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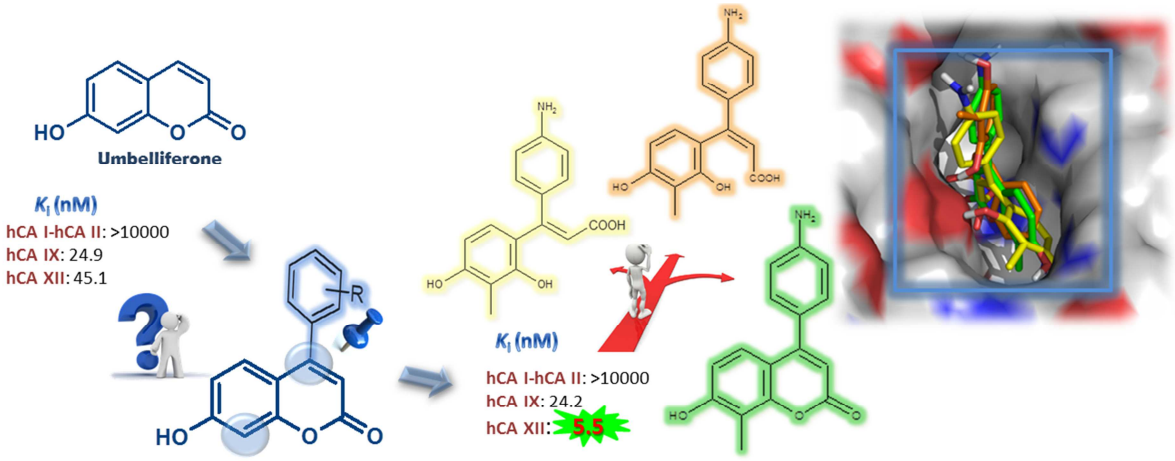


Inhibitory Effects and Structural Insights for a Novel series of Coumarin-based Compounds that Selectively Target human CA IX and CA XII Carbonic Anhydrases

Laura De Luca[1,] Francesca Mancuso,[1,2] Stefania Ferro,[1] Maria Rosa Buemi,[1] Andrea Angeli,[3] Sonia del Prete,[4] Clemente Capasso,[4] Claudiu T. Supuran,[3] Rosaria Gitto[1,*]*

[1] Dipartimento di Scienze Chimiche, Biologiche, Farmaceutiche ed Ambientali (CHIBIOFARAM), Università degli Studi di Messina, Viale Annunziata, I-98168, Messina, Italy
[2] Fondazione Prof. Antonio Imbesi, Piazza Pugliatti 1, 98100, Messina, Italy
[3] Dipartimento NEUROFARBA, Università di Firenze, Via Ugo Schiff, I-50019, Sesto Fiorentino, Italy
[4]Istituto di Bioscienze e Biorisorse - CNR ,Via Pietro Castellino 111- 80131, Napoli, Italy,

Corresponding authors:
Laura De Luca, PhD, Associate professor on Medicinal Chemistry, Dipartimento di Scienze Chimiche, Biologiche, Farmaceutiche ed Ambientali (CHIBIOFARAM), Università degli Studi di Messina, Viale Annunziata, I-98168, Messina, Italy
Tel 00390906766410, Fax 00390906766404, e-mail: ldeluca@unime.it
Rosaria Gitto, PhD, Associate professor on Medicinal Chemistry, Dipartimento di Scienze Chimiche, Biologiche, Farmaceutiche ed Ambientali (CHIBIOFARAM), Università degli Studi di Messina, Viale Annunziata, I-98168, Messina, Italy
Tel 00390906766412, Fax 00390906766404, e-mail: rgitto@unime.it

Keywords: coumarins; hCAs; hCA IX, hCA XII; antitumor agents; molecular docking

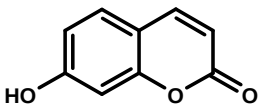
Abbreviations: hCA: human Carbonic Anhydrases; AAZ: acetazolamide; MBG: metal binding groups; CARP: CA related-proteins; SG: sticky group; FC: Flash Chromatography; PDB: RCSB Protein Data Bank;

Abstract

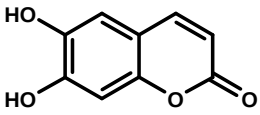
Coumarin derivatives are a peculiar class of inhibitors of the family of metalloenzymes carbonic anhydrases (CA, EC 4.2.1.1). Several coumarins display higher affinity and selectivity toward most relevant and druggable CA isoforms. By decorating the natural compound umbelliferone (**1**) we have identified a new series of coumarin-based compounds demonstrating high CA inhibitory effects with nanomolar affinity for hCA IX and hCA XII isoforms that were considered a target amenable to develop antitumor agents. The most active tested compounds proved to be potent inhibitors with K_i values equal to that of the well-known inhibitor acetazolamide (AAZ), that lacks selectivity over ubiquitous hCA I and hCA II. As suggested by docking studies the coumarins, that are lacking of the canonical metal binding groups, do not interact with Zinc ion within the catalytic site as found for classical sulfonamide type inhibitors of CAs. Thus, the studied inhibitors might possess a non-classical inhibitory mode of action preventing the carbon dioxide to entry into catalytic cavity and its conversion into bicarbonate ion. Specifically, the most active inhibitor of hCA XII compound **18i** (K_i value of 5.5 nM) and its supposed hydrolytic products could establish a web of H-bond interactions within the enzymatic cavity.

1. Introduction

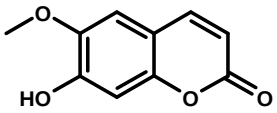
Coumarins are a very large series of polyphenolic derivatives widely diffuse throughout the plant kingdom. Their structural diversity led to the classification into different categories such as the simple coumarin prototype and many other groups of polycyclic analogues (furanocoumarins and pyranocoumarins). The most widespread derivatives natural compounds are umbelliferone (**1**), esculetin (**2**) and scopoletin (**3**), that belong to the simple coumarin scaffold (see Chart 1). The wide range of biological functions attributed to coumarin derivatives made them an attractive heterocyclic system as mould for further chemical derivatization to identify novel therapeutic agents. To date, several studies have proven multiple potential roles which include antimicrobial,[1] antiviral,[2-4] anti-inflammatory,[5, 6] anti-oxidant,[5] anti-coagulant,[7] lipid lowering,[8] anti-proliferative activity in some cancer cells lines,[9] and so on.



umbelliferone, 1



esculetin, 2



scopoletin, 3

Chart 1. Chemical structures of coumarin derivatives (**1-3**)

In the last decades, several coumarin derivatives have shown to constitute a new class of inhibitors of the carbonic anhydrase (CA, EC 4.2.1.1) family [10-13]. The family of human carbonic anhydrases (hCAs) comprises 16 α–CA isozymes that differ for kinetic properties, tissue and cellular distribution. Among these isoforms, five are cytosolic (CA I-III, CA VII and CA XIII), two are mitochondrial (CA VA and CA VB), four are membrane-bound (CA IV, CA IX, CA XII and CA XIV) and one is secreted into saliva and milk (CA VI). Three acatalytic isoforms are also known, the CA related-proteins (CARP), VIII, X and XI which are located in the cytosol. CAs catalyze the simple, but crucial, reversible reaction of hydration of carbon dioxide to bicarbonate ion and protons. Therefore, 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, and tumorigenicity. On this basis, hCA inhibitors (hCAIs) could exert therapeutic potential for the treatment of a plethora of pathologies. In addition to the validated role of the well-known hCAIs as diuretic and anti-glaucoma agents, they also emerged as potential anticonvulsant, antiobesity, anti-infective and anticancer drugs. However, the main drawback of the clinically used CAIs is the lack of isoform selectivity, which may lead to side effects due to the inhibition of the ubiquitous hCA I and II isoforms.

