Journal of Medicinal Chemistry

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J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.5b01720 • Publication Date (Web): 19 Dec 2015

ACS Publications

Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Thioxocoumarins show an alternative carbonic anhydrase inhibition mechanism compared to coumarins

Marta Ferraroni,[1] Fabrizio Carta,[1] Andrea Scozzafava,[1] and Claudiu T. Supuran[1,2] *

[1] Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino (Florence), Italy.

[2] Università degli Studi di Firenze, NEUROFARBA Dept., Sezione di Scienze Farmaceutiche, Via Ugo Schiff 6, 50019 Sesto Fiorentino (Florence), Italy.

Abstract: A series of coumarins and the corresponding 2-thioxocoumarines were prepared and tested for their inhibition profiles against four physiologically relevant human carbonic anhydrases (hCAs, EC 4.2.1.1), isoforms hCA I, II, IX and XII. The X-ray crystal structure of 6-hydroxy-2-thioxocoumarin bound to hCA II revealed an unprecedented and unexpected inhibition mechanism for this new class of inhibitors, when compared to isostructural coumarins. Unlike coumarins which are hydrolyzed by the esterase CA activity to the corresponding 2-hydroxy-cinnamic acid derivatives, the 2-thioxocoumarin was observed intact when bound to hCA II, with its *exo*-sulphur atom anchored to the zinc-coordinated water molecule, whereas the scaffold establishing favorable contacts with amino acid residues from the active site. This inhibition mechanism is very different from the one observed for hydrolyzed coumarins, which occlude the entrance of the active site cavity. This versatility in the binding mode of coumarins/thioxocoumarins has important consequences for the design of isoform-selective CA inhibitors, some of which are in clinical use or clinical development for various pathologies, among which glaucoma, edema, epilepsy, neuropathic pain and hypoxic tumors.

Key words: thioxocoumarin, coumarin, metalloenzyme, carbonic anhydrase; isoforms I, II, IX, XII, X-ray crystallography

Introduction.

Among the metallo-enzymes possessing a crucial physiologic function, the carbonic anhydrases (CAs, EC 4.2.1.1) represent an interesting case, as they act on very simple substrates, such as CO_2, COS, CS_2 or cyanamide[1-3] generating products which are either involved in pH regulation (bicarbonate and protons), biosynthetic processes (bicarbonate, urea) or in other important phenomena such as for example chemosensing (in vertebrates and invertebrates),[4] sexual development (in pathogenic fungi),[5] pH and CO_2-sensing, pathogenicity, and survival in ambient air of many bacteria, fungi and/or protozoa.[6-8] There are six genetic families encoding such enzymes in virtually all organisms known to date, the α-, β-, γ-, δ-, ζ- and η-CAs, with the last class reported very recently.[9] All CAs known so far are metal ion-dependent enzymes, with a metal-hydroxide species within the enzyme cavity acting as a nucleophile in the catalytic cycle, and a second step (usually rate-determining) involving a proton transfer reaction from a water molecule coordinated to the active site metal ion to the environment, for regenerating the nucleophile.[10] Metal ions employed at the active site of the different CAs include Zn(II) (in all classes), Cd(II) (in ζ-CAs), Co(II) (in the δ class) or Fe(II) (for γ-CAs, in anaerobic conditions).[11,12] This ping-pong mechanism makes some of the members of the CA superfamily among the most effective enzymes known in nature, with k_{cat}/K_M values close to the limit of the diffusion-controlled processes.[13]

Only α-CAs have been reported in vertebrates, but in most investigated species a large number of different isoforms were described.[1-3] For example in humans, 15 CA isoforms are known, CA I - CA VA, CA VB, CA VI - CA XIV, with 12 of them being catalytically active and three (CA VIII, X and XI) devoid of activity but still playing significant functions in tumorigenesis and other physiologic as well as pathologic processes.[14]

Due to the fact that the substrates/reaction products of α-CAs (CO_2, bicarbonate and protons) are simple molecules/ions involved in a host of physiologic processes, their up- or down-regulation is associated with a range of diseases.[1-3,15-18] Indeed, CA inhibitors (CAIs) are clinically used for decades as diuretics,[15b] antiglaucoma agents,[1b,d,3d] antiepileptics,[16] or more recently anti-obesity agents,[17] whereas compounds targeting the tumor-associated isoforms CA IX and XII are in clinical development as anticancer agents/diagnostic tools for hypoxic, metastatic tumors.[3,18] CA activators (CAAs) may have potential for developing agents for Alzheimer's disease or aging, as in these pathologies a diminishing of the activity of some physiologically relevant isoforms (such as CA I and II) has been reported.[19]

One of the main hurdles connected with the use of CAIs in the treatment of diverse conditions as those mentioned above, is related to the off-target inhibition of isoforms other than the

desired one.[1-3] In fact the various pharmacological applications of the CAIs are due to the high number of isoforms and their involvement in different pathologies.[15-18]

Recently a number of important advances in the field of designing isoform-selective CAIs targeting various isoforms has been achieved, mainly by using structure-based drug design approaches.[1-3] Among them the so-called tail approach is one of the most employed one for such purposes.[20,21] This approach was initially reported for the sulfonamide CAIs,[20] and consists in attaching tails (moieties) able to interact with the middle and the rim part of the active site cavity, which is the most variable region among the 15 CA isoforms known in humans.[1-3] Thereafter this approach was extended to all other classes of CAIs, such as the coumarins,[22] sulfocoumarins,[23] and dithiocarbamates.[24]

It has been demonstrated that coumarins, a class of CAIs reported in 2009, do possess highly selective CA inhibition profiles, which rely on their particular inhibition mechanism.[25] In fact the coumarin itself acts as a prodrug, whereas its hydrolysis products (formed due to the esterase CA activity which opens the lactone ring of the coumarin) represents the real inhibitor (Fig. 1).[25] Indeed, coumarins **A** or **B** in complex with hCA II were crystallized allowing the evidence of their hydrolysis products **A1** and **B1** (2-hydroxy-cinnamic acid derivatives) bound at the entrance of the active site cavity, occluding it.[25b]

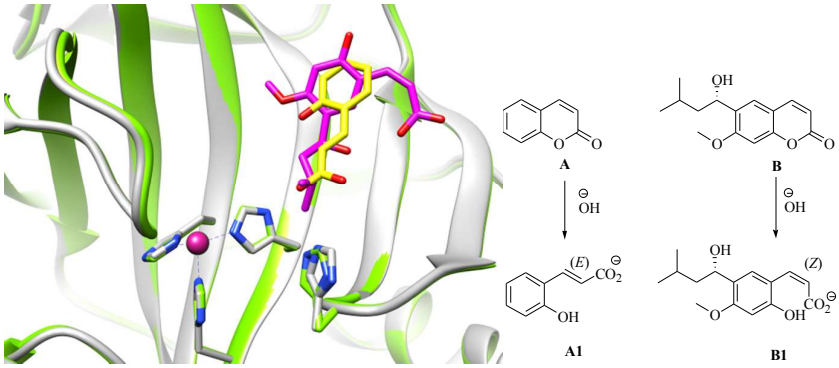


Figure 1. Superposition of the coumarin **A** hydrolysis product (*trans*-2-hydroxy-cinnamic acid **A1** in yellow) with the coumarin **B** hydrolysis product (*cis*- 2-hydroxycinnamic acid **B1**, magenta)-hCA II adducts (PDB code 3F8E and 5BNL, respectively). The protein backbone is shown as green (PDB code 3F8E) and grey (PDB code 5BNL) ribbon, the catalytic Zn (II) ion as violet sphere, with its three protein ligands (His94, 96, and 119) also evidenced.[25b]

