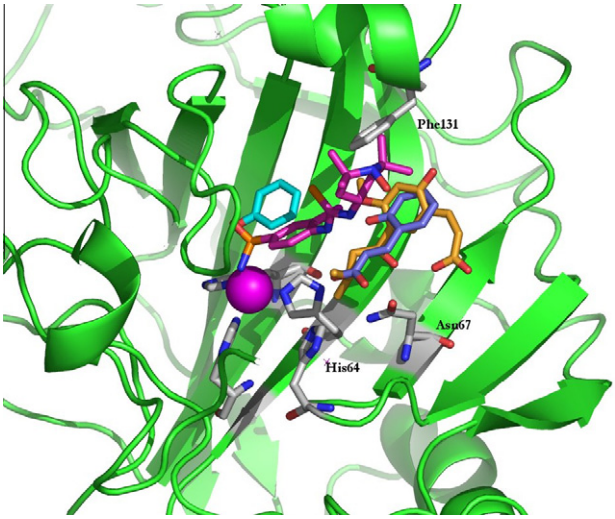


Figure 1. Superposition of the hCA II—**A1** adduct (gold, PDB file 3F8E), hCA II—**B1** adduct (dark blue, PDB file 4F8E) with the hCA II—phenol adduct **C** (sky blue, PDB file not deposited) and hCA II—sulfonamide **D** adduct (magenta, PDB file 3EFT).

enzyme,[1,2] at the entrance of the active site cavity, plugging the entire entrance to it, as shown in Figure 1. Only very recently, some fullerene derivatives were shown to bind in a similar way to the CAs.[8] Occlusion of the CA active site entrance, by hydrolyzed coumarins (i.e., *cis*- or *trans*-2-hydroxy-cinnamic acids)[1,2] or fullerenes[8] thus constitutes a totally novel mechanism of CA inhibition, which may be exploited to design compounds with various applications.[2]

As seen in Figure 1, the binding site of the hydrolyzed coumarins, at the entrance of the CA active site, is very different from the site where phenol **C** binds (deep within the active site, interacting with the zinc-bound solvent molecule)[5] or from the sulfonamide binding site, exemplified in our case by the spin-labeled benzenesulfonamide **D**, for which the X-ray crystal structure in adduct with the physiologically dominant isoform human (h) hCA II has been recently reported.[9] Indeed, the scaffold of this sulfonamide extends throughout the entire active site, whereas the sulfamoyl group (in deprotonated state) is coordinated to the Zn(II) ion. It may be observed the total lack of overlap of the coumarin-, phenol- or sulfonamide-binding sites when these inhibitors are within the enzyme cavity (Fig. 1).

CAIs of the sulfonamide type are clinically used for decades, for various classes of diuretics and systemically acting antiglaucoma agents.[3,4,10] In the last years novel applications emerged for this class of pharmacological agents, such as the topically acting antiglaucoma agents, anticonvulsants, antiobesity, antipain, and antitumor drugs/diagnostic tools.[3,4,10–13] However critical barriers to the use of CAIs as therapeutic agents are related to the high number of isoforms in humans (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.[3,4,10–13] Thus, there is a stringent need of CAIs with a more selective inhibition profile compared to the sulfonamides and their isosteres, and the coumarins represent an interesting class due to several reasons: (i) the inhibition mechanism is different from those of other CAIs, and due to their binding at the entrance of the enzyme active site, where there is a rather large variability in the 3D structure and amino acid sequence between the various isoforms, may lead to compounds with isoform-selective behavior. Indeed, we have already evidenced several such CA IX-selective, CA XIII-selective, and CA VII-selective coumarin CAIs in the previous work;[2] (ii) coumarins are widespread natural products[14] and are also easily prepared synthetically with a large range of substitution patterns, incorporating moieties of diverse chemical nature, in different numbers of such moieties and in diverse positions of the coumarin ring. This has as a consequence the possibility to generate rather large and diverse libraries of compounds to be investigated for their interactions with the various CA isoforms, with the goal to search isoform-selective compounds targeting all the mammalian isozymes with medicinal chemistry applications (such as CA II and XII for antiglaucoma drugs; CA VA and VB for antiobesity agents; CA VII for antiepileptics; CA IX and XII for antitumor drugs/diagnostic tools for cancer, etc.).[3,4] Continuing our interest in investigating coumarins as CAIs, we report here a study of a series of simple coumarin derivatives incorporating hydroxyl- and chloromoieties in various positions of the heterocyclic ring, which behave as weak or highly ineffective inhibitors of the house-keeping (off-target) cytosolic isoforms hCA I and II, whereas acting as effective inhibitors against the transmembrane, tumor-associated isoforms hCA IX and XII, which are established anticancer drug targets.[12,15]