To obtain new theranostic tools useful in cancer, an attractive strategy is recently focused on the development of new highly selective molecules targeting human CA IX/XII [14-17]. Specifically, the dimeric membrane bound hCA IX isoform is overexpressed in hypoxic tumors, whilst it is not generally abundant in normal tissue. The catalytic activity of hCA IX isoform is maximum at low pH values, that characterize hypoxic tumors not responsive to

chemotherapy and radiotherapy. Therefore, it seems that hCA IX contributes to acidification of the extracellular environment maintaining a moderately alkaline intracellular pH. In a similar way, the transmembrane tumor-associated isozyme hCA XII decreases the extracellular pH cooperating with hCA IX [14].

Typically, the CA family is inhibited by compounds anchoring the Zinc ion, through metal binding groups (MBG) such as sulfonilamides and their bioisosteres (sulfamates, sulfamides, N-substituted sulfonilamides, and so on) [18-22]. Due to the lack of the MBG, the coumarins could represent a class of CAIs characterized by unusual mechanism of enzyme inhibition [23]. The analysis of the X-ray crystal structure of the adduct hCA II with the natural product 6-(1-*S*-hydroxy-3-methylbutyl)-7-methoxy-2*H*-chromen-2-one (**4**), isolated from *Leionema Ellipticum* (Rutaceae), demonstrated the presence in to the enzyme active site of its hydrolytic product as the substituted *(Z)*-2-hydroxycinnamic acid derivative [12]. A similar behaviour it has been observed for the unsubstituted coumarin (**5**) [10] for which the (*E*)-2-hydroxycinnamic acid is bound to the hCA II catalytic site. Moreover, it has been demonstrated that several (thioxo)coumarins (*e.g.* **6**) might occlude the entrance of the enzyme active site [24].

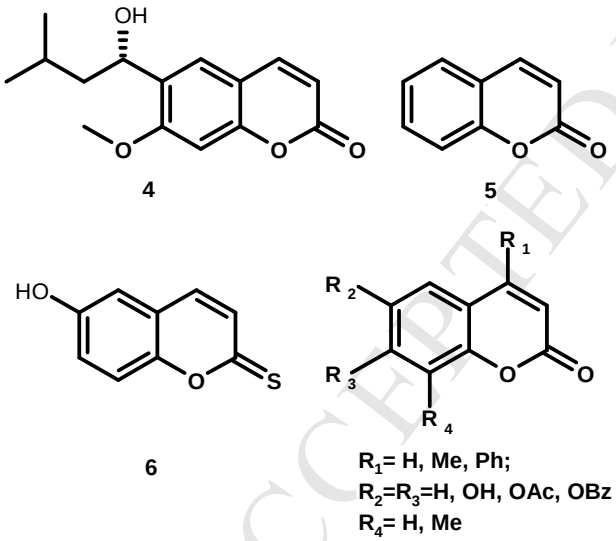


Chart 2. Chemical structures of coumarin-based carbonic anhydrase inhibitors **4-6** and new designed compounds

Therefore, several coumarins bind the enzyme cavity through a sticky group (SG) attached to the heterocyclic scaffold which can also incorporate a *tail* which makes additional interaction away from the catalytic hole. It is well known that both the rim part of active site cavity and the middle region are the most variable region of among the 16 isoforms

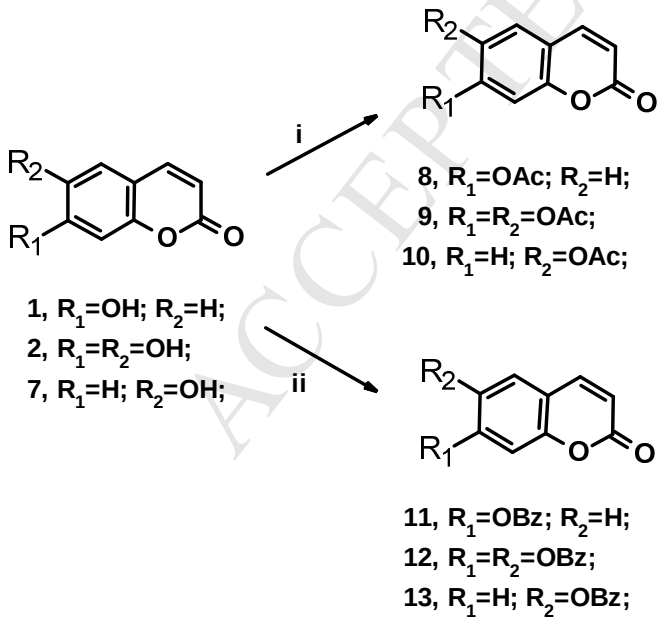
controlling the selectivity profile of coumarin derivatives over abundant hCA I and hCA II isoforms. Specifically, the 7-hydroxycoumarin (umbelliferone, **1**) and some of its derivatives [11] are inhibitors of the tumor-associated isoforms CA IX and XII.

In the last decade, we have been engaged in the development of potent and selective inhibitors from synthetic source and we have identified a large class of isoquinoline-sulfonamides being CAIs at nanomolar concentration [25-34]. With the aim to identify new CAIs structurally related to natural products, we now report a novel series of coumarin derivatives that were designed to study the influence of R_1, R_2, R_3, R_4-substituents on coumarin scaffold (see Chart 2). To evaluate the inhibitory activity and selectivity toward hCA IX/XII over ubiquitous hCA I and hCA II, all obtained coumarins were *in vitro* assayed against these selected isoforms. Finally, to in-depth study the binding poses of the most active inhibitors the docking simulations have been carried out.

2. Results and discussion

Firstly, we have focused our interest on 7-hydroxycoumarin (**1**), 6,7-dihydroxycoumarin (**2**) and 6-hydroxycoumarin (**7**) that were also converted in to the corresponding esters **8-13** following previously reported procedures (Scheme 1) [35].

Scheme 1.

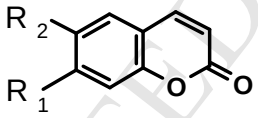


Reagents and conditions: i) Ac_2O, H_2SO_4, TEA, rt; ii) BzCl, Et_3N, DCM, rt.

The inhibitory effects of this subseries of hydroxyl coumarins **1-2, 7** and corresponding esters **8-13** were evaluated against the tumor-associated hCA IX and hCA XII and the ubiquitous isoforms hCA I and hCA II. The K_is data are summarized in Table 1 in comparison with well-known inhibitor acetazolamide (AAZ).

From the analysis of K_i values we observed that all the compounds show inhibitory activity against hCA IX and hCA XII with affinity in the range of 23.1-116.2 nM and 9.3-814.4 nM, respectively. Most of tested compounds were potent inhibitors which proved to be equi-active when compared with AAZ. In contrast, the tested coumarins were ineffective inhibitors of the hCA I and hCA II (K_i values > 10,000 nM), thus showing high selectivity over these isoforms that are considered responsible of side-effects of CAIs. In terms of structure-affinity relationships it could be observed that the natural compounds 7-hydroxy and 6,7-dihydroxy-coumarins **1**-**2** were generally more active inhibitors when compared with corresponding ester derivatives **8-9** and **11-12**. Whereas, by masking the 6-hydroxyl group of coumarin **7** we obtained esters **10** and **13** that showed a slight reduction of inhibitory effects.

Table 1. K_i values of coumarin derivatives (**1-2, 7-13**) against hCA I, hCA II, hCA IX, and hCA XII. For comparison purpose data of AAZ were also reported.