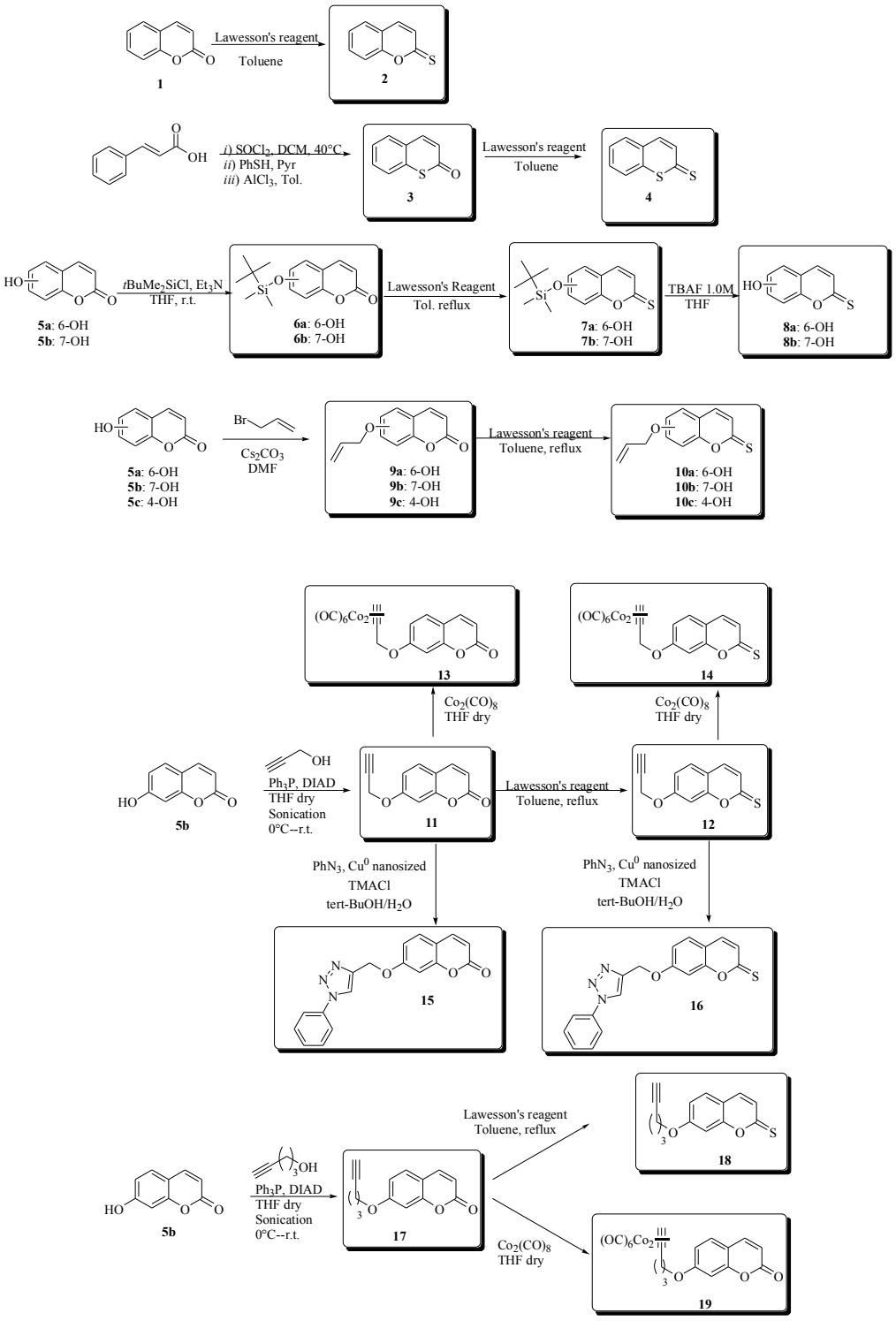
With the aim to pursue the identification of new potent and selective coumarine-based CAIs we report here a series of coumarins and their corresponding thioxocoumarines **1-19** which were tested *in vitro* for their inhibition profiles against four most physiologically important hCAs, such as

the hCA I, II, IX and XII. The X-ray crystal structure adduct of 6-hydroxy-2-thioxocoumarin **8a** bound hCA II at 1.1 Å resolution, is also reported. This data reveals an unprecedented and unexpected inhibition mechanism of the thioxocoumarins when compared to the structurally related coumarin scaffold.

Results and Discussion

Compound design and synthesis. Coumarins were discovered to act as prodrug inhibitors of the metalloenzyme carbonic anhydrase by this group.[25] The first compound for which such an activity has been reported was the natural product coumarin **B** (reported in figure 1), which was isolated from the Australian plant *Leionema elipticum*.[25a] Its X-ray crystal structure in adduct with the ubiquitous cytosolic isoform hCA II surprisingly showed that the lactone ring of the inhibitor was hydrolysed due to the esterase activity of the CA, with formation of the *Z*-hydroxycinnamic acid derivative **B1**, which was observed bound at the entrance of the CA active site thus occluding it.[25b] No other inhibitors were ever observed in that region of the CA active site,[26] which has been always associated with the binding of the CA activators.[26-28] A similar behaviour was thereafter observed for the simple coumarin derivative **A**, which again by means X-ray crystallography, was found bound in the same active site region as **B1**, but in the case of **A1** the *E*-hydroxycinnamic acid was observed (figure 1).[25b] The very new mechanism of CA inhibition revealed for coumarins inspired much research in this field, mainly because a large number of such derivatives possessing various substitution patterns at the coumarin ring proved to act as highly isoform-specific CAIs,[25,29] a phenomenon never observed for the main class of clinically used such agents, the sulfonamides and their isosteres (sulfamates, sulfamides, etc.).[30]

In a previous work[31a] we reported the 2*H*-chromene-2-thione **2** (2-thioxocoumarin) as well as the 2*H*-thiochromen-2-one **3** (thiocoumarin) and the thiochromene-2-thione **4** (dithiocoumarin) as CAIs isosters of the simple coumarin **1**, which was also used for their preparation as depicted in Scheme 1.



Scheme 1. Synthesis of coumarin and thioxocoumarin derivatives **2-19**.

Moreover 4-, 6- and 7- substituted derivatives **6-10** were also prepared[31b] and assayed as CAIs (Table 1 and Scheme 1). As an extension of our previous studies we report here new derivatives,

such as compounds **11-19**, which were obtained by means of known synthetic procedures. As shown in Scheme 1, the introduction of a terminal alkyne chain, such as in compounds **11** and **17**, was accomplished by means of sonicated-mediated Mitsunobu coupling reactions, which allow fast and cleaner reaction procedures when compared to the standard thermal conditions (data not shown). The presence of a terminal alkyne moiety also allowed us to explore the effect of various moieties, also considering the liker lengths between the main scaffold and the alkyne functionality. Thus we investigated lipophylic bulky moieties such as in **13** and **19** or a phenyltriazole moiety (compound **15**), which is expected to interact through hydrogen bonds with amino acid residues located at the rim of the enzymatic cavity. All obtained compounds were treated with Lawesson's reagent to afford the corresponding thioxo derivatives **12**, **14**, **16** and **18**.

Carbonic anhydrase inhibition. As shown in Table 1, compounds **1-19** were tested *in vitro* for their inhibition profiles against four physiologycally relevant hCA enzymes, the cytosolic isoforms I and II and the trans-membrane, tumor associated IX and XII.

Table 1. CA inhibition data against isoforms hCA I, II, IX and XII with compounds **2-19** and acetazolamide (**AAZ**) as standard, by a stopped-flow CO_2 hydrase assay.[32]

Compound	K_I (μM)*			
	hCA I	hCA II	hCA IX[a]	hCA XII[a]
1**	3.1	9.2	>100	>100
2	>100	>100	6.7	95.2
3	>100	>100	0.97	26.5
4	>100	>100	19.6	96.0
5a**	>100	>100	0.19	0.68
5b**	58.4	>100	0.48	0.75
5c**	95.0	>100	0.41	6.30
6a**	8.78	>100	0.80	0.28
6b**	8.32	>100	0.85	0.83
7a**	7.57	>100	0.86	0.31
7b**	8.18	>100	0.96	0.35
8a**	7.17	>100[#]	0.80	0.34
8b**	8.02	>100	0.78	0.32

9a[***]	30.3	>100	0.93	0.80
9b[***]	72.9	>100	0.73	0.64
9c[***]	43.2	>100	0.21	0.88
10a[***]	8.51	>100	3.26	1.25
10b[***]	7.60	>100	3.23	2.83
10c[***]	9.24	>100	3.04	1.27
11	72.0	>100	1.35	0.73
12	950	>100	41.6	38.9
13	85.8	>100	61.2	31.0
14	>200	>100	47.3	30.2
15	>200	>100	0.008	0.005
16	>200	>100	0.004	0.027
17	124	>100	0.83	0.37
18	>200	>100	0.22	0.41
19	>200	>100	52.3	61.2
AAZ	0.20	0.012	0.025	0.006

[*]Errors in the range of ±5 % of the reported values, from three different assays. [**] From Ref.[16b] [***]From Ref.[25a] ;[#]A K_I of 285 μM has been measured, working with higher concentrations of inhibitor **8a**.[a] Catalytic domain.

