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Non-Zinc Mediated Inhibition of Carbonic Anhydrases: Coumarins Are a New Class of Suicide Inhibitors#

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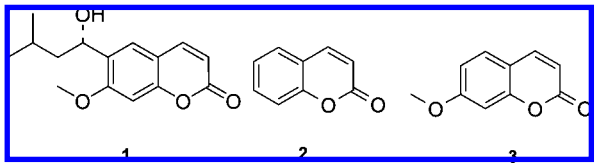
Abstract: The X-ray crystal structure of the adduct between the zinc metalloenzyme carbonic anhydrase II (CA, EC 4.2.1.1) with the recently discovered natural product coumarin derivative 6-(1*S*-hydroxy-3-methylbutyl)-7-methoxy-2*H*-chromen-2-one showed the coumarin hydrolysis product, a *cis*-2-hydroxy-cinnamic acid derivative, and not the parent coumarin, bound within the enzyme active site. The bound inhibitor exhibits an extended, two-arm conformation that effectively plugs the entrance to the enzyme active site with no interactions with the catalytically crucial zinc ion. The inhibitor is sandwiched between Phe131, with which it makes an edge-to-face stacking, and Asn67/Glu238sym, with which it makes several polar and hydrogen bonding interactions. This unusual binding mode, with no interactions between the inhibitor molecule and the active site metal ion is previously unobserved for this enzyme class and presents a new opportunity for future drug design campaigns to target a mode of inhibition that differs substantially from classical inhibitors such as the clinically used sulfonamides and sulfamates. Several structurally simple coumarin scaffolds were also shown to inhibit all 13 catalytically active mammalian CA isoforms, with inhibition constants ranging from nanomolar to millimolar. The inhibition is time dependent, with maximum inhibition being observed after 6 h.

Introduction

In a recent communication[1] one of our groups identified a novel natural product inhibitor of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1)[2] following screening of a large number of natural product extracts using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) as a bioaffinity screening tool. To the general surprise, this CA inhibitor (CAI) isolated from the Australian plant *Leionema ellipticum* Paul G. Wilson (Rutaceae) was a coumarin derivative, namely 6-(1*S*-hydroxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one (**1**). Coumarins represent a novel structural motif compared to all CAIs investigated to date.[2]



The CA family of enzymes is widespread all over the phylogenetic tree with 16 different α-CA isozymes presently known in mammals, among which 13 show catalytic activity.[2] CAs catalyze a simple, yet critical, reaction: the reversible hydration of carbon dioxide to a bicarbonate anion and a proton, i.e., $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$. This reaction does not proceed with an appreciable rate under physiological conditions in the absence of CA. The Zn^{2+} core of CA serves an essential function: it is a strong Lewis acid that binds to and activates the hydrating water molecule. The Zn^{2+}coordinated water has a pK_a of ∼7 compared to the pK_a of bulk water of ∼14. CA thus facilitates deprotonation of water to generate the strongly basic hydroxide anion (OH^-) which is the reactive species in the hydration of CO_2 leading to formation and release of HCO_3^-.[2] CAs are inhibited by sulfonamides, their bioisosteres

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The X-ray coordinates of the hCA II−**4** adduct are available in PDB with the ID code 3F8E.

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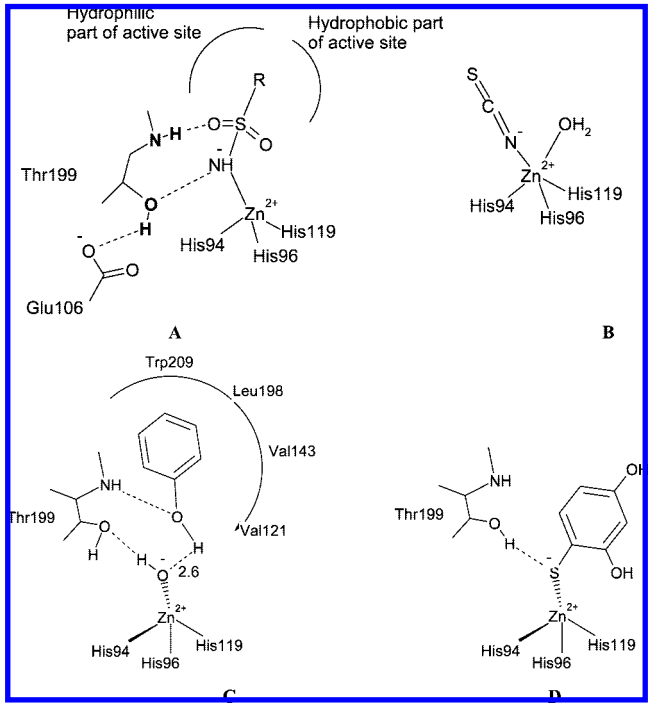