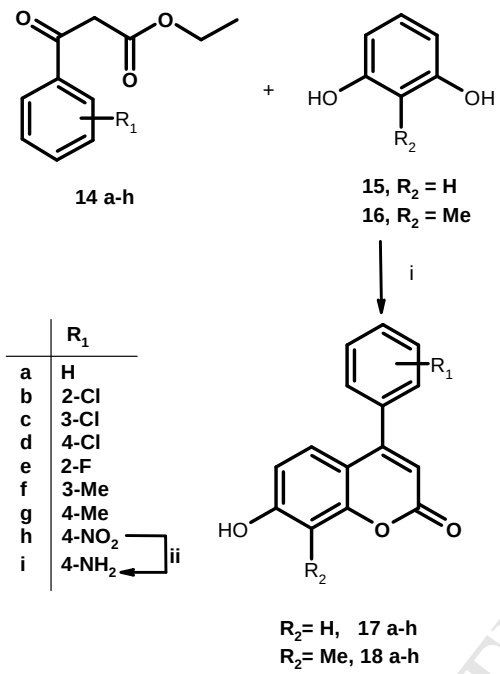
			K_i(nM)[a]			
	R_1	R_2	hCA I	hCA II	hCA IX	hCA XII
1	OH	H	>10000	>10000	24.9	45.1
2	OH	OH	>10000	>10000	36.4	9.3
7	H	OH	>10000	>10000	30.5	33.9
8	OAc	H	>10000	>10000	40.2	573.2
9	OAc	OAc	>10000	>10000	116.2	48.2
10	H	OAc	>10000	>10000	23.1	47.0
11	OBz	H	>10000	>10000	51.4	814.4
12	OBz	OBz	>10000	>10000	38.4	292.4
13	H	OBz	>10000	>10000	29.9	87.4
AAZ[b]	-	-	250	12.1	25.0	5.7

[a]Errors are in the range of ± 5-10% of the reported value, from 3 different assays. [b]Data are taken from Reference 25.

From the first subseries of coumarin-based compounds, the umbelliferone (**1**) has been selected for further structural optimization. Therefore, the second and third subseries of 4-aryl-7-hydroxy-2*H*-chromen-2-ones (**17a-i**) and 4-aryl-7-hydroxy-8-methyl-2*H*-chromen-2-ones (**18a-i**) have been designed by introducing aryl group as well as methyl group at C-4 and C-8 position of coumarin nucleus. To synthesize compounds **17a-h** and **18 a-h**, the

classical Pechmann condensation has been employed (see Scheme 2). Thus, selected commercially available ethyl benzoylacetates **14 a-h** and the resorcinol **15** or 2-methylresorcinol **16** reacted with in strong acid conditions to provide the desired coumarins **17a-h** and **18 a-h**, respectively. To study the influence of the introduction of a polar group on phenyl ring, it was also performed the nitro-reduction of compounds **17h** and **18h** in to the corresponding aniline derivatives **17i** and **18i.** All compounds were carefully structurally characterized by spectroscopic techniques (see experimental section).

Scheme 2

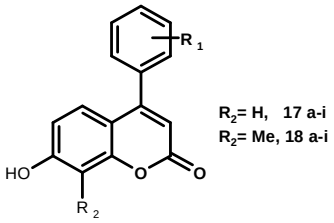


Reagents and conditions: i) H_2SO_4, rt; ii) NH_2NH_2-H_2O, EtOH, Pd/C, 70° C.

Table 2 collects the K_i inhibition data determined for coumarin-derived compounds belonging to the **17a-i** and **18 a-i** subseries that differs for the presence of the C-8 methyl substituent. As you can see in Table 2, the two series of 4-aryl-7-hydroxy-2*H*-chromen-2-ones proved to be active and selective inhibitors over ubiquitous hCA I and hCA II isoforms. Among the first subseries of compounds **17a-i** we found that that the introduction of an unsubstituted phenyl ring at C-4 position (*i.e.* **17a**) resulted in the decrease of inhibitory effects toward hCA IX and hCA XII isoforms when compared with K_i values of prototype **1** (see Table 1). Concerning the inhibitory effects hCA IX isoform, with exception of compound **18e** (K_i value of 95.9 nM) a flat SAR generally characterizes the inhibitory profile of the two subseries of compounds **17** and **18** for which the K_i values fall in a

narrow range from 17.0 to 47.2 nM. Therefore, we can hypothesize that the 4-arylsubstituent occupies a very large area lacking in specific hydrophilic/hydrophobic contacts with crucial aminoacid residues.

Table 2. K_i values of coumarin derivatives **17a-i** and **18 a-i** against hCA I, hCA II, hCA IX, and hCA XII.



			K_i(nM)[a]			
	R_1	R_2	hCA I	hCA II	hCA IX	hCA XII
17a	H	H	>10000	>10000	47.2	90.8
17b	2-Cl	H	>10000	>10000	30.8	7.8
17c	3-Cl	H	>10000	>10000	31.0	7.6
17d	4-Cl	H	>10000	>10000	31.5	8.9
17e	2-F	H	>10000	>10000	31.8	8.0
17f	3-Me	H	>10000	>10000	17.0	7.8
17g	4-Me	H	>10000	>10000	35.8	64.7
17h	4-NO_2	H	>10000	>10000	20.3	6.5
17i	4-NH_2	H	>10000	>10000	21.6	7.7
18a	H	Me	>10000	>10000	39.5	402.2
18b	2-Cl	Me	>10000	>10000	27.5	31.0
18c	3-Cl	Me	>10000	>10000	36.6	369.2
18d	4-Cl	Me	>10000	>10000	35.3	293.7
18e	2-F	Me	>10000	>10000	95.9	9.0
18f	3-Me	Me	>10000	>10000	37.4	89.1
18g	4-Me	Me	>10000	>10000	38.2	44.9
18h	4-NO_2	Me	>10000	>10000	30.9	9.3
18i	4-NH_2	Me	>10000	>10000	24.2	5.5

[a]Errors are in the range of ± 5-10% of the reported value, from 3 different assays.

Notably significant inhibitory effects toward hCA XII are shown by the subseries of the 4-aryl-7-hydroxy-2*H*-chromen-2-ones **17a-f** and **17h-i** (K_i values ranging from 6.5-8.9 nM). In contrast, the 7-hydroxy-4-(4-methylphenyl)-2*H*-chromen-2-one (**17g**, R = 4-Me) displayed moderate inhibitory effects toward this isoform. Introduction of a methyl group at C-8 position generally induces a decrease of hCA XII inhibitory effects for compounds belonging to the subseries of 4-aryl-7-hydroxy-8-methyl-2*H*-chromen-2-ones **18** with exception of derivatives **18e**, **18h** and **18i** that were potent inhibitors sharing a similar activity with analogs **17e**, **17h** and **17i**. Surprisingly, for compounds **18a**, **18c** and **18d** we found up to 50-fold decrease of affinity. Among the two subseries of coumarins, the best active hCA XII inhibitors were compounds **17f**, **17h, 17i** and **18i** which displayed a very similar potency when compared with well-known inhibitor AAZ and demonstrated high selectivity over hCA I/II isoforms.

To gain more information about the putative binding mode in to the hCA XII cavity for 4-aryl-7-hydroxy-2H-chromen-2-one derivatives (**17a-i** and **18 a-**i) we have performed docking studies by means of AutoDock program and using the crystal structure of the hCA XII (PDB code 1JCZ). Considering that CAs could exert an esterase activity which causes the hydrolytic process of coumarin system, we chose to analyze the binding pose of the tested coumarins as well as their corresponding 2-hydroxycinnamic acid derivatives as *Z*- and *E*-isomers. Specifically, panels B-D in Figure 1 display the docking results for the most active compound **18i** and corresponding plausible hydrolytic products **(*Z*)-18i-A** and **(*E*)-18i-B,** which were generated by a well-known pathway summarized in panel A of Figure 1.