In general all compounds reported showed to be low- medium potency inhibitors of the slow cytosolic isoform hCA I (K_Is of 7.17 μM or > 100 μM), were inactive against the hCA II isoform with the only exception represented by the simple coumarin **1**, and were highly potent inhibitors of the tumor associated isoforms hCA IX and XII, with K_Is spanning between 0.004-47 μM (hCA IX) and 0.005-95.2 μM (hCA XII), respectively. In particular the following structure-activity-relationship (SAR) considerations for each hCA tested can be drawn:

i) For the hCA I the replacement of one or both of the oxygen atoms into the simple coumarin **1** (K_I 3.1 μM) to afford compounds **2-4** resulted in a complete loss of the inhibitory activity (K_Is > 100 μM). The introduction of the phenolic moiety into the coumarin **1** at positions 4, 6 and 7 also spoiled the inhibition potency with K_Is of 95.0, >100 and 58.4 for **5c**, **5a** and **5b** respectively. Conversely the manipulation of the phenol moiety in compounds **5a-c** through the introduction of a TBDMS or an allyl group restored the inhibition potencies against the hCA I to low-medium micromolar values. In particular silylation of **5a** and **5b** (K_Is >100 and 58.4 μM) to afford

compounds **6a** and **6b** resulted in a significant reduction of the K_I values to 8.78 and 8.32 μM respectively. The introduction of the allyl group in **5a-c** resulted in a marked enhancement of the inhibition potencies only for the **5a** and **5c** derivative to afford **9a** and **9c** (K_Is 30.3 and 42.9 μM), as for the 7-*O*- allyl susbstituted derivative **9b** a 1.25 fold decrease of the inhibitory activity was observed (K_I 72.9 μM). The same trend was also observed when coumarin **5b** was propargylated (compounds **11** and **17**, K_Is 72 and 124 μM) and the terminal acetylenic moiety was further elaborated with a metallorganic species (compound **13** K_I 85.8 μM) or subjected to a copper catalyzed click chemistry reaction to afford compound **15** (K_I >200 μM). Interestingly, the introduction of an *exo*-sulfur atom into the species previously discussed strongly influenced the inhibitory potencies. As reported in Table 1 both silyl derivatives **7a,b**, their corresponding phenolic derivatives **8a,b** as well as the allyl substituted compounds **10a-c** showed K_Is in the low micromolar range and comprised between 7.17-9.24 μM, which make them among the most potent compounds within the series against hCA I. Conversely the substitution of the *exo*- lactonic oxygen with a sulphur in **11** to afford compound **12** resulted in a significant increase of the K_I (> 100 μM) and all the other derivatives such as **14**, **16** and **18** were inactive.

ii) Contrary to hCA I, the replacement of one or both oxygen atoms within the coumarin **1** scaffold to afford the compounds **2-4** resulted in a significant increase of the inhibition activity against hCA IX with K_I values of 6.7, 0.97 and 19.6 μM respectively. Also the introduction of hydroxyl moieties into the coumarin ring at positions 4, 6 and 7 led to a marked increase of the inhibition potency (K_Is of 0.41, 0.19 and 0.48 μM for **5a-c**, respectively). The conversion of the hydroxyl moieties in **5a** and **5b**, to the corresponding silylated derivatives, such as **6a** and **6b**, resulted in a slight increase of the K_Is of up to 0.80 and 0.85 μM, respectively. In analogy, the introduction of an *O*-allyl group at position 6 and 7 to afford **9a** and **9b** resulted in a 4.9 and 1.5-fold reduction of the inhibition activities, respectively. The only exception in this case was represented by the *O*-allyl derivative at 4 position (compound **9c**), which K_I was halved (0.41 μM for **5c** and 0.21 μM for **9c**). The introduction of a terminal alkyne moiety in **5b** to afford compounds **11** and **17** resulted in a 2.8 and 1.73 K_I fold increase of the inhibitory power respectively (compared to the lead **1**), which was further enhanced when the cobalt(II)-based protection of the terminal alkyne was installed (61.2 and 52.3 μM for compounds **13** and **19** respectively). Interestingly, the introduction of the phenyl triazole moiety in **11** to afford compound **15** led to a great reduction of the K_I to 8.0 nM, thus making it one of the most active inhibitors against hCA IX within the series herein reported. In general the replacement of the *exo*-oxygen atom of the coumarins with a sulphur, resulted in reduction of the inhibitory activity, which make the 2-thioxocoumarins a highly interesting class of CAIs. As shown in Table 1, the thioxo derivatives **8a** and **8b** had K_Is 4.2 and 1.6- fold higher when

compared to the coumarin progenitors **5a** and **5b**. Analogous inhibition profiles were also observed for the silyl derivatives **7a** and **7b** (K_I 0.86 and 0.96 μM), for the *O*-allyl thioxo derivatives **10a-c** (K_Is of 3.26, 3.23 and 3.04 μM respectively) and the propargyl derivative **12** (K_I 41.6 μM). Interestingly, a slight improvement in the inhibition potency was reported for the pentyne derivative **18** (K_I 0.22 μM), for the bulky protected alkyne **14** (K_I 47.3 μM) and for the phenyltriazolyl derivative **16,** which was the most potent inhibitor reported within this series against the hCA IX (K_I 4 nM). The phenyltriazolyl-containing compounds **15** and **16** were even more potent CAIs compared to the standard sulfonamide acetazolamide (K_I 25 nM)

iii) The inhibition profiles of the compounds reported here against hCA XII isoform were more intricate compared to what discussed above for the other three investigated isoforms, and a clear-cut structure-activity relationship is rather difficult to draw. As for hCA IX, the introduction of one or more sulphur atoms within the simple coumarine scaffold **1** (K_I >100 μM) accounted for a restoration of the inhibition activity (K_Is 95.2, 26.5 and 96.0 μM for **2-4,** respectively). Also the introduction of the hydroxyl moiety at the 4, 6 and 7 positions of the coumarin ring resulted in a marked enhancement of the inhibitory activity (K_Is 6.30, 0.68 and 0.75 μM for compounds **5c**, **5a** and **5b,** respectively). The functionalisation of the hydroxyl moieties, as for the silyl derivatives **6a** and **6b**, determined different behaviours. Thus the 6-*O*-TBDMS derivative **6a** was more active when compared to its progenitor **5a** (K_I 0.28 μM for **6a** and 0.68 μM for **5a**); conversely the 7-*O*-TBDMS derivative **6b** showed just a modest 1.1 fold decrease of its activity (K_I 0.83 μM). The introduction of the *O*-allyl moiety into the coumarins **5a-c** resulted in a reduction of the inhibition potency for the 4 and 7-substituted derivatives **9b** and **9c** (K_Is 0.64 and 0.88 μM respectively) whilst the 6-*O*-allyl derivative **6a** showed a 1.2 times increase of its inhibitory potency. Conversely to the hCA I and IX enzymes in which the introduction of the *O*-propargyl and *O*-pentenyl chains at the 7-position of the coumarin scaffold determined a decrease of the inhibitory potencies, in the case of hCA XII a slight K_I decrease was observed for compound **11** (K_I 0.73 μM) and a 2 fold decrease for the longer-chain derivative **17** (K_I 0.37 μM). The protection of the terminal alkyne moieties in **11** and **17**, to afford compounds **13** and **19**, spoiled their inhibition potencies by a 42.5 and 165.4 fold K_I increase, respectively. Interestingly the introduction of the phenyltriazolyl moiety in **11** to afford compound **15** resulted in a drastic reduction of the K_I up to 5.0 nM, thus making this compound as the most active in the series (against hCA XII) and comparable to the inhibition value of the sulfonamide **AAZ** (K_I 6.0 nM), which however is a promiscuous CAI unlike the coumarins/thioxocoumarins. Introduction of the *exo*-sulphur moiety into the simple hydroxyl coumarins **5a** and **5b**, as in compounds **8a** and **8b**, led to an enhancement of the inhibition potency (K_I 0.34 and 0.32 μM respectively) compared to the corresponding coumarins. As for the silyl

derivatives **7a** and **7b** only a slight K_I increase was observed for the former (K_I 0.31 μM) whereas the latter showed a 0.42 fold increase of its potency (K_I 0.35 μM). The insertion of the sulphur atom in compounds **9a-c** to afford **10a-c**, determined a reduction of the inhibition against the hCA XII (K_Is 1.25, 2.83 and 1.27 μM, respectively). In analogy the conversion of **11** and **17** to their corresponding thioxo derivatives **12** and **18** resulted in reduction of the inhibition potencies (K_Is 38.9 and 0.41 μM respectively). Only a small K_I reduction was observed for compound **14** when compared to its oxo-analogue **13** (K_I 31.0 μM for **13** and 30.2 μM for **14**). Finally the thioxo derivative of **15**, i.e., **16**, showed a 5.4 fold increase of its K_I, thus making it the second most potent inhibitor against the hCA XII within this series.