Figure 1. Schematic representation for the interactions of the four classes of known CAIs with the enzyme active site: (A) Sulfonamides (and their isosteres, sulfamates, sulfamides, etc.). (B) Inorganic anion inhibitors (thiocyanate as an example). (C) Phenol(s).[7] (D) Thiophenols (4-mercapto-benzene-1,3-diol).[8]

(sulfamates, sulfamides, etc), metal complexing anions, phenols, and thiophenols.[2–4] These inhibitors bind as shown schematically in Figure 1.

CAs are involved in numerous physiological and pathological processes, including respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiological and pathological processes in humans, as well as the growth and virulence of various fungal/bacterial pathogens.[3,4,9−11] In addition to the established role of CAIs as diuretics and antiglaucoma drugs, it has recently emerged that they have potential as anticonvulsant, antiobesity, anticancer, and antiinfective drugs.[2,4] Many of the CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited or activated to treat a wide range of disorders.[2,4,9–11] However a critical barrier to the design of CAIs as therapeutic agents is related to the high number of isoforms, their diffuse localization in many tissues/organs, and the lack of isozyme selectivity of the presently available inhibitors.[2–4] Thus the coumarin **1**, a completely new structural motif for the inhibition of this enzyme class,[1] constitutes a valuable lead for researchers working in the design of CAIs.

As coumarin **1** does not possess any of the zinc-anchoring groups present in known CAIs, it appeared of great interest to elucidate the CA inhibition mechanism of this new type of derivative as well as to investigate the inhibition profile against the mammalian catalytically active isoforms (13 such CAs are known up until now).[2] Herein we report a detailed inhibition study with the simple coumarin **2**, its 7-methoxy derivative **3** (the 7-methoxy groups is present in inhibitor **1** identified in the Australian plant),[1] and the originally reported CAI belonging to this class, i.e., 6-(1*S*-hydroxy-3-methylbutyl)-7-methoxy-2*H*-chromen-2-one **1**.

Table 1. Inhibition of Mammalian Isozymes CA I−XV with Coumarins **1**−**3** and *trans*-2-Hydroxy-cinnamic acid **5E** by a Stopped-Flow, CO_2 Hydration Assay Method[13]

	K_I (μM)[c]			
isozyme[a]	**1**[d]	**2**[d]	**3**[d]	**5E**[e]
hCA I	0.08	3.1	5.9	3.4
hCA II	0.06	9.2	0.07	9.3
hCA III	>1000	>1000	161	nt
hCA IV	3.8	62.3	7. 8	nt
hCA VA	96.0	>1000	645	nt
hCA VB	17.7	578	48.6	nt
hCA VI	35.7	>1000	61.2	nt
hCA VII	27.9	>1000	9.1	nt
hCA IX[b]	54.5	>1000	767	nt
hCA XII[b]	48.6	>1000	167.4	nt
mCA XIII	7.9	>1000	6.0	nt
hCA XIV	7.8	>1000	9.7	nt
mCA XV	93.1	>1000	>1000	nt

[a] h = human; *m* = murine isozyme; nt = not tested. [b] Catalytic domain. [c] Errors in the range of ±5% of the reported data from three different assays. [d] Preincubation of 6 h between enzyme and inhibitor. [e] Preincubation 15 min between enzyme and inhibitor.

Results and Discussion

CA Inhibition. Inhibition data for compounds **1**−**3** against the 13 catalytically active mammalian (*h* = human, *m* = murine) CA isoforms CA I−IV, VA, VB, VI, VII, IX, XII−XV are presented in Table 1. The data have been obtained by a stopped-flow technique monitoring the physiological reaction catalyzed by these enzymes, i.e., CO_2 hydration to bicarbonate and protons.[13] When assaying all other types of CAIs, such as sulfonamides, inorganic complexing anions, phenols, or thiophenols, an incubation time of 10−15 min is allowed for the formation of the enzyme−inhibitor adduct.[6,7,12,13] Working with the same conditions, only a weak micromolar to millimolar inhibition was observed with coumarins **1**−**3** (data not shown). These observations prompted us to investigate the effect of the incubation time between the enzymes and coumarins **1**−**3** on