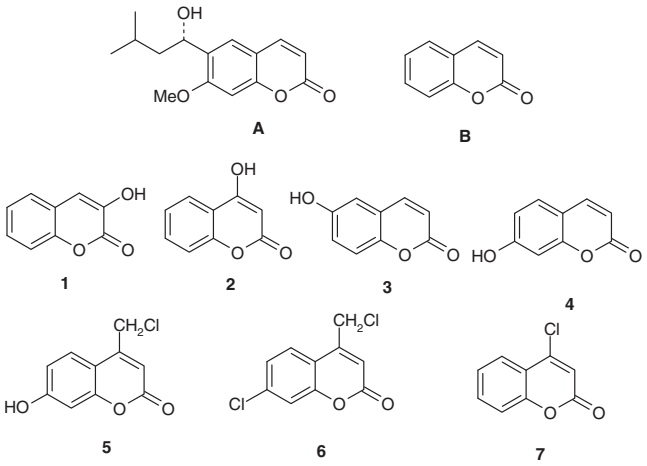
Inhibition data with simple hydroxy-, chloro- and/or chloromethyl-substituted (in various positions of the heterocyclic ring) coumarins **1–7**[16] against four CA isozymes, that is, hCA I, II, IX, and XII,[17] are shown in Table 1. Inhibition data of the two coumarins **A** and **B**, investigated earlier in detail for their interaction with these enzymes,[1,2] are also provided in Table 1, for comparison reasons. The following structure–activity relationship (SAR) observations can be drawn from data of Table 1:

(i) The slow cytosolic isoform hCA I was weakly inhibited by coumarins **1–7**, with inhibition constants in the range of 58.4 to >100 μM, unlike the natural product **A** which was a very effective inhibitor (K_I of 0.078 μM) or the simple lead **B**, which was a medium potency inhibitor (K_I of 3.1 μM). The 'best' hCA I inhibitor among the newly investigated derivatives was 7-hydroxy-coumarin **4** (K_I of 58.4 μM) which was anyhow 748 times weaker than **A**. Thus, irrespective of the position of the hydroxy moiety in the coumarin ring, in the 2-, 3-, 6- or 7-position, or whether this groups is replaced by a chlorine atom or a chloromethyl group, these compounds were very weak or totally ineffective hCA I inhibitors. This is an extremely desirable feature since hCA I is not a drug target, but an offtarget, being a widely expressed isoform in many tissues and cell types and possessing house-keeping physiological functions.[3,4]

(ii) The second offtarget isoform, hCA II, which is in fact the physiologically dominant one due to the fact that it has the highest catalytic efficiency among the α-CAs,[3] and is present in many organs/tissues in which is responsible both for pH homeostasy and electrolyte secretion, among others,[3,4] was not inhibited at all by coumarins **1–7** investigated here (K_Is >100 μM). This is a very significant result, especially considering the fact that **A** was an effective hCA II inhibitor (K_I of 0.059 μM) whereas other such derivatives, including the unsubstituted coumarin **B** showed medium-weak hCA II inhibition (for example **B** showed a K_I of 9.2 μM).[1,2] As far as we know, this is the first example in the literature of a class of CAIs which do not inhibit at all hCA II, an isoform extremely sensitive to sulfonamide and sulfamate inhibitors, which leads to a range of side effects reported for these drugs.[3,4]

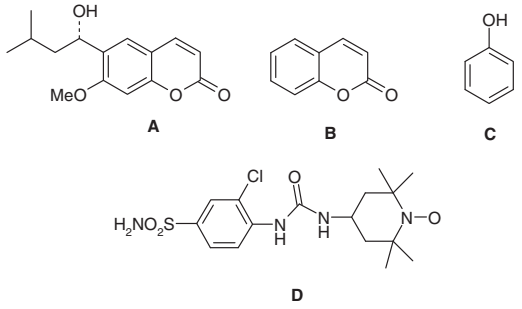
(iii) Although the tumor-associated hCA IX is not significantly inhibited by coumarins **A** and **B**,[1,2] most of the derivatives **1–7** investigated here showed effective inhibition, with K_Is in the range of 0.198–4.92 μM (Table 1). The most effective hCA IX inhibitors were 6-hydroxycoumarin **3** and 7-chloro-4-chloromethyl-coumarin **6**, with K_Is of 0.198–0.359 μM.