In summary among all the compounds reported here, the phenyltriazolyl bearing derivatives **15** and **16** were the most potent and selective inhibitors of the tumor associated hCA IX and XII with K_Is comparable or lower of the standard sulfonamide **AAZ**. In particular the introduction of the *exo*-sulphur atom within the coumarine scaffold of **15**, to afford **16**, halved the K_I against the tumor associated isoform hCA IX. Such an inhibition profile was not observed against hCA XII. However both compounds **15** and **16** were highly active and selective for the tumor-associated isoforms among the derivatives belonging to this series.

X-ray crystallography. In order to understand the structural elements which led to such interesting inhibitory profiles, as well as to dissect the inhibition mechanism with thioxocoumarins, we report here the high resolution (1.1 Å) crystal structure of the adduct of hCA II with thioxocoumarin **8a. A** very interesting binding mode for this compound within the enzyme active site (Figure 2**A** and **B**, and experimental section Table 2) has been thus revealed.

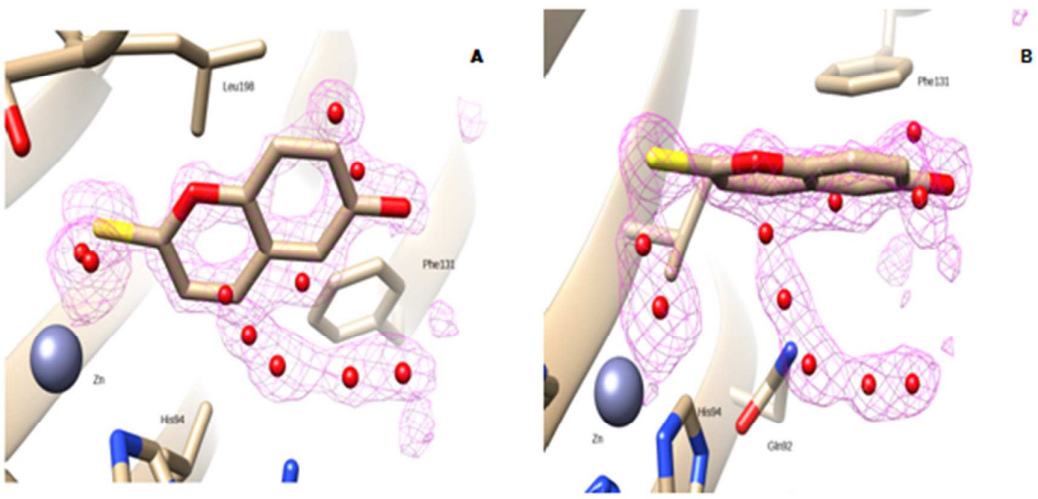


Figure 2. A: Fo-Fc omit map of **8a** and water molecules within the hCA II active site in the hCA II – **8a** adduct; **B**: Tilted view of the electron density of 8a and water molecules within the hCA II active site.

The sulphur (S1) atom of inhibitor **8a** is hydrogen bonded to the water coordinating the zinc ion (Wat348 – S1 2.90 Å). The Wat348 to Zn(II) distance is of 1.91 Å, as in most X-ray structures of CA alone or in complex with inhibitors in which the non-protein zinc ligand is a water molecule/hydroxide ion.20 Furthermore, S1 forms a hydrogen bond with the Thr199 peptide nitrogen (S1···H– N Thr199 of 2.64 Å). This is very different from inhibitors which directly coordinate to the metal ion, in which a hydrogen bond with the OH of Thr199 is usually observed.[21,22] A disordered second water molecule (Wat394) was observed in the electron density nearby the Zn(II) coordinated water molecule (Wat348). This is probably due to the fact that the occupancy of the inhibitor in the adduct is of 50% (also due to its low affinity for isoform hCA II; see Table 1) and to the high degree of disorder observed for the water molecules in the adduct (see below). This water is too far from the Zn(II) (Wat394 – Zn 2.58 Å) to be considered as coordinated, and too close to the S1 atom of the inhibitor (Wat394 – S1 1.71 Å). The bicyclic ring system of inhibitor **8a** resides in a hydrophobic pocket formed by residues Phe131, Val121, Leu198 and Pro202 (Figures 3, 4). The inhibitor forms two C-H···O hydrogen bonds with Leu198 (CB Leu198···H–OAK, of 2.44 Å, and CD2 Leu198···H–OAK Leu198, and one with Thr200, OG1Thr200···H–CAL of 2.24 Å). The inhibitor was introduced at 0.5 of occupancy, as mentioned above (see also Supplementary Table 3 for the B factors of the inhibitor/water molecules). Some water molecules occupy the same position of the inhibitor and they were also introduced at partial occupancy. Interestingly they form in the active site the same hydrogen bonding network usually observed in native hCA II structures.

Thus, unlike coumarins investigated in detail by X-ray crystallography (and kinetic measurements) the structurally-related 2-thioxocoumarins possess a CA binding mode which resembles the phenols,[33a] polyamines,[33b] or sulfocoumarins,[34] which all anchor to the zinc-coordinated water molecule/hydroxide ion, with the scaffold participating in supplementary interactions with the active site, thus stabilizing the enzyme-inhibitor adduct. In fact as seen from Figure 3, where the present structure was superimposed on that of the hCA II – coumarin **B** adduct, the active site regions occupied by the two structurally similar inhibitors are quite distinct, with the main difference being that the coumarin **B** is hydrolyzed whereas the 2-thioxocoumarin **8a** was observed intact within the enzyme active site.

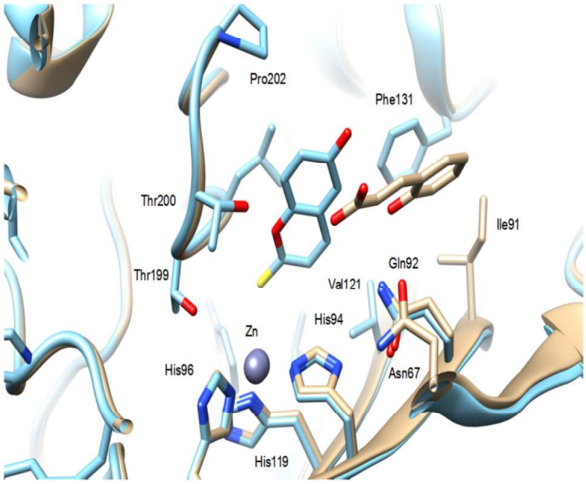


Figure 3. Superposition of the hCA II - **8a** adduct (sky blue, 4WL4) with the hCA II - hydrolyzed coumarin **B1** adduct (5BNL) (silver). The zinc ion, its three His ligands and amino acid residues involved in the binding of inhibitors are shown.