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CA inhibition. Inhibition constants were measured after 30 min, 1 h, 4 h, 6 h, and 24 h of incubation. For all investigated CA isoforms we observed a progressive decrease of the inhibition constants with the increase of the incubation time up to 6 h, while from 6 to 24 h of incubation no further changes of K_I were evidenced. Table 1 reports the inhibition constants for coumarins **1**−**3** following 6 h of incubation time with the enzymes. Against isoforms I and II (ubiquitous, cytosolic CAs)[2] **1**−**3** show effective inhibition, with K_I's in the range 80 nM−5.9 μM (hCA I) and 60 nM−9.2 μM (hCA II), respectively. The best hCA I and II inhibitor was always the natural product coumarin **1**. The remaining CA isoforms were typically inhibited only weakly by the simple coumarin derivatives **2** and **3** (many of them showed K_I's > 1000 μM) with few exceptions, notably compound **3** against CA IV, VII, XIII, and XIV (K_I's in the range 6.0−9.1 μM). The natural product **1** was an effective inhibitor of all CAs investigated here (except CA III, K_I > 1000 μM), with inhibition constants in the range 3.8−93.1 μM against isoforms CA IV−XV (Table 1). The results observed with this panel of coumarin derivatives show that there is a wide distribution of inhibition constants for *the same compound* against the various CA isoforms (e.g., **1** shows inhibition constants in the range 59 nM−1000 μM). The inhibitory properties of **1**−**3** are also dependent on the substitution pattern of the coumarin ring, with the number of these groups influencing inhibitory activity. The least active coumarin **2** lacks substitution on the aromatic coumarin scaffold and coumarin **1** has two substituents (in the 6 and 7 positions), while coumarin **3** has a single substituent (7 position). Results presented in Table 1 demonstrate that substitution leads to an increase in the CA inhibitory activity against many of the CA isozymes. Thus, this newly identified chemotype in the context of CAIs is indeed very promising for obtaining novel types of potent and potentially more isozyme-selective inhibitors as compared to the classical sulfonamide class of CA targetting drugs.

X-Ray Crystallography and Mass Spectrometry. To understand the inhibitory mechanism with this new class of CAI, we resolved the X-ray crystal structure (at a resolution of 2.0 Å) of the most novel and potent of these inhibitors—the natural product coumarin **1**—in adduct with the physiologically dominant CA isoform,[2] hCA II.[17−22]

Inspection of the electron density maps (Figure 2) at various stages of the refinement showed features compatible with the presence of one molecule of inhibitor bound within the active site, but compound **1** could not be fitted in the observed electron density. Instead, its hydrolysis product, the *cis*-2-hydroxy-4-(1*S*-3-methylbutyl)-3-methoxy-cinnamic acid **4Z** (Chart 1), perfectly fits within this electron density (Figure 2).

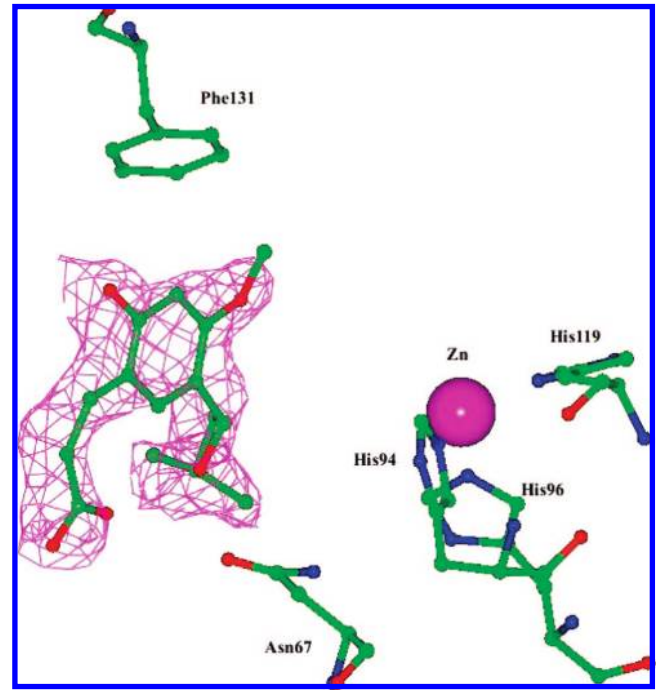
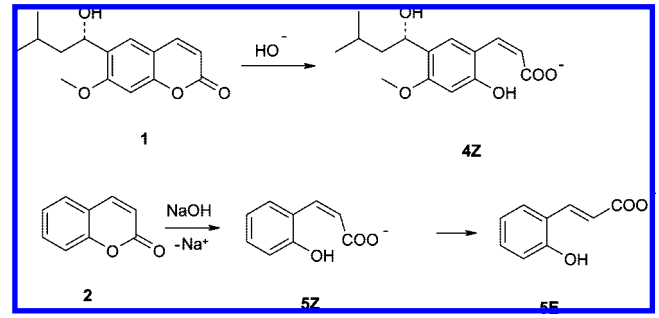


Figure 2. Omit map corresponding to the hydrolyzed coumarin **1** (i.e., the *cis*-2-hydroxy-cinnamic acid derivative **4Z**), of some relevant CA II active site residues and the Zn(II) ion (violet sphere).