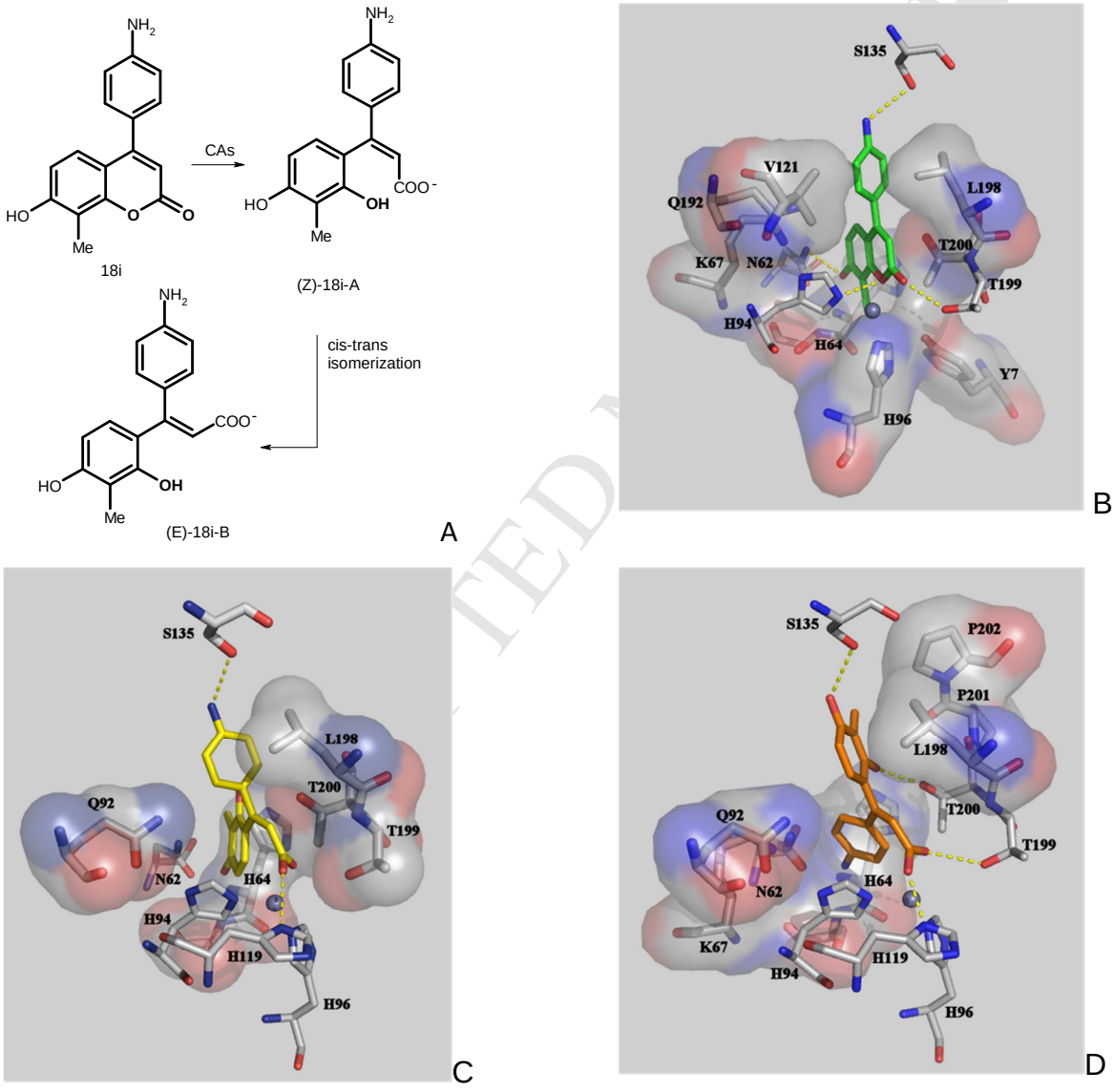


Figure 1. In panel A is shown the supposed CA-mediated hydrolysis of coumarin derivative **18i** leading to *Z*- or *E*-2-hydroxycinnamic acid derivatives **(*Z*)-18i-A** and **(*E*)-18i-B**. Docked poses of compound **18i** (B), isomer **(*Z*)-18i-A** (C) and isomer **(*E*)-18i-B** (D) within hCA XII cavity retrieved from RCSB Protein Data Bank (PDB: 1JCZ). Key residues of the hCAXII are presented. The hydrogen bonds are displayed in dotted lines. The Zinc ion is shown as gray sphere.

As expected for coumarin **18i** as well as related compounds **(*Z*)-18i-A** and **(*E*)-18i-B,** there was the lack of coordination of Zinc ion within catalytic site. From the comparison of the docked poses for the three ligands it emerged that **18i**, **(*Z*)-18i-A** and **(*E*)-18i-B** occupy the hCA XII cavity and establish profitable contacts with hydrophilic/hydrophobic crucial residues. A very similar binding mode was observed for coumarin derivative **18i** (panel B) and isomer **(*Z*)-18i-A** (panel C), for which the phenyl ring at C-4 position of coumarin nucleus is located in proximity of the hydrophobic residue L198; whereas the 4'-amino-functional group engages H-bond donor interaction with S135 in the top of the cavity. Furthermore, for the **18i** (panel B) and isomer **(*Z*)-18i-A** (panel C) there is a dense network of hydrogen bonds with N62, H94, H119, and T199 residues.

As depicted in Figure 1 (panel D), the hypothetic **(*E*)-18i-B** isomer binds the hCA XII cavity assuming a different orientation for the 4-aminophenyl substituent that is located in the bottom of the cavity. The 2,4-dihydroxylphenyl ring assumes a peculiar disposition for which two relevant hydrogen bond interactions with S135 and T200 mantain the aromatic ring leaned against the hydrophobic wall (L198, P201 and P202) of the cavity. Finally, the carboxylate moiety creates a strong bidentate interaction with H119 and T199 residues and shows an orientation similar to that of precursor **18i**.

3. Conclusions

Based on the proof-of-principle that coumarins derivatives could be selective inhibitors of tumor-associated hCA IX and hCA XII isoforms, we have planned the synthesis of new umbelliferone-based compounds that resulted potent inhibitors at low nanomolar concentration. Moreover, all tested compounds showed relevant selectivity over ubiquitous hCA I and hCA II isoforms. This experimental evidence confirms that coumarin derivatives represent useful heterocyclic system for further chemical optimization to obtain CAIs characterized by a peculiar mechanism of CA inhibition which does not involve the coordination of Zinc ion within catalytic site.

4. Experimental section

4.1 Chemistry

Starting materials and reagents were purchased from commercial sources (Sigma-Aldrich Milan Italy and Alfa Aesar Karlsruhe Germany) and were used without further purification. Melting points were determined on a BUCHI Melting Point B-545 (BUCHI Labortechnik AG Flawil, Switzerland) apparatus and are uncorrected. Elemental analysis (C, H, N) were carried out on a Carlo Erba Model 1106 Elemental Analyzer and the results were within ± 0.4% of the theoretical values. ^{1}H-NMR spectra were recorded in $CDCl_3$ and DMSO with TMS as internal standard or DMSO-d_6 on a Varian Gemini-300 spectrometer. Chemical shifts were expressed in δ (ppm) and coupling constants (J) in hertz (Hz). R*f* values were determined on TLC plates using a mixture of Cy/EtOAc 8:2 as eluent.