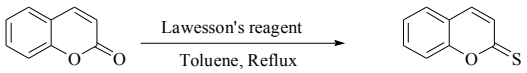
Conclusions. Coumarins and their isosteres represent very interesting classes of CAIs which led to highly isoform-selective compounds. Such derivatives investigated by X-ray crystallography and kinetic measurements allowed the discovery of new CA inhibition mechanisms, i.e., occlusion of the active site entrance.[1-3] Here we report that the structurally-related sulfur containing coumarin derivatives, such as the 2*H*-chromene-2- thiones, possess a CA inhibition mechanism different form the parent oxygen-bearing compounds. The hCA II-**8a** adduct revealed the *exo*-sulfur atom of the inhibitor anchored to the zinc-coordinated water molecule/hydroxide ion, with the scaffold participating in supplementary interactions within the active site, thus contributing in stabilizing the enzyme-inhibitor adduct. Thus, the main difference of the binding modes between coumarins and 2*H*-chromene-2-thione derivatives is that the first were observed hydrolyzed when bound to the enzyme, whereas the latter ones are not. This different behavior is amenable to drug design campaigns, also considering the simplicity of the scaffold of **8a** and the relative facility with which some of its derivatives could be obtained. In fact the click chemistry applied to this class of compounds afforded low nanomolar inhibition of the tumor-associated isoforms hCA IX/XII with thioxocoumarins, these compounds being not inhibitory against the offtarget cytosolic isoforms hCA I and II. As one hCA IX-selective sulfonamide inhibitor is in Phase I clinical trials for the treatment of hypoxic, metastatic solid tumors, we estimate that the present findings may lead to even more interesting drug candidates for the treatment of this condition.

Experimental protocols

Chemistry

General. Anhydrous solvents and all reagents were purchased from Sigma-Aldrich, Alfa Aesar and TCI. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes techniques to transfer solutions. Nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR) spectra were recorded using a Bruker Advance III 400 MHz spectrometer in DMSO-d_6. Chemical shifts are reported in parts per million (ppm) and the coupling constants (*J*) are expressed in Hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; brs, broad singlet; dd, double of doublets. The assignment of exchangeable protons (O*H* and N*H*) was confirmed by the addition of D_2O. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel F-254 plates. Flash chromatography purifications were performed on Merck Silica gel 60 (230-400 mesh ASTM) as the stationary phase and ethyl acetate/*n*-hexane were used as eluents. Melting points (mp) were carried out in open capillary tubes using a Gallenkamp MPD350.BM3.5 apparatus and are uncorrected. 2*H*-Chromen-2-one **1** and *trans*-cinnamic acid were commercially available from Sigma-Aldrich, Milan, Italy. All compounds reported here were > 98% pure.

Synthesis of 2*H*-chromene-2-thione **2.**[31a]

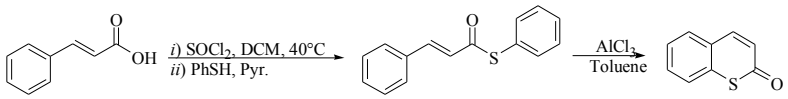


1 **2**

2*H*-Chromen-2-one **1** (0.5 g, 1.0 eq) was dissolved in dry toluene (20 ml) and treated with Lawesson's reagent (2.0 eq). The reaction mixture was refluxed until consumption of the starting material (TLC monitoring). Then the solvent was removed under *vacuo* and the obtained residue was purified by silica gel column chromatography eluting with 20% *v/v* ethyl acetate/*n*-hexane to afford the titled compound **2** as a yellow solid.

2*H*-Chromene-2-thione **2**: 60% yield; silica gel TLC R_f 0.27 (ethyl acetate/*n*-hexane 20% *v/v*); ν_{max} (KBr) cm^{-1} 1765, 1518, 1220; δ_H (400 MHz, DMSO-d_6) 7.31 (1H, d, *J* 10.0, 3-H), 7.61 (1H, dt, *J* 7.6, 1.2, 6-H), 7.64 (1H, d, *J* 8.4, 5-H), 7.74 (1H, dt, *J* 7.6, 1.2, 7-H), 7.85 (1H, d, *J* 8.4, 8-H), 7.96 (1H, d, *J* 10.0, 4-H); δc (100 MHz, DMSO-d_6), 198.5 (*C*=S), 157.0, 137.0, 133.6, 130.0, 129.6, 126.8, 121.2, 117.1; Anal. Calc. C, 66.64; H, 3.73; S, 19.77; Anal. Found. C, 66.15; H, 3.43; S, 12.38.

Synthesis of 2*H*-thiochromen-2-one **3.**[31a]



Intermediate 1 **3**

(*E*)-S-Phenyl 3-phenylprop-2-enethioate

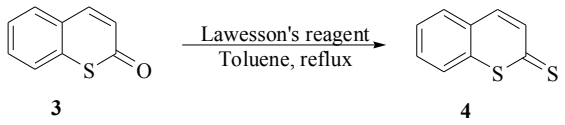
trans-Cinnamic acid (1.0 g, 1.0 eq) was dissolved in dry DCM (20 ml) and thionyl chloride (10.0 eq) was added drop-wise at 0 °C. The solution was refluxed until starting material was consumed (TLC monitoring), then the solvents were removed under *vacuo* to give a sticky oily residue that was dissolved in dry pyridine (10 ml) at 0 °C and thiophenol (0.74 g, 1.0 eq) was added drop-wise. The yellow solution was stirred at r.t. for 2 h, quenched with H_2O (30 ml), extracted with ethyl acetate (3 x 15 ml) and the combined organic layers were dried over Na_2SO_4, filtered and concentrated under *vacuo* to give a residue that was purified by silica gel column chromatography eluting with 5% *v/v* ethyl acetate/*n*-hexane to afford intermediate **1** as a pale yellow solid.

(*E*)-S-Phenyl 3-phenylprop-2-enethioate (intermediate **1)**: 62% yield; 94-96 °C; silica gel TLC R_f 0.17 (ethyl acetate/*n*-hexane 5% *v/v*); ν_{max} (KBr) cm^{-1}, 1670 (C=O), 1515 (aromatic); δ_H (400 MHz, DMSO-d_6) 7.16 (1H, d, *J* 16.0, 2-H), 7.49 (3H, m, 2 x 6-H, 7-H), 7.54 (5H, s, S-Ar-H), 7.70 (1H, d, *J* 16.0, 3-H), 7.84 (2H, m, 2 x 5-H); δc (100 MHz, DMSO-d_6), 188.0 (*C*=O), 142.5, 135.4, 134.6, 132.0, 130.5, 130.3, 130.0, 129.9, 128.2, 125.2.

(*E*)-S-Phenyl 3-phenylprop-2-enethioate (0.2 g, 1.0 eq) was dissolved in dry toluene (5.0 ml) and $AlCl_3$ (0.56 g, 5.0 eq) was added. The orange solution was stirred at 70 °C for 5 h (TLC monitoring), cooled down to r.t., quenched with slush and extracted with ethyl acetate (3 x 20 ml). The combined organic layers were washed with H_2O (2 x 20 ml), dried over Na_2SO_4, filtered-off and concentrated under *vacuo* to give an orange residue that was purified by silica gel column chromatography eluting with 5% *v/v* ethyl acetate/*n*-hexane to afford the titled compound **2** as a pale yellow solid.

2*H*-Thiochromen-2-one **3**: 55% yield; 77-78 °C; silica gel TLC R_f 0.11 (ethyl acetate/*n*-hexane 5% *v/v*); ν_{max} (KBr) cm^{-1}, 1660 (C=O), 1515 (aromatic); δ_H (400 MHz, DMSO-d_6) 6.65 (1H, d, *J* 10.8, 3-H), 7.64 (3H, m, 5-H, 6-H, 7-H), 7.92 (1H, d, *J* 8.0, 8-H), 8.12 (1H, d, *J* 10.8, 4-H); δc (100 MHz, DMSO-d_6), 185.1 (*C*=O), 145.8, 137.2, 133.0, 131.4, 127.8, 126.8, 126.7, 124.4; Anal. Calc. C, 66.64; H, 3.73; S, 19.77; Anal. Found. C, 62.96; H, 3.63; S, 12.08.

Synthesis of thiochromene-2-thione **4**

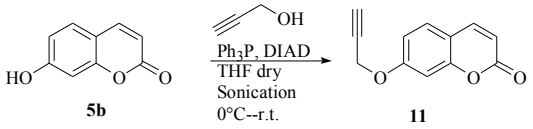


3 **4**

2*H*-Thiochromen-2-one **3** (0.03 g, 1.0 eq) was dissolved in dry toluene (10 ml) and treated with Lawesson's reagent (2.0 eq). The reaction mixture was refluxed until consumption of the starting material (TLC monitoring). Then the solvent was removed under *vacuo* and the obtained residue was purified by silica gel column chromatography eluting with 10% *v/v* ethyl acetate/*n*-hexane to afford the titled compound **4** as a red solid.