Chart 1. Hydrolysis of Coumarins **1** and **2** to the Corresponding Hydroxy-cinnamic Acid Derivatives **4Z** and **5Z/5E**



Thus, the zinc bound hydroxide anion of the CA enzyme that is responsible for the various catalytic activities of CAs[2−4] including the esterase activity[14] appears likely to have hydrolyzed the lactone ring of **1** leading to the formation of **4Z**. Interactions between the protein and Zn^{2+} ion were entirely preserved in the hCA II−**4Z** adduct (data not shown).[6,7] The inhibitor **4Z** was found bound at the entrance of the active site cavity (Figure 3) with the two bulky arms of **4Z** in an extended conformation effectively plugging this entrance. The hydroxypentyl arm orients toward the hydrophilic half of the active site, while the *cis*-carboxyethylene arm orients toward the hydrophobic half.[6,7] The methoxy and carboxyl moieties of the inhibitor participate in several hydrogen bonds with the active site (amino acid residues and water molecules). Moreover, the 1*S*-OH moiety of the hydroxypentyl arm makes a strong dipole−dipole interaction with the carbonyl oxygen of the side chain of Asn67. An edge-to-face (CH−π)[15] stacking between the aromatic ring of **4Z** and the phenyl group of Phe131 was also evidenced (with a distance of ∼3.5 Å between them). This

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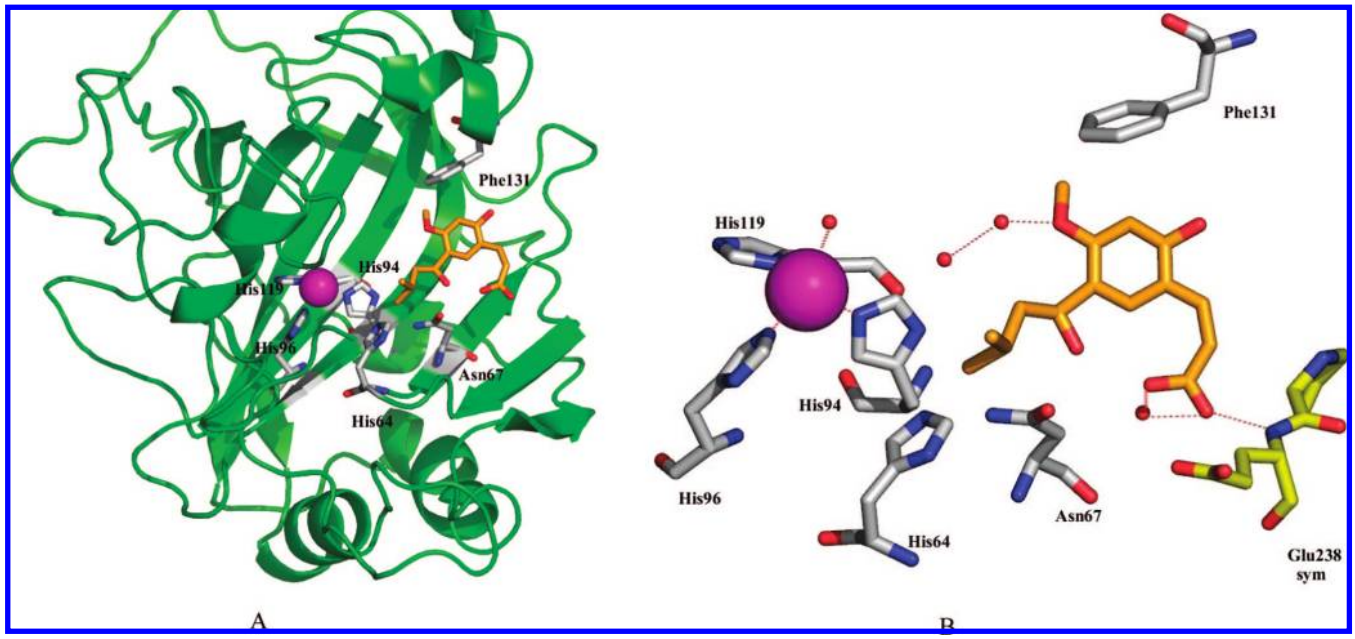