*4.1.1 General procedures for the synthesis of acetates **8-10***

The compounds **1**, **2** or **7** (respectively 1.2, 1.1 mmol and 1.1) were stirred with acetic anhydride (3 mL) in ice bath and a catalytic amount of 96% sulfuric acid was added dropwise. After that, 2.5 molar equivalents of Et_3N were added to the mixture and agitated until the disappereance of starting compounds (analyzed by TLC). After reaction was completed it was cooled into ice and the solid was filtered off and dried to afford the corresponding compounds **8-10**. Chemical and spectral data for **8-9** are in good agreement with literature [35].

*4.1.1.1 (2-Oxochromen-6-yl)acetate (**10**)* Mp: 143-145 °C (Et_2O), yield 64%; reaction time: 1.5 hours; R*f*: 0.16; ^{1}H NMR ($CDCl_3$): 2.32 (s, 3H, CH_3), 6.45 (d, *J*=10.1, 1H, CH), 7.23-7.25 (m, 2H, ArH), 7.36 (d, 1H, ArH), 7.65 (d, *J*=10.1, 1H, CH). Anal. Calcd for $C_{11}H_8O_4$: C:64.7; H:3.9. Found: C:64.3; H:3.6.

*4.1.2 General procedures for the synthesis of benzoates **11-13***

To a well-stirred solution of compounds **1**, **2** or **7** (1mmol) in DCM (2 mL), Et_3N (2.5 molar equivalents) and benzoyl chloride (5 molar equivalents) were added. The mixture was stirred until the end of the reaction (monitored by TLC) at room temperature and then cooled into ice. The solid in suspension was filtered off and recrystallized from the suitable solvent to give esters **11-13**. Chemical and spectral data for **11-12** are in good agreement with literature [35].

*4.1.2.1 (2-Oxochromen-6-yl)benzoate (**13**)*

Mp: 137-139 °C (EtOH), yield 60%; reaction time: 20 h; R*f*: 0.23; 1H NMR (DMSO-d_6): 6.53 (s, *J*=2.1, 1H, CH), 6.56 (s, *J*=2.1, 1H, CH), 7.50-7.60 (m, 3H, ArH), 7.72-7.74 (m, 2H, ArH), 8.02-8.06 (m, 1H, ArH), 8.11-8.14 (m, 2H, ArH). Anal. Calcd for $C_{16}H_{10}O_4$: C:72.1; H:3.7. Found: C:72.3; H:3.9.

4.1.3 General procedures for the synthesis of 4-arylcoumarins ***17a-h*** *and* ***18a-h***

The title compounds were prepared as previously described [4] with slight modifications. To a stirred solution of the appropriate ethylbenzoylacetate (**14a-h**) (1 mmol), resorcinol (**15**) or 2-methylresorcinol (**16**) (1 mmol) and 96% sulfuric acid (1.5 ml) was added dropwise at 0° C. The resulting mixture was stirred for different reaction times at room temperature and monitored by TLC until completion. The reaction was quenched with ice and extracted with EtOAc (3 x 10 mL). The organic phase was dried with Na_2SO_4 and concentrated until dryness under reduced pressure. The residue was purified by crystallization with suitable solvent to afford the desired compounds **17a**-**h** and **18a-h**.

Chemical and spectral data for **17a**, **17e**, **18a-b** and **18d** are in good agreement with literature [4, 36].

4.1.3.1 4-(2-Chlorophenyl)-7-hydroxy-2*H*-chromen-2-one ***(17b)***

Mp: 218-220°C (Et_2O), yield 22%; reaction time: 2.5 hours; R*f*: 0.16*;* ^{1}H NMR (DMSO-d_6): 6.16 (s, 1H, CH), 6.72-6.77 (m, 3H, ArH), 7.45-7.50 (m, 3H, ArH), 7.52-7.62 (m, 1H, ArH), 10.69 (bs, 1H, OH). Anal. Calcd for $C_{15}H_9ClO_3$: C:66.1; H:3.3. Found: 65.8; H: 3.0.

4.1.3.2 4-(3-Chlorophenyl)-7-hydroxy-2*H*-chromen-2-one ***(17c)***

Mp: 237-239°C (EtOH), yield 31%; reaction time: 2 hours; R*f*: 0.16; ^{1}H NMR (DMSO-d_6): 6.18 (s, 1H, CH), 6.74-6.78 (m, 2H, ArH), 7.18 (d, 1H, ArH), 7.40 (m, 1H, ArH), 7.55-7.58 (m, 3H, ArH). Anal. Calcd for $C_{15}H_9ClO_3$: C:66.1; H:3.3. Found: C:65.9; H:3.4.

4.1.3.3 4-(4-Chlorophenyl)-7-hydroxy-2H-chromen-2-one ***(17d)***

Mp: 280-282°C (EtOH), yield 33%; reaction time: 2.5 hours; R*f*: 0.16; ^{1}H NMR (DMSO-d_6): 6.16 (s, 1H, CH), 6.76- (d, *J*=8.8, 2H, ArH), 7.22 (d, 1H, ArH), 7,53 (d, *J*=8.8, 2H, ArH), 7.59 (m, 2H, ArH), 10.70 (bs, 1H, OH). Anal. Calcd for $C_{15}H_9ClO_3$: C:66.1; H:3.3. Found: C:65.8; H:3.1.

4.1.3.4 7-Hydroxy-4-(3-methylphenyl)-2H-chromen-2-one ***(17f)***

Mp: 210-212 °C (Et_2O), yield 32%;reaction time: 3 hours; R*f*: 0.18*;* ^{1}H NMR (DMSO-d_6): 2.35 (s, 3H, CH_3), 5.98 (s, 1H, CH), 6.65 (m, 2H, ArH), 7.19 (d, 1H, ArH), 7.24-7.28 (m,

3H, ArH), 7.39 (m, 1H, ArH). Anal. Calcd for $C_{16}H_{12}O_3$: C:76.2; H:4.8. Found: C:75.9; H:4.5.

4.1.3.5 7-Hydroxy-4-(4-methylphenyl)-2H-chromen-2-one ***(17g)***

Mp: 236-237 °C (Et_2O/EtOH), yield 13%; reaction time: 4 hours; R*f*: 0.16; ^{1}H NMR (DMSO-d_6): 2.37 (s, 3H, CH_3), 6.09 (s, 1H, CH), 6.74-6.76 (m, 2H, ArH), 7.35-7.37 (m, 5H, ArH). Anal. Calcd for $C_{16}H_{12}O_3$: C:76.2; H:4.8. Found: C:76.0; H:4.6.

*4.1.3.6 7-Hydroxy-4-(4-nitrophenyl)-2H-chromen-2-one (**17h**)*

Mp: 178-180 °C (EtOAc), yield 89%; reaction time: 1.5 hours; R*f*: 0.33; ^{1}H NMR (DMSO-d_6): 6.24 (s, 1H, CH), 6.77 (dd, J_1=8.5, J_2=2.1, 1H, CH), 6.84 (d,1H, CH), 7.15 (d, *J*=9.1 1H, CH), 7.79 (d, *J*=8.5, 2H, ArH), 8.35 (d, *J*=8.5, 2H, ArH). Anal. Calcd for $C_{15}H_9NO_5$: C:63.6; H:3.2 N:4.9. Found: C:63.4; H:3.3; N:4.7.

4.1.3.7 4-(3-Chlorophenyl)-7-hydroxy-8-methyl-2H-chromen-2-one ***(18c)***

Mp: 315-317°C (EtOH), yield 79%; reaction time: 2.5 hours; R*f*: 0.22; ^{1}H NMR (DMSO-d_6): 2.18 (s, 3H, CH_3), 6.18 (s, 1H, CH), 6.83 (d, *J*= 8.5, 1H, ArH), 7.04 (d, *J*= 9.0, 1H, ArH), 7.44 (d, *J*= 8.5, 1H, ArH), 7.53-7.59 (m, 3H, ArH), 10.59 (bs, 1H, OH). Anal. Calcd for $C_{16}H_{11}ClO_3$: C:67.0; H:3.9. Found: C:66.7; H:3.6.