2*H*-Thiochromene-2-thione **4**: 33% yield; 103-105 °C; silica gel TLC R_f 0.20 (ethyl acetate/*n*-hexane 10% *v/v*); ν_{max} (KBr) cm^{-1}, 1770, 1520, 1230; δ_H (400 MHz, DMSO-d_6) 7.43 (1H, d, *J* 10.0, 3-H), 7.61 (1H, dt, *J* 8.0, 1.6, 5-H), 7.28 (2H, m, 6-H, 7-H), 7.90 (1H, d, *J* 10.0, 4-H), 8.00 (1H, d, *J* 8.0, 8-H); δc (100 MHz, DMSO-d_6), 209.7 (*C*=S), 140.2, 136.9, 136.3, 133.0, 131.9, 129.2, 128.5, 124.6; Anal. Calc. C, 60.63; H, 3.39; S, 35.97; Anal. Found. C, 59.48; H, 3.05; S, 21.27.

Synthesis of 7-(prop-2-ynyloxy)-2*H*-chromen-2-one **11.**



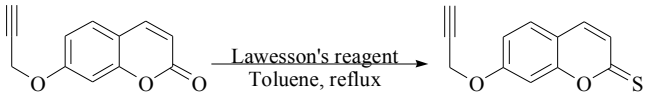
5b **11**

7-Hydroxy coumarin **5b** (1.0 g, 1.0 eq), propargyl alcohol (1.0 eq) and triphenylphoshine (1.0 eq) were dissolved in dry THF (90 ml). Then the temperature was lowered to 0 °C and diisopropylazadicarboxylate (1.1 eq) was added drop-wise under sonication. The orange solution was sonicated at r.t. under a nitrogen atmosphere using a water bath sonication system working at 40 kHz, until starting material was consumed (TLC monitoring). Solvents were removed under *vacuo* to give a white solid that was recrystallized from MeOH to give **2** as a white solid.

7-(Prop-2-ynyloxy)-2*H*-chromen-2-one **11**: 67% yield; m.p. 118 °C (Lit.[35] 120 °C); silica gel TLC R_f 0.53 (ethyl acetate/*n*-hexane 50% *v/v*); ν_{max} (KBr) cm^{-1}, 3310 (C≡C-H), 2160 (C≡CH), 1765 (C=O), 1604 (Aromatic); δ_H (400 MHz, DMSO-d_6) 3.69 (1H, t, *J* 2.4, 3'-H), 4.97 (2H, d, *J* 2.4, 1'-H_2), 6.36 (1H, d, *J* 9.6, 3-H), 7.03 (1H, dd, *J* 8.5, 2.3, 6-H), 7.09 (1H, d, *J* 2.3, 8-H), 7.69 (1H, d, *J* 8.5, 5-H), 8.03 (1H, d, *J* 9.6, 4-H); δ_C (100 MHz, DMSO-d_6) 161.1 (C-2), 161.0 (C-7), 156.0 (C-8a),

145.1 (C-4), 130.4 (C-5), 113.9 (C-3), 113.8 (C-4a), 113.7 (C-6), 102.7 (C-8), 79.8 (C-2'), 79.4 (C-3') and 57.0 (C-1'). Data in agreement with reported data.[35]

Synthesis of 7-(prop-2-ynyloxy)-2*H*-chromene-2-thione **12**

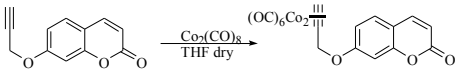


11 **12**

7-(Prop-2-ynyloxy)-2*H*-chromen-2-one **11** (0.2 g, 1.0 eq) and Lawesson's Reagent (1.5 eq) were dissolved in dry toluene (10 ml) and the yellow solution was refluxed until starting material was consumed (TLC monitoring). Then the solvent was removed under *vacuo* and the orange residue was partitioned between H_2O and ethyl acetate. The organic phase was washed with H_2O (2 x 20 ml), brine (3 x 20 ml), dried over Na_2SO_4, filtered-off and concentrated under *vacuo* to give a red sticky oil that was purified by silica gel column chromatography eluting with 10 % ethyl acetate in *n*-hexane to afford the title compound **12** as a yellow solid.

7-(Prop-2-ynyloxy)-2*H*-chromene-2-thione **12**: 72% yield; m.p. 97-101 °C; silica gel TLC R_f 0.27 (ethyl acetate/*n*-hexane 10% *v/v*); ν_{max} (KBr) cm^{-1}, 3300 (C≡C-H), 2165 (C≡C-H), 1601 (Aromatic); δ_H (400 MHz, DMSO-d_6) 3.72 (1H, t, *J* 2.4, 3'-H), 5.02 (2H, d, *J* 2.4, 1'-H_2), 7.13 (1H, dd, *J* 9.2, 2.4, 6-H), 7.18 (1H, d, *J* 9.2, 3-H), 7.31 (1H, d, *J* 2.4, 8-H), 7.80 (1H, d, *J* 9.2, 5-H), 7.90 (1H, d, *J* 9.2, 4-H); δ_C (100 MHz, DMSO-d_6) 198.1 (C-2), 161.8 (C-7), 158.6 (C-8a), 137.4 (C-4), 130.6 (C-5), 127.4 (C-3), 115.7 (C-4a), 115.6 (C-6), 102.3 (C-8), 80.0 (C-2'), 79.2 (C-3') and 57.3 (C-1'). Anal. Calc%. C, 66.65; H, 3.73; S, 14.83; Anal. Found. C, 65.36; H, 3.71; S, 9.37.

Synthesis of 7-(prop-2-ynyloxy)-2*H*-chromen-2-one hexacarbonyldicobalt **13**

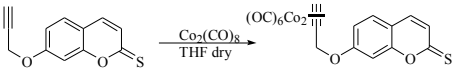


11 **13**

7-(Prop-2-ynyloxy)-2*H*-chromen-2-one **11** (0.1 g, 1.0 eq) was dissolved in THF (10ml) and then cobalt carbonyl (1.05 eq) was added. The black solution was stirred at r.t. for 40 min. Then SiO_2 (0.3 g) was added and solvent removed under *vacuo* to give a black solid that was purified by silica gel column chromatography eluting with 20% *v/v* ethyl acetate/*n*-hexane to afford **6** as a red solid.

7-(Prop-2-ynyloxy)-2*H*-chromen-2-onehexacarbonyldicobalt **13**: 94% yield; silica gel TLC R_f 0.22 (ethyl acetate/*n*-hexane 20% *v/v*); ν_{max} (KBr) cm^{-1} 1752 (C=O), 1600 (Aromatic); δ_H (400 MHz, DMSO-d_6) 5.50 (2H, s, 1'-H_2), 6.35 (1H, d, *J* 9.4, 3-H), 6.89 (1H, s, 3'-H), 7.00 (1H, dd, *J* 8.8, 2.4, 6-H), 7.11 (1H, d, *J* 2.4, 8-H), 7.70 (1H, d, *J* 8.8, 5-H), 8.04 (1H, d, *J* 9.4, 4-H); δ_C (100 MHz, DMSO-d_6) 200.9 (C=O), 161.7 (C-2), 161.0 (C-7), 156.2 (C-8a), 145.1 (C-4), 130.5 (C-5), 113.7, 113.6, 113.4, 102.4 (C-8), 90.8 (C-3'), 73.9 and 69.4.