Figure 3. (A) The hCA II−**4Z** adduct. The protein is represented as green ribbon, the catalytic Zn(II) ion is represented as the violet sphere, and its three protein ligands (His94, 96, and 119) are in CPK colors, as stick models. The inhibitor **4Z** (in gold) and amino acid residues interacting with it (Phe131, Asn67) are also evidenced, as well as His64, a residue involved in the catalytic cycle and acting as a proton shuttle [2–4] between the zinc-bound water molecule and the buffer. (B) Detailed interactions between CA II and inhibitor **4Z** when bound to the enzyme. The catalytic Zn(II) ion is shown as violet sphere, with its three His ligands (His94, 96, and 119) and coordinated water molecule (red smaller sphere) also evidenced. The inhibitor molecule (gold) interacts with three active site ordered water molecules (red spheres), with Phe131 and Asn67 (CPK colors) from the active site as well as with Glu238sym (yellow) from a symmetry related enzyme molecule. The proton shuttle residue His64 is also shown (CPK colors).

latter amino acid residue is also important in the binding mode of classical sulfonamide inhibitors to this enzyme.[16] In summary, hydrolyzed coumarin inhibitor **4Z** is sandwiched between the phenyl moiety of Phe131 and the side chains of Asn67 and Glu238sym (the last amino acid residue from a symmetry related enzyme molecule in the crystal packing) with three active site water molecules further stabilizing the interaction of **4Z** with the enzyme, Figure 3. The hydrolysis product of coumarin **1**, the *cis*-2-hydroxy-cinnamic acid derivative **4Z**, binds very differently to the hCA II active site as compared the four classes of known CAIs which interact with the metal ion or the water coordinated to it (cf. Figure 1 and discussion above). This different type of binding has not been evidenced before for any CAI.

Several experiments afforded supplementary confirmation regarding the proposed CA inhibition mechanism by coumarins, as follows:

(i) Incubation of compounds **1**−**3** for 30 min−24 h with the buffer used in the enzyme assay (in the absence of enzyme) allowed us to observe the same weak inhibition measured with the original compounds **1**−**3** when a 15 min incubation time between enzyme and test inhibitor was employed (data not shown). Thus, coumarins **1**−**3** are not hydrolyzed spontaneously by the buffer used in the enzyme inhibition assay.

(ii) The hydrolysis product of coumarin **2**, the simple *trans*-2-hydroxy-cinnamic acid **5Z** (as the sodium salt), has been prepared by hydrolysis of **2** with sodium hydroxide, as described in the literature.[23] Compound **5Z** was then investigated for its inhibition of hCA I and II (Table 1). This hydrolyzed compound inhibits the two CAs with the same magnitude as the parent coumarin **2** (within the limits of experimental errors of the assay). This observation is consistent with our proposal that the true CA enzyme inhibitor is the hydrolyzed compound (e.g., **4Z** and **5Z** and/or **5E**), not the parent coumarins **1** and **2**. It should be mentioned that in solution the stable form of hydrolyzed **2** is the *E*-isomer **5E**,[23] but literature data[24] show that the *cis*−*trans* isomerization of cinnamic acids is a readily observed process, in various experimental conditions, mainly in the presence of nucleophiles. It is noteworthy that, in the X-ray crystal structure discussed above, we observe only the *Z*-isomer of cinnamic acid **4Z** to be stabilized when bound to the enzyme active site, although in solution probably the *E*-isomer would be favored.[23,24] It should be also mentioned that treatment of the sodium salt **5Z/5E** with dilute acid leads to the immediate formation of the original coumarin **2** (data not shown) as described in the literature.[23,24] Based upon these data we propose the following to explain our experimental observation that coumarins act as suicide inhibitors of CA. Coumarins may undergo hydrolysis by the Zn^{2+}-activated water molecule/hydroxide ion of the enzyme cavity, which acts as a very potent nucleophile.[2] It is thus possible that the *cis*-2-hydroxy-4-(1*S*-3-methylbutyl)-3-methoxy-cinnamic acid **4Z** is formed within the enzyme cavity by hydrolysis of bound **1**, which thereafter cannot bind effectively in the restricted space near the Zn^{2+} ion due to its bulky pendant arms. A rearrangement of the enzyme−inhibitor adduct may then occur (with the movement of the bulky **4Z** toward the exit of the active site), leading to the effective binding of the hydrolyzed species of **1**, namely compound **4Z** in the active site region in which we observed it by X-ray crystallography. Thus, the initially weak inhibitor **1** (a K_I of 10.8 μM was measured for **1** when an incubation time of 15 min between enzyme and inhibitor was used; data not shown), by acting as a substrate to the enzyme