4.1.3.8 4-(2-Fluorophenyl)-7-hydroxy-8-methyl-2H-chromen-2-one ***(18e)***

Mp: 256-258°C (EtOH), yield 60%; reaction time: 12 hours; R*f*: 0.19; ^{1}H NMR (DMSO-d_6): 2.15 (s, 3H, CH_3), 6.17 (s, 1H, CH), 6.78-6.87 (m, 2H, ArH), 7.36-7.43 (m, 3H, ArH), 7.57 (m, 1H, ArH), 10.58 (bs, 1H, OH). Anal. Calcd for $C_{16}H_{11}FO_3$: C:65.0; H:4.0. Found: C:64.7; H:3.75.

4.1.3.9 7-Hydroxy-8-methyl-4-(3-methylphenyl)-2H-chromen-2-one ***(18f)***

Mp: 185-187° C (EtOAc), yield 99%; reaction time: 3 hours; R*f*: 0.24; ^{1}H NMR (DMSO-d_6): 2.19 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 6.10 (s, 1H, CH), 6.82 (d, *J*=8.8, 1H, ArH), 7.12 (d, *J*=8.8, 1H, ArH), 7.27-7.43 (m, 4H, ArH), 10.56 (bs, 1H, OH). Anal. Calcd for $C_{17}H_{14}O_3$: C: 76.7; H: 5.3. Found: C:76.6; H:5.1.

4.1.3.10 7-Hydroxy-8-methyl-4-(4-methylphenyl)-2H-chromen-2-one ***(18g)***

Mp: 259-261°C (EtOH), yield 63%; reaction time: 2.5 hours; R*f*: 0.22; ^{1}H NMR (DMSO-d_6): 2.19 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 6.09 (s, 1H, CH), 6.82 (d, *J*= 8.5, 1H, ArH), 7.13 (d, *J*= 8.5, 1H, ArH), 7.35-7.37 (m, 4H, ArH), 10.53 (bs, 1H, OH). Anal. Calcd for $C_{17}H_{14}O_3$: C: 76.7; H: 5.3. Found: C:76.8; H:4.9.

*4.1.3.11 7-hydroxy-8-methyl-4-(4-nitrophenyl)chromen-2-one (**18h**)*

Mp: 360 °C dec. (EtOAc), yield 98%; reaction time: 1.5 hours; R*f*: 0.15; ^{1}H NMR (DMSO-d_6): 2.17 (s, 3H, CH_3), 6.22 (s, 1H, CH), 6.80 (d, *J*= 8.8, 1H, ArH), 6.90 (d, *J*= 8.8, 1H,

ArH,) 7.75 (d, *J*= 7.0, 2H, ArH), 8.34 (d, *J*= 7, 2H, ArH). Anal. Calcd for $C_{16}H_{11}NO_5$: C:64.6; H:3.7; N:4.7. Found: C:64.2; H:3.3; N:4.4

*4.1.4 Synthesis of 4-(4-aminophenyl)-7-hydroxychromen-2-one (**17i**) and 4-(4-aminophenyl)-7-hydroxy-8-methyl-chromen-2-one (**18i**)*

To a suspension of the arylnitro derivatives **17h** or **18h** (1 mmol) and a catalytic amount of Pd/C in EtOH (15 mL), hydrazine hydrate (10 mmol) was slowly added. The reaction was stirred and refluxed (70 °C) under nitrogen atmosphere. Then, the mixture was filtered through Celite which was later washed with EtOH. The solution was evaporated *in vacuo* to give the crude product. The residue was purified by crystallization from the suitable solvent.

*4.1.4.1 4-(4-Aminophenyl)-7-hydroxychromen-2-one (**17i**)*

Mp: 307-309 °C (EtOH), yield 83%; reaction time: 1 hour; R*f*: 0.03; ^{1}H NMR (DMSO-d_6): 5.60 (bs, 2H, NH_2), 5.96 (s, 1H, CH), 6.65 (d, *J*=5.9, 2H, ArH), 6.73 (m, 2H, ArH), 7.17 (d, *J*=5.9, 2H, ArH), 7.43 (m, 1H, ArH), 10.67 (bs, 1H, OH). Anal. Calcd for $C_{15}H_{11}NO_3$: C:71.1; H:4.4; N:5.5. Found C:70.8; H:4.1; N:5.2.

4.1.4.2 4-(4-Aminophenyl)-7-hydroxy-8-methyl-chromen-2-one (***18i***)

Mp: 250-252°C (EtOAc), yield 90%; reaction time: 1.5 hours; R*f*: 0.03; ^{1}H NMR (DMSO-d_6): 2.15 (s, 3H, CH_3), 5.99 (bs, 2H, NH_2), 5.96 (s, 1H, CH), 6.64 (d, *J*= 8.5, 2H, ArH), 6.81 (d, *J*= 9.0, 1H, ArH), 7.17 (d, *J*= 8.5, 2H, ArH), 7.29 (d, *J*= 9, 1H, ArH). Anal. Calcd for $C_{16}H_{13}NO_3$: C:71.9; H:4.9; N:5.2. Found: C:71.5; H:4.4; N:4.9.

4.2 CA Inhibition Assay

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 10 – 20 mM Hepes (pH 7.5) or Tris (pH 8.3) as buffers, and 20 mM Na_2SO_4 or 20 mM $NaClO_4$ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10-100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5-10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 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 at

room temperature prior to assay and to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier, and represent the mean from at least three different determinations. CA isoforms were recombinant ones obtained as reported earlier by this group [11,12].

4.3 Molecular docking

Automated docking was carried out by means of the program AUTODOCK 4.2. [37] The crystal structure of was retrieved from the RCSB Protein Data Bank (PDB: 1JCZ) [38]. The ligand and water molecules were discarded, and hydrogen atoms were added to protein with Discovery Studio 2.5.5. Structures of the ligands were constructed using Discovery Studio 2.5.5 and energy minimized using the Powel protocol (1000 steps). The regions of interest used by AUTODOCK were defined by considering the suitable ligand docked into the CA XII receptor as the central group; in particular, a grid of 60, 60, and 60 points in the x, y, and z directions was constructed centered on the center of the mass of Zn metal ion. A grid spacing of 0.375 Å and a distance-dependent function of the dielectric constant were used for the energetic map calculations. Using the Lamarckian Genetic Algorithm, all docked compounds were subjected to 100 runs of the AUTODOCK search, in which the default values of the other parameters were used. Cluster analysis was performed on the docked results using an RMS tolerance of 2.0 Å. The Lamarckian genetic algorithm (LGA) was applied to model the interaction between ligands and hCA XII active site. For the Lamarckian genetic algorithm: 27,000 maximum generations; a gene mutation rate of 0.02 and; a crossover rate of 0.8 were used. Cluster analysis was performed on the docked results using an RMSD (Root Mean Square Deviation) tolerance of 2 Å. All the compounds were docked according to the afore mentioned parameters. The hCAXII/ligand complex obtained by docking studies was minimized using 1000 iterations of SD and 1000 interaction of Polak-Ribiere Conjugate Gradient. Interactions were identified using the LigPlot software [39] and the figures were prepared using PyMOL [40].