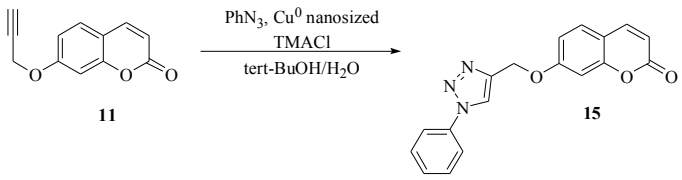
Synthesis of 7-(prop-2-ynyloxy)-2*H*-chromene-2-thione hexacarbonyldicobalt **14**



12 **14**

7-(Prop-2-ynyloxy)-2*H*-chromene-2-thione **12** (0.1 g, 1.0 eq) was dissolved in THF (10ml) and then cobalt carbonyl (1.05 eq) was added. The black solution was stirred at r.t. for 40 min. Then SiO_2 (0.3 g) was added and solvent removed under *vacuo* to give a black solid that was purified by silica gel column chromatography eluting 10% *v/v* ethyl acetate/*n*-hexane to afford **14** as a red solid.
7-(prop-2-ynyloxy)-2*H*-chromene-2-thione hexacarbonyldicobalt **14**: 79% yield; silica gel TLC R_f 0.18 (ethyl acetate/*n*-hexane 10% *v/v*); ν_{max} (KBr) cm^{-1} 1775 (C=O), 1530 (aromatic); δ_H (400 MHz, DMSO-d_6) 5.55 (2H, s, 1'-H_2), 6.90 (1H, s, 3'-H), 7.09 (1H, dd, *J* 8.8, 2.4, 6-H), 7.18 (1H, d, *J* 9.2, 3-H), 7.36 (1H, d, *J* 2.4, 8-H), 7.80 (1H, d, *J* 8.8, 5-H), 7.90 (1H, d, *J* 9.2, 4-H); δc (100 MHz, DMSO-d_6), 200.7 (*C*≡O), 198.3 (*C*=S), 166.5, 162.4, 158.9, 137.2, 130.0, 127.1, 115.4, 101.9, 73.9, 69.7, 57.4 ; Anal. Calc. C, 44.12; H, 2.14; S, 6.20; Anal. Found. C, 44.75; H, 2.08; S, 3.94.

Synthesis of 7-[(1-phenyl-1*H*-1,2,3-triazol-4-yl)methoxy]-2*H*-chromen-2-one **15**

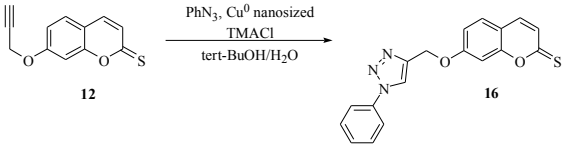


11 **15**

7-(Prop-2-ynyloxy)-2*H*-chromen-2-one **11** (0.08 g, 1.0 eq) and phenylazide (1.1 eq) were dissolved in *tert*-ButOH/H_2O (1/1, 2.0 ml) and then tetramethylamonium chloride (1.0 eq) and copper nanosize (5% mol) were added. The mixture was vigorously stirred at r.t. until starting material was consumed (TLC monitoring). Solvents were removed under *vacuo* (temperature has not to exceed 40 °C) and the brown residue was purified by silica gel column chromatography eluting with 25% *v/v* ethyl acetate/*n*-hexane to afford **15** as a white solid.

7-[(1-Phenyl-1*H*-1,2,3-triazol-4-yl)methoxy]-2*H*-chromen-2-one **15**: 54% yield; m.p. 170-174 °C silica gel TLC R_f 0.11 (ethyl acetate/*n*-hexane 25% *v/v*); ν_{max} (KBr) cm^{-1} 1750 (C=O), 1602 (Aromatic); δ_H (400 MHz, DMSO-d_6) 5.40 (2H, s, 1'-H_2), 6.35 (1H, d, *J* 9.6, 3-H), 7.10 (1H, dd, *J* 9.6, 2.4, 6-H), 7.24 (1H, d, *J* 2.4, 8-H), 7.55 (1H, tt, *J* 7.6, 1.2, Ar-H), 7.65 (2H, d, *J* 7.6, 2 x Ar-H), 7.7 (1H, d, *J* 9.6, 5-H), 7.95 (2H, d, *J* 7.6, 2 x Ar-H), 8.04 (1H, d, *J* 9.6, 4-H), 9.04 (1H, s, 3'-H); δ_C (100 MHz, DMSO-d_6) 162.0 (C-2), 161.2 (C-7), 156.2 (C-8a), 145.2 (C-2'), 144.1 (C-4), 138.0, 130.9, 130.5, 129.8, 124.1, 121.2, 113.8, 113.7, 113.6, 102.6 (C-8) and 63.0 (C-1').

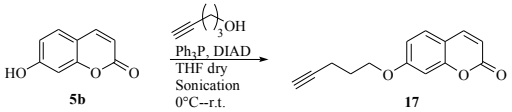
Synthesis of 7-[(1-phenyl-1*H*-1,2,3-triazol-4-yl)methoxy]-2*H*-chromene-2-thione **16**



7-(Prop-2-ynyloxy)-2*H*-chromene-2-thione **12** (0.1 g, 1.0 eq) and phenylazide (1.1 eq) were dissolved in *tert*-ButOH/H_2O (1/1, 2.0 ml). Then tetramethylamonium chloride (1.0 eq) and copper nanosize (10% mol) were added. The mixture was vigorously stirred at r.t. until starting material was consumed (TLC monitoring). Solvents were removed under *vacuo* (temperature has not to exceed 40 °C) and the brown residue was purified by silica gel column chromatography eluting with 50% *v/v* ethyl acetate/*n*-hexane to afford **16** as a yellow solid.

7-[(1-Phenyl-1*H*-1,2,3-triazol-4-yl)methoxy]-2*H*-chromene-2-thione **16**: silica gel TLC R_f 0.50 (ethyl acetate/*n*-hexane 10% *v/v*); ν_{max} (KBr) cm^{-1}, 1604 (Aromatic); δ_H (400 MHz, DMSO-d_6) 5.50 (2H, s, 1'-H_2), 7.12 (1H, dd, *J* 9.6, 2.4, 6-H), 7.26 (1H, d, *J* 9.6, 3-H), 7.35 (1H, d, *J* 2.4, 8-H), 7.58 (1H, tt, *J* 7.6, 1.2, Ar-H), 7.70 (2H, d, *J* 7.6, 2 x Ar-H), 7.72 (1H, d, *J* 9.6, 5-H), 7.95 (2H, d, *J* 7.6, 2 x Ar-H), 8.02 (1H, d, *J* 9.6, 4-H), 9.01 (1H, s, 3'-H); δ_C (100 MHz, DMSO-d_6) 198.0 (C-2), 162.0 (C-7), 157.0 (C-8a), 146.3 (C-2'), 144.0 (C-4), 136.0, 132.0, 131.0, 1230, 124.6, 121.0, 115.0, 114.0, 113.7, 103.0 (C-8) and 63.0 (C-1').

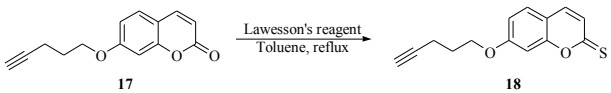
Synthesis of 7-Pent-4-ynyloxy-chromen-2-one **17**



7-Hydroxy coumarin **5b** (1.0 g, 1.0 eq), pent-4-yn-1-ol (1.0 eq) and triphenylphoshine (1.0 eq) were dissolved in dry THF (90 ml). Then the temperature was lowered to 0 °C and diisopropylazadicarboxylate (1.1 eq) was added drop-wise under sonication. The orange solution was sonicated at r.t. under a nitrogen atmosphere until starting material was consumed (TLC monitoring). Solvents were removed under *vacuo* to give a white solid that was purified by silica gel column chromatography eluting with 50% *v/v* ethyl acetate/*n*-hexane to afford the title compound **17** as white solid.

7-Pent-4-ynyloxy-chromen-2-one **17**: 52% yield; m.p. 112 °C; silica gel TLC R_f 0.50 (ethyl acetate/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6) 1.96 (2H, pent, *J* 6.4), 2.38 (2H, m), 2.89 (1H, t, *J* 2.8, ≡C*H*), 4.18 (2H, t, *J* 6.4), 6.32 (1H, d, *J* 9.6, Ar-*H*), 6.98 (1H, d, *J* 9.6, Ar-*H*), 7.03 (1H, d, *J* 2.4, 8-H), 7.66 (1H, d, *J* 9.6, Ar-*H*), 8.01 (1H, d, *J* 9.6, Ar-*H*); δ_C (100 MHz, DMSO-d_6) 15.3, 28.4, 67.7, 72.6, 84.4, 102.1, 113.3, 113.4, 113.6, 130.4, 145.2, 156.3, 161.2, 162.6.