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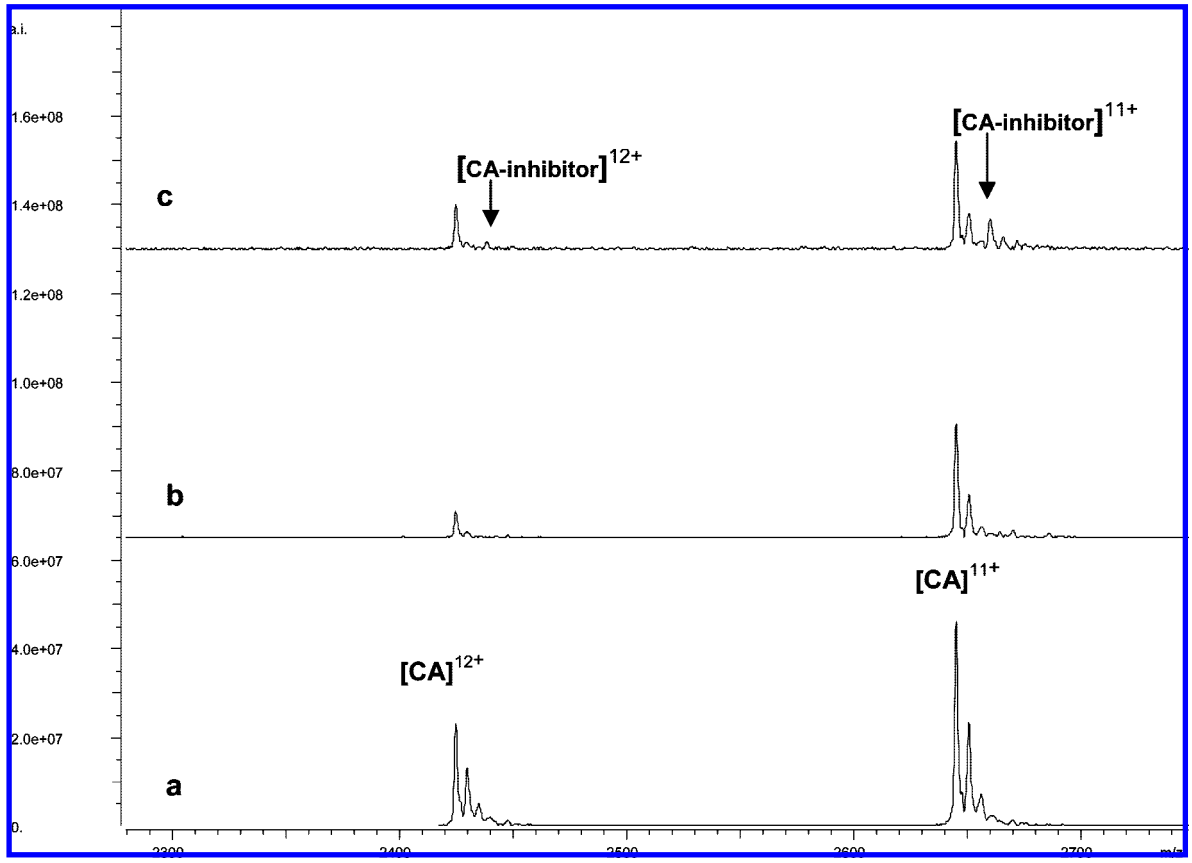

Figure 4. (a) ESI mass spectrum of bCA II (30.9 μM) at charge state 12^+ and 11^+. (b) Mass spectrum of bCA II (30.9 μM)−coumarin **2** (1.2 mM) at charge state 12+ and 11+ following incubation for 15 min. (c) Mass spectrum of bCA II (30.9 μM)−coumarin **2** (1.2 mM) at charge state 12+ and 11+ following 23 h of incubation.

is transformed into an effective inhibitor **4Z** (K_I of 59 nM) mediated by the esterase activity of CA II, leading us to propose coumarins as suicide CA inhibitors.