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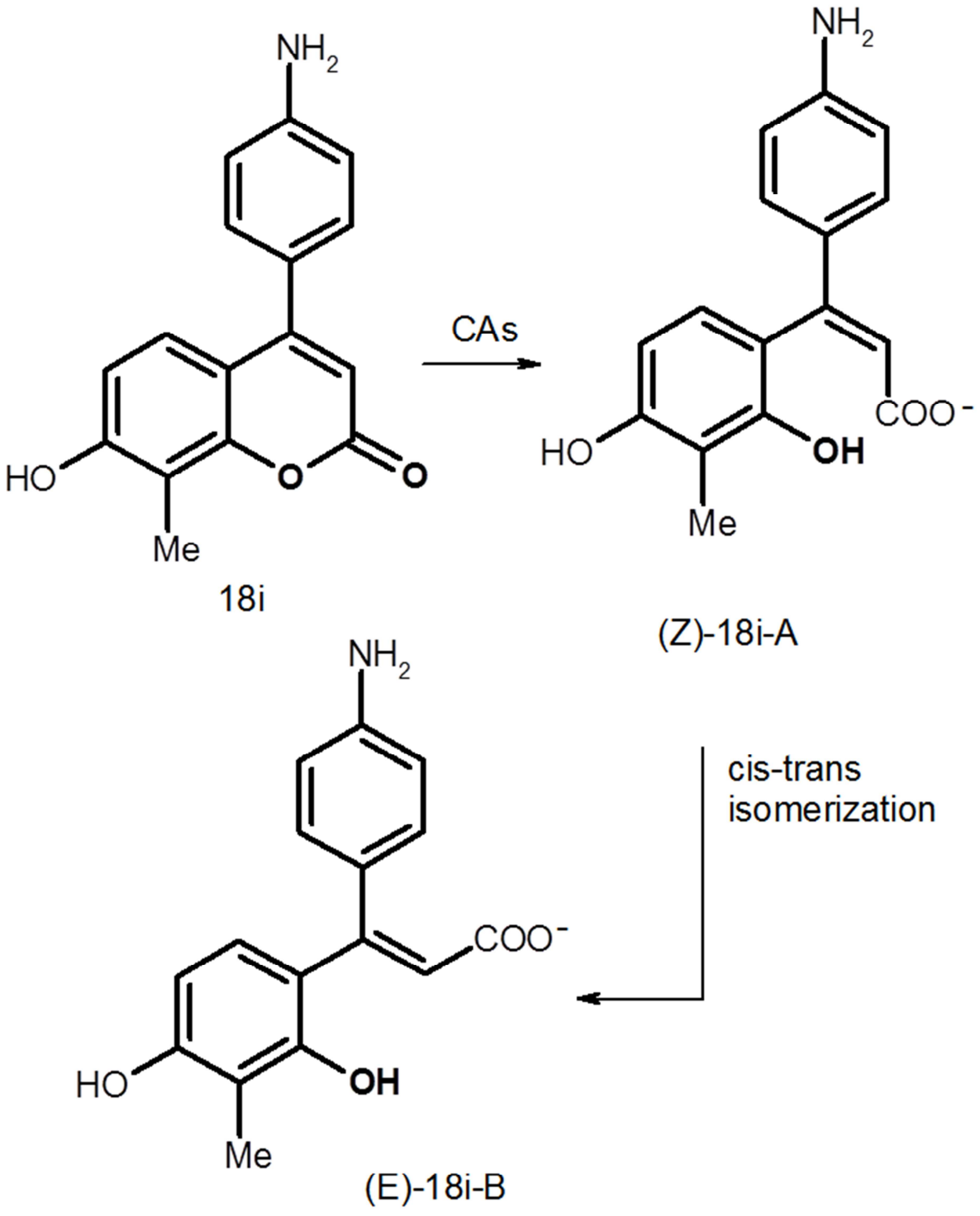
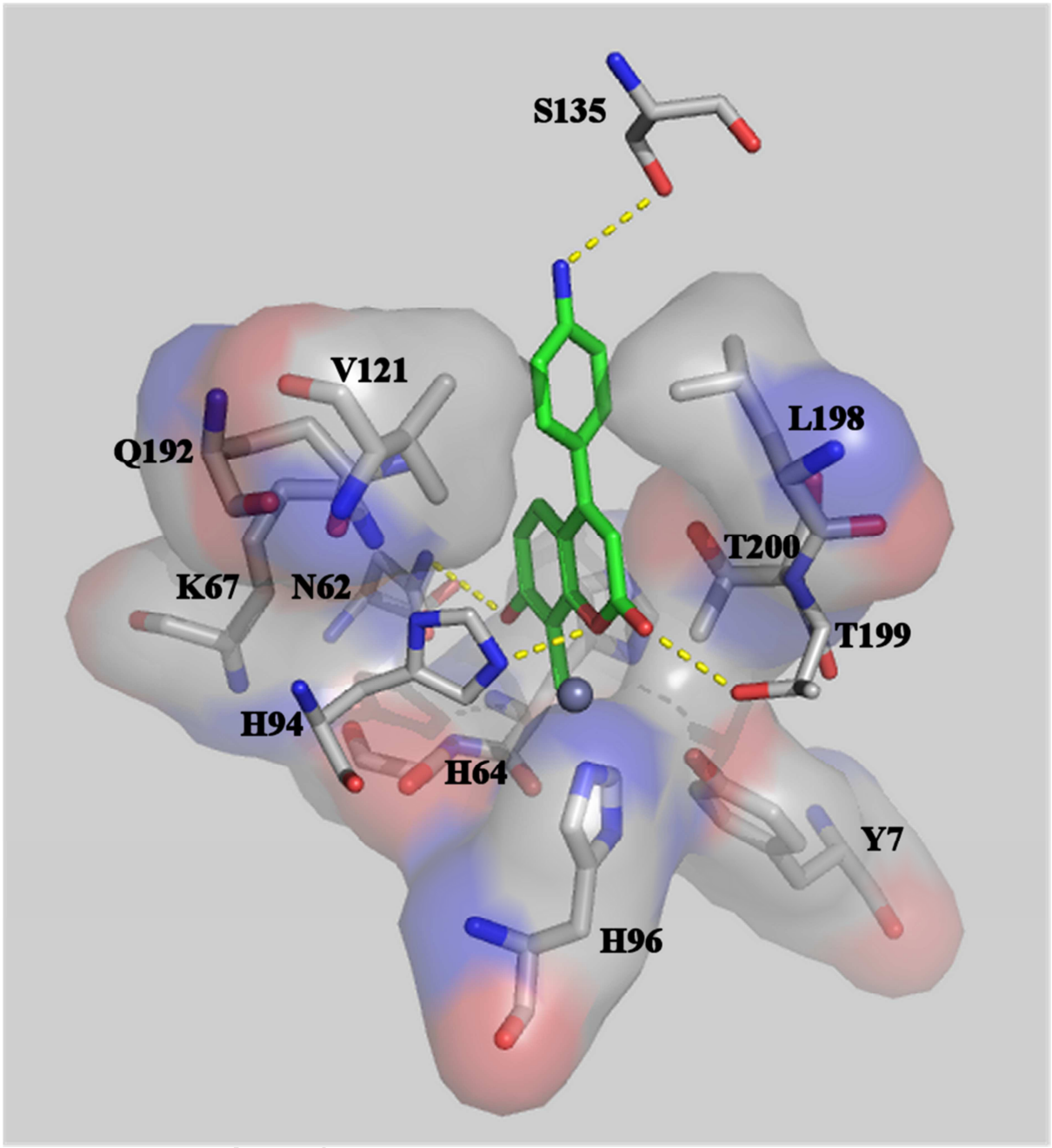