Synthesis of 7-pent-4-ynyloxy-chromene-2-thione **18**

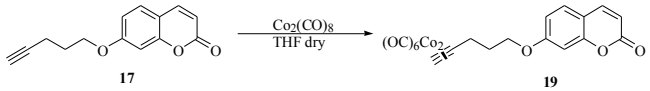


17 18

7-Pent-4-ynyloxy-chromen-2-one **17** (0.2 g, 1.0 eq) and Lawesson's Reagent (1.5 eq) were dissolved in dry toluene (10 ml) and the yellow solution was refluxed until starting material was consumed (TLC monitoring). Then the solvent was removed under *vacuo* and the orange residue was partitioned between H_2O and ethyl acetate. The organic phase was washed with H_2O (2 x 20 ml), brine (3 x 20 ml), dried over Na_2SO_4, filtered-off and concentrated under *vacuo* to give a red sticky oil that was purified by silica gel column chromatography eluting with 10% *v/v* ethyl acetate/*n*-hexane to afford the title compound **18** as a yellow solid.

7-Pent-4-ynyloxy-chromene-2-thione **18**: 35% yield; silica gel TLC R_f 0.54 (ethyl acetate/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6) 1.94 (2H, pent, *J* 6.4), 2.40 (2H, m), 2.76 (1H, t, *J* 2.8, ≡C*H*), 4.19 (2H, t, *J* 6.4), 6.99 (1H, s, Ar-*H*), 7.04 (1H, dd, *J* 8.8, 2.4, Ar-*H*), 7.19 (1H, d, *J* 9.2, Ar-*H*), 7.35 (1H, d, *J* 2.4, Ar-*H*), 7.80 (1H, d, *J* 8.8, Ar-*H*), 7.92 (1H, d, *J* 9.2, Ar-*H*); δ_C (100 MHz, DMSO-d_6) 15.3, 28.4, 67.7, 72.6, 84.4, 102.1, 113.3, 113.4, 113.6, 130.4, 145.2, 156.3, 161.2, 198 (*C*=S).

Synthesis of 7-(pent-4-ynyloxy)-2*H*-chromen-2-one hexacarbonyldicobalt **19**



17 19

7-(Pent-4-ynyloxy)-2H-chromen-2-one **17** (0.05 g, 1.0 eq) was dissolved in THF (10 ml) and then cobalt carbonyl (1.05 eq) was added. The black solution was stirred at r.t. for 40 min. Then SiO_2 (0.3 g) was added and solvent removed under *vacuo* to give a black solid that was purified by silica gel column chromatography eluting with 20% *v/v* ethyl acetate/*n*-hexane to give **19** as a red solid.
7-(Pent-4-ynyloxy)-2*H*-chromen-2-one hexacarbonyldicobalt **19**: 92 % yield; silica gel TLC R_f 0.20 (ethyl acetate/*n*-hexane 20% *v/v*); ν_{max} (KBr) cm^{-1} 1762 (C=O), 1530 (aromatic); δ_H (400 MHz, DMSO-d_6) 2.10 (2H, quint, *J* 6.8, 2'-H_2), 3.09 (2H, t, *J* 6.8, 3'-H_2), 4.28 (2H, t, *J* 6.8, 1'-H_2), 6.33 (1H, d, *J* 9.6, 3-H), 6.84 (1H, s, 5'-H), 7.01 (1H, dd, *J* 8.8, 2.0, 6-H), 7.06 (1H, d, *J* 2.0, 8-H), 7.66 (1H, d, *J* 8.8, 5-H), 8.03 (1H, d, *J* 9.6, 4-H); δc (100 MHz, DMSO-d_6), 200.9 (*C*≡O), 162.7 (*C*=O), 161.3, 156.5, 145.4, 130.6, 113.8, 113.5, 102.3, 98.5, 75.5, 72.5, 68.4, 31.8, 31.0; ; Anal. Calc. C, 47.66; H, 2.86; Anal. Found. C, 46.74; H, 2.27.

Co-crystallization and X-ray data collection. Crystals of native hCA II were obtained using the hanging drop vapor diffusion method. 2 µl of the protein solution were mixed with 2 µl of a solution of 1.6 M sodium citrate, 50 mM Tris pH 8.0 and were equilibrated against the same solution at 296 K. Protein concentration was 0.4 mM in 50 mM Tris pH=8.0. Crystals of the complex with **8a** were obtained by soaking the hCAII crystals in a saturated solution of the compound dissolved in 1.2 M sodium citrate, 50 mM Tris pH 8.0 and 15% glycerol.
A crystal of the complex was harvested from this solution and flash-frozen at 100K. A data set on a crystal of the complex hCAII-inhibitor **8a** was collected to a maximum resolution of 1.10 Å, using synchrotron radiation at the ID23-1 beamline at ESRF (Grenoble, France) with a wavelength of 1.000 Å and a DECTRIS Pilatus 6M detector. Data were integrated and scaled using the program XDS.[36] Data processing statistics are showed in Table 1.

Structure determination. The crystal structure of hCA II (PDB accession code: 3P58) without solvent molecules and other heteroatoms was used to obtain initial phases of the structures using Refmac5[37] 5% of the unique reflections were selected randomly and excluded from the refinement data set for the purpose of Rfree calculations. Inspection of the difference electron-density maps indicated the presence of an inhibitor molecule bound to the water that coordinate the catalytic zinc ion. Atomic models for the inhibitor were calculated and energy minimized using the program JLigand 1.0.39. A fractional occupancy factor of 0.5 was attributed to all the inhibitor atoms. After

the introduction of the inhibitor positive residual densities were present in the difference electron-density maps close to the inhibitor and were attributed to disordered water molecules (occupancy factors 0.5).

During the refinement anisotropic temperature factors were introduced and hydrogen atoms were added to the model. Manual building of the atomic model were carried out using COOT[38] Solvent molecules were introduced automatically using the program ARP[39] working in the default solvent building mode. The quality of the final models were assessed with PROCHECK.[40] Crystal and refinement data are summarized in Table 2. Graphical representations were generated with Chimera.[41]

Table 2. Summary of Data Collection and Atomic Model Refinement Statistics.*

	hCA II+ 8a
PDB ID	4WL4
Wavelength (Å)	1.000
Space Group	P21
Unit cell (a,b,c,□) (Å, °)	42.26, 41.37, 72.28, 104.21
Limiting resolution (Å)	29.11-1.10 (1.17-1.10)
Unique reflections	78925 (3784)
Rsym (%)	4.8 (42.1)
Redundancy	3.5 (2.1)
Completeness overall (%)	80.7 (27.2)
<I/(I)>	12.90 (1.73)
Refinement statistics	
Resolution range (Å)	29.11-1.10
Unique reflections, working/free	75094 (3784)
Rfactor (%)	10.90
Rfree(%)	12.99
No. of protein atoms	4682
No. of water molecules	395

No. of compound atoms	18
r.m.s.d. bonds(Å)	0.0066
r.m.s.d. angles (°)	1.311
Ramachandran statistics (%)	
Most favored	96.5
additionally allowed	3.5
generously allowed regions	0
Average B factor (Å^2)	
main-chain protein atoms	12.72
side chain protein atoms	14.88
compound	15.80
solvent	33.69

*Values in parentheses are for the highest resolution shell.

Table 3. Occupancy and B factors of the zinc ion, the inhibitor **8a** atoms and water molecules in the active site of the hCA II complex.

atom	occupancy	B isotropic
Zn	1.0	7.36
S1	0.50	16.09
CAF	0.50	13.79
CAI	0.50	15.56
HAI	0.50	14.05
CAH	0.50	13.93
HAH	0.50	13.97
CAN	0.50	15.08
CAM	0.50	15.99
HAM	0.50	16.21

OAK	0.50	15.45
CAO	0.50	14.83
CAL	0.50	15.48
HAL	0.50	15.39
CAG	0.50	16.35
Wat343	0.50	35.87
Wat344	0.50	18.95
Wat349	0.50	38.00
Wat350	0.50	33.12
Wat351	0.50	12.46
Wat352	0.50	22.01
Wat353	0.50	20.66
Wat354	0.50	22.75
Wat344	0.50	18.95
Wat394	0.50	12.08

Accession Codes. Coordinates and structure factors for CA II complexes with **8a** have been deposited in the Protein Data Bank (PDB) accession code: 4WL4.

Acknowledgements. This research was financed by two EU grants of the 7th framework program (Metoxia and Dynano projects to AS and CTS).

Nonstandard abbreviations. CA, carbonic anhydrase; CAI, CA inhibitor; K_I, inhibition constant; TBDMS, *tert*-butyldimethylsylil.

***Corresponding Author:** Prof. Claudiu T. Supuran (CTS) Phone: +39-055-4573005; fax: +39-055-4573385; E-mail: claudiu.supuran@unifi.it

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