(iii) ESI-MS analysis of bCA II (b = bovine) in aqueous ammonium acetate yielded the mass spectrum of Figure 4, entry a. Peaks corresponding to the 11+ to 12+ charge states of bCA II were observed as originally reported by Whitesides' group,[25] with the 11+ charge state predominating. This charge state envelope (low charge states and few charge states) is typical for bCA II when in a compact, tightly folded native structure.[25,26] Next we incubated simple coumarin **2** with bCA II under conditions of excess coumarin and obtained the mass spectrum, Figure 4, entry b (15 min incubation) and entry c (23 h incubation). If an inhibitor is combined with its target protein then the noncovalent complex of [protein + inhibitor] may be observed in the ESI mass spectrum. The mass difference between the peaks for the unbound protein and the protein−inhibitor complex ($\Delta m/z$) can be multiplied by the charge state to give directly the molecular weight of the binding inhibitor, i.e., $MW_{inhibitor} = \Delta m/z \times z$. A noncovalent complex of bCA II is not observed with either parent coumarin **2** (MW = 146) or hydrolyzed coumarin (**5Z** or **5E**) after 15 min, and the mass spectrum looks similar to the free protein, Figure 4, entry b. After 23 h we do however observe a strong noncovalent complex of bCA II in the mass spectrum, Figure 4, entry c. The same charge state envelope as that for bCA II (Figure 4, entry a) was observed; however each charge state now consisted of a grouping of peaks: a peak that corresponded to native bCA II and, at a higher m/z value, a group of peaks that corresponded to bCA II- complexes [bCA II-**5Z/5E**]. For example using the 11+ charge state, $MW_{inhibitor}$ is calculated as follows:

$$MW_{inhibitor} = \Delta m/z \times z = (2660.06 - 2645.13) \times 11 = 164.2$$

Thus the observed $MW_{inhibitor}$ is 164; this corresponds to the hydrolysis product of coumarin **2**, i.e., **5Z** and/or **5E** as the carboxylic acid.

(iv) Experiments were performed in which hCA II was precomplexed with the low nanomolar sulfonamide inhibitor acetazolamide (K_I = 12 nM)[2] which was incubated for 1−3 days with coumarins **1** and **2**. No hydrolysis products **4Z** and **5Z/5E** could be evidenced in these reaction mixtures by MS (data not shown). This supports our premise that the catalytically active enzyme is needed for the proposed lactone hydrolysis which converts the less effective coumarins **1** and **2** to the potent CA inhibitors **4Z** and **5Z/5E**. Inhibition is mediated by the active site esterase chemistry of CA II.

Conclusion

6-(1*S*-Hydroxy-3-methylbutyl)-7-methoxy-2*H*-chromen-2-one (**1**) and structurally related, simple coumarins (**2**−**3**) act as suicide inhibitors of 13 mammalian isoforms of the zinc metalloenzyme CA, with inhibition constants from the nanomolar to the millimolar range. The inhibition is time dependent,

(25) Gao, J.; Cheng, X.; Chen, R.; Sigal, G. B.; Bruce, J. E.; Schwartz, B. L.; Hofstadler, S. A.; Anderson, G. A.; Smith, R. D.; Whitesides, G. M. *J. Med. Chem.* **1996**, *39*, 1949–1955.
(26) Cheng, X.; Chen, R.; Bruce, J. E.; Schwartz, B. L.; Anderson, G. A.; Hofstadler, S. A.; Gale, D. C.; Smith, R. D. *J. Am. Chem. Soc.* **1995**, *117*, 8859–8860.

being observed after incubation of enzyme and coumarins for 4 h or longer. The X-ray crystal structure of the hCA II−6-(1*S*-hydroxy-3-methylbutyl)-7-methoxy-2*H*-chromen-2-one (**4Z**) adduct showed the hydrolysis product of this coumarin bound within the enzyme cavity in a region not observed earlier for other classes of CA inhibitors, such as the sulfonamides, complexing anions, phenols, and thiophenols—all of which bind in a direct/indirect manner to the active site zinc cation. The coumarin lacks classical zinc binding functional groups and exhibits an unusual binding mode that may be exploited to design isoform selective CA inhibitors. The inhibitor exhibits an extended two-arm conformation that effectively plugs the entrance to the active site. The inhibitor is sandwiched between Phe131, with which it makes an edge-to-face stacking, and Asn67/Glu238sym, with which it makes several polar interactions and hydrogen bonds. Our results indicate that the development of nonclassical CAIs based on the coumarin scaffold has promise for obtaining isoform selective inhibitors and hence exhibit lower side effects than currently marketed CAIs. As the entrance of the active site cavity (where the hydrolyzed coumarins bind) in the various CA isoforms is the region with least conserved amino acid residues among the investigated isozymes,[3–6] this binding mode offers indeed the possibility to design inhibitors with selectivity for some isoforms with medicinal chemistry applications.