18i

(Z)-18i-A

(E)-18i-B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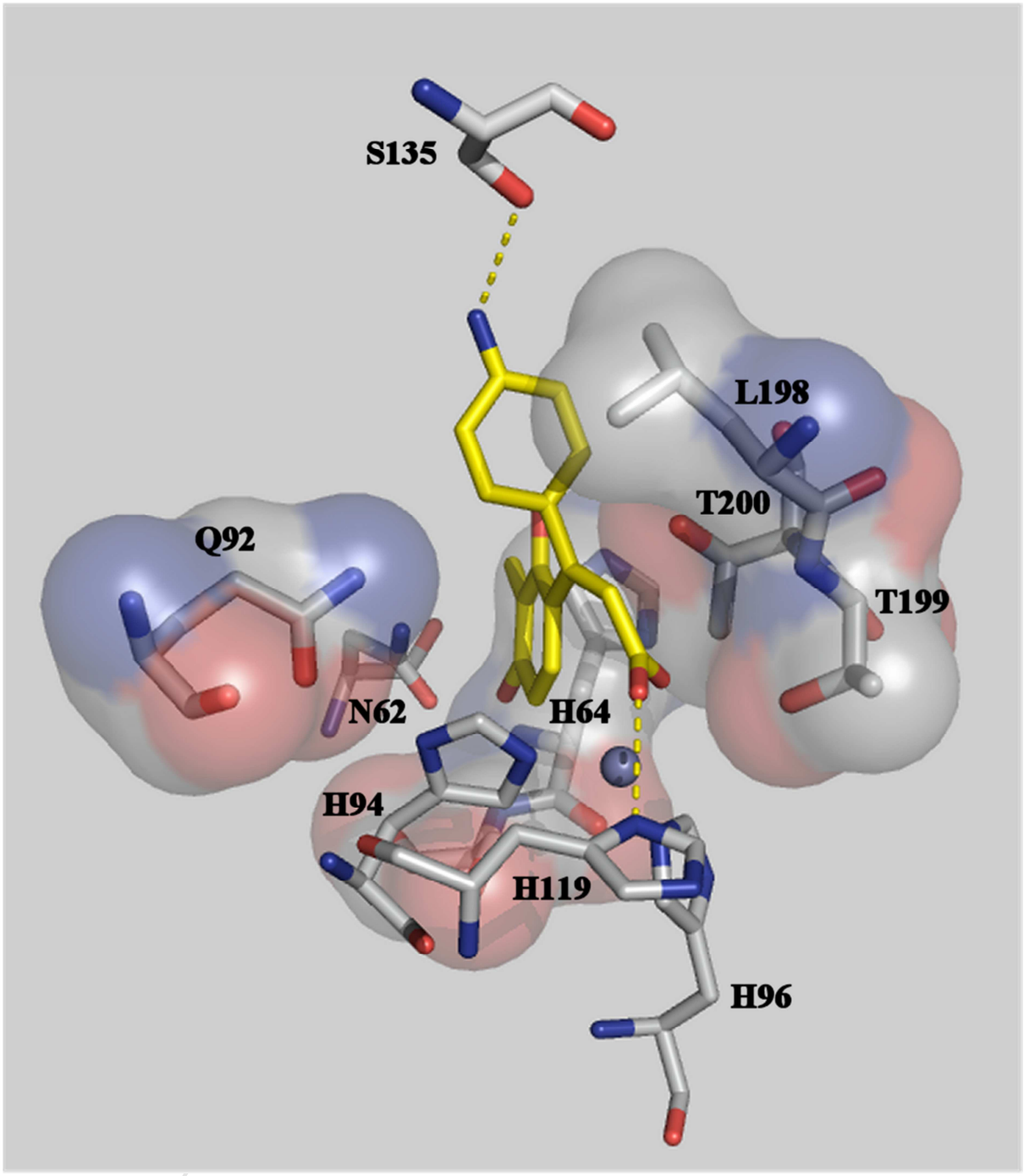
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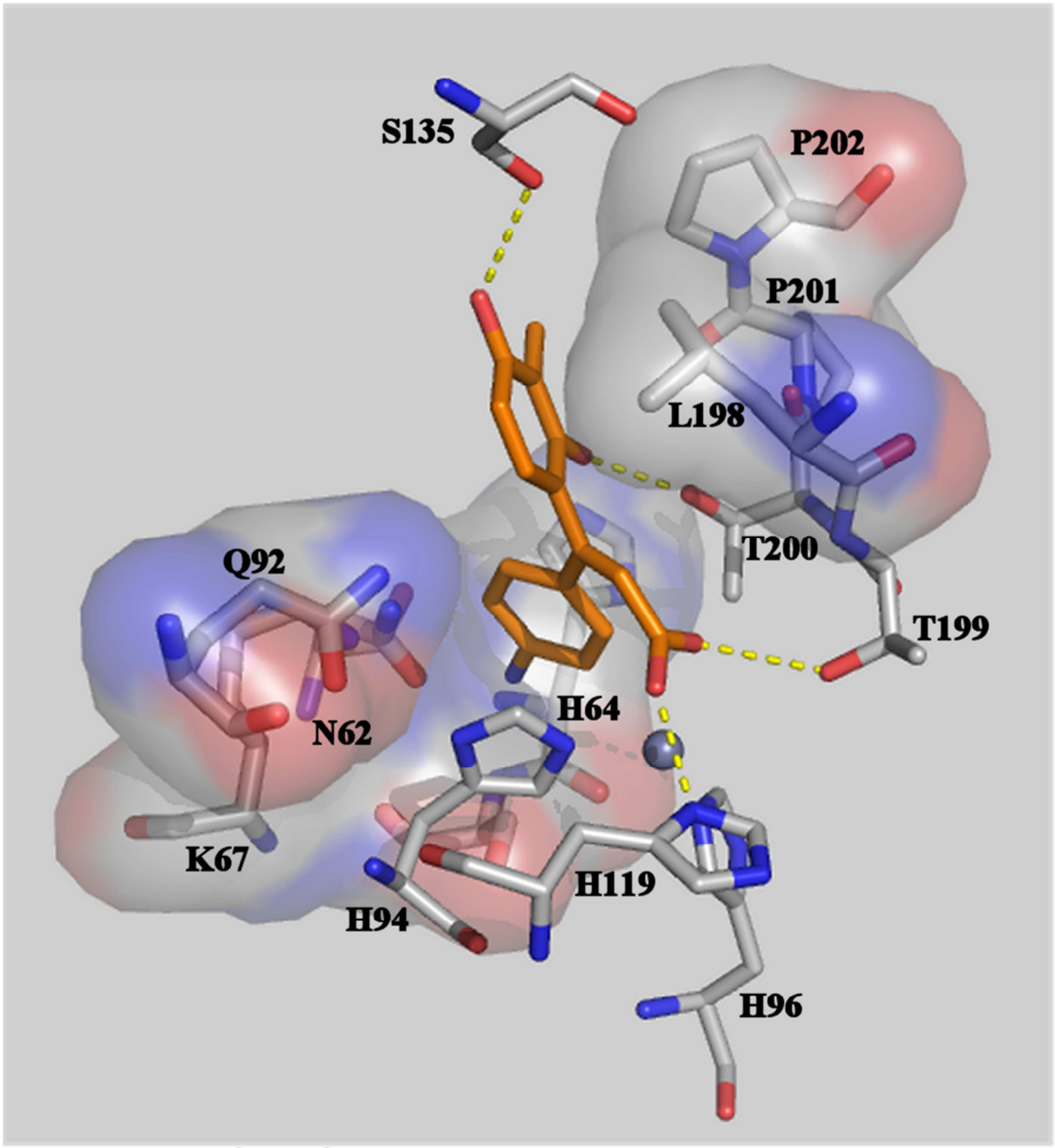
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RESEARCH HIGHLIGHTS

- New coumarin-based derivatives as CA inhibitors were designed and synthesized.
- Several cumarins were effective inhibitors of tumor-associated hCA IX and hCA XII
- Developed cumarins were selective inhibitors over ubiquitous hCA I and hCA II.
- Docking studies suggested the binding mode of active compound (**18i**) and hCA XII