Table 1
hCA I, II, IX and XII inhibition data with coumarins **1–7** and **A**, **B** (as standard inhibitors), by a stopped-flow, CO_2 hydration assay method (6 h incubation time between enzyme and coumarin)[17]



Compound	K_I (μM)			
	hCA I[a]	hCA II[a]	hCA IX[b]	hCA XII[b]
A	0.078	0.059	54.5	48.6
B	3.1	9.2	>100	>100
1	79.4	>100	0.508	9.60
2	95.0	>100	0.418	6.30
3	>100	>100	0.198	0.683
4	58.4	>100	0.482	0.754
5	>100	>100	0.478	8.02
6	72.8	>100	0.359	0.735
7	86.9	>100	4.92	7.03

[a] Full length, cytosolic isoform.
[b] Catalytic domain, recombinant enzyme.



4-Chlorocoumarin **7** was the least effective one, with a K_I of 4.92 μM, whereas the remaining derivatives (**1, 2, 4**, and **5**) had inhibition constants in the submicromolar range (K_Is of 0.418–0.508 μM, Table 1). Thus, the nature of the substituents and their position on the coumarin ring strongly influence activity as CAIs, as we already reported earlier for another series of coumarins with various substituents in diverse positions of the heterocyclic ring.[2] From these data it can be observed that the best substitution patterns for hCA IX inhibition were those present in **2–6**, with the OH moiety in the 4-, 6- or 7-position of the heterocyclic ring. These moieties are also easily amenable to derivatization, since these phenolic OH moieties show a high reactivity.[1,2,14]

(iv) The same behavior as that observed for hCA IX was also detected for the inhibition of the second tumor-associated isoform, hCA XII; with the coumarins investigated here. Thus, the leads **A** and **B** were ineffective as hCA XII inhibitors (K_Is >48.6 μM) whereas **1–7** behaved as much more effective inhibitors, with K_Is in the range of 0.683–9.60 μM. Coumarins **3, 4**, and **6** showed submicromolar hCA XII inhibition (K_Is of 0.683–0.754 μM) whereas the remaining ones were low micromolar inhibitors (K_Is of 6.30–9.60 μM, Table 1). SAR is thus different compared to the inhibition of hCA IX, but again the nature and the position of the substituting groups at the coumarin ring influence activity significantly. Compound **3** was one of the most interesting leads detected here, as it did not act as hCA I and II inhibitor, but had submicromolar activity against the tumor-associated isoforms hCA IX and XII, with K_Is of 0.198–0.683 μM. Furthermore, it can be easily derivatized at the phenolic moiety and may lead to a large number of derivatives to be investigated for their interaction with various CA isoforms.

In conclusion, we report here that a series of coumarins incorporating hydroxy-, chloro- and/or chloromethyl- moieties in positions 3-, 4-, 6- and 7- of the heterocyclic ring show interesting CA inhibitory properties. These coumarins were very weak or ineffective as inhibitors of the house-keeping, offtarget isoforms CA I and II, but showed effective, submicromolar inhibition of the transmembrane, tumor-associated isoforms CA IX and XII. The nature

and position of the groups substituting the coumarin ring greatly influenced CA inhibitory properties. For example, 6-hydroxycoumarin showed K_Is >100 μM against CA I and II, of 0.198 μM against CA IX and of 0.683 μM against CA XII, being thus a selective, efficient inhibitor for the tumor-associated over cytosolic isoforms. These compounds are thus excellent leads for designing isoform-selective enzyme inhibitors.

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17. 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 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 (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min to 72 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 6 h incubation, which led to the completion of the in situ hydrolysis of the coumarin and formation of the hydroxy-cinnamic acid.[1,2] The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[1,2] and represent the mean from at least three different determinations. CA isoforms were recombinant ones obtained in house as reported earlier.[1,2]