Experimental Protocols

Chemistry and CA Inhibition. Compound **1** was isolated from the Australian plant as reported earlier,[1] whereas other chemicals (coumarins **2**, **3**; buffers; inorganic salts; bCA II; etc.) were the highest purity available reagents from Sigma-Aldrich (Milan, Italy) and used without further purification. All other enzymes (hCA I−mCA XV) were recombinant ones produced in our laboratories as described earlier.[6–9,12,14]

An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as an indicator, working at an 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−24 h at room temperature (15 min) or 4 °C (all other incubation times) prior to assay, to allow for the formation of the E−I complex or for the eventual active site mediated hydrolysis of the inhibitor. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, as reported earlier[6,7,18] and represent the mean from at least three different determinations.

Crystallization, X-ray Data Collection, and Refinement. Crystals of the hCA II−**4Z** complex were obtained by using the hanging-drop method for cocrystallizing the protein with the ligand, as previously described.[5b,6] A monochromatic experiment at the Cuα wavelength was performed on a crystal of CA II grown in the presence of **1** (10 μM) by the rotation method on a PX-Ultra sealed-tube diffractometer (Oxford Diffraction) at 100 K. The crystal was diffracted up to a 2.0 Å resolution (resolution: 20.0−2.0 Å), with 15 970 (3194) unique reflections out of 75 057 (11 663) reflections, and belonged to space group *P*21 (a = 42.2 Å, b = 41.6 Å, c = 72.2 Å, and $\alpha = \gamma = 90°$, $\beta = 104.6°$). The overall completeness was 99.4 (98.0)%, Rsym=15.5 (31.9)%, multiplicity: 4.7 (3.7), F2/sig(F2)=23.0 (3.4). The numbers in parentheses refer to the values in the highest resolution shell (2.1−2.0 Å). Data were processed with CrysAlis RED (Oxford Diffraction 2006).[18,19] The structure was analyzed by a difference Fourier technique, using the PDB file 1CA2 as a starting model. The refinement was carried out with the program REFMAC5;[20] model building and map inspections were performing using the COOT program.[21] The final model of the complex had an *R*-factor of 19.9% and *R*-free 26.8% in the resolution range 20.0−2.0 Å, with an rms deviation from standard geometry of 0.011 Å in bond lenghts and 1.36° in angles. The correctness of stereochemistry was finally checked using PROCHECK.[22] Crystallographic parameters and refinement statistics are shown in Table 2.

Table 2. Crystallographic Parameters and Refinement Statistics for the hCA II−**4Z** Adduct

parameter	value
Crystal Parameter	
space group	$P2_1$
cell parameters	a = 42.2Å
	b = 41.6 Å
	c = 72.2 Å
	β = 104.6°
Data Collection Statistics (20.0−2.0 Å)	
no. of total reflections	25 057
no. of unique reflections	15 970
completeness (%)[a]	99.4 (98.8)
$F^2/\sigma(F^2)$	23.0 (3.4)
R-sym (%)	15.5 (31.9)
Refinement Statistics (20.0−2.0 Å)	
R-factor (%)	19.9
R-free (%)[b]	26.9
rmsd of bonds from ideality (Å)	0.011
rmsd of angles from ideality (deg)	1.36

[a] Values in parentheses relate to the highest resolution shell (2.1−2.0 Å). [b] Calculated using 5% of data.

Mass Spectrometry. Mass spectrometry was performed on an APEX III 4.7 T FTICR mass spectrometer (Bruker Daltonics, Billerica, MA, USA) fitted with an Apollo ESI source operated in positive ion mode. XMASS NT V7.0.2 mass spectrometry software on a PC platform was used for data acquisition. Broadband excitation was used to analyze a mass range from m/z 100−4500, with 256K, 128K, or 32K data points acquired. Samples were infused into the ESI source at 2 μL min^{-1}. Nitrogen was used as both the drying gas (125 °C) and nebulizing gas. An Agilent ES tuning mix (catalogue number G2421A) was used for an external four-point calibration. Samples were prepared as follows: (i) bCA II (30.9 μM) in 10 mM ammonium bicarbonate buffer (pH = 8) and (ii) bCA II (30.9 μM) in 10 mM ammonium bicarbonate buffer (pH = 8) + coumarin **2** (1.2 mM) (b = bovine).

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