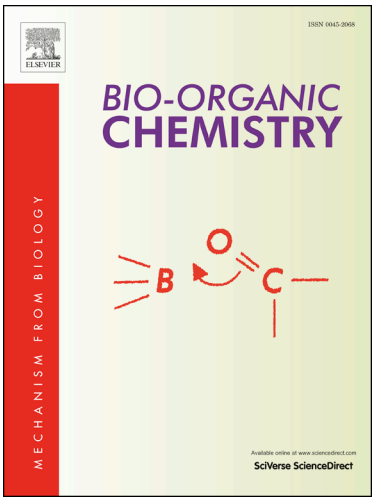

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Design, synthesis and biological evaluation of coumarin-3-carboxamides as selective carbonic anhydrase IX and XII inhibitors

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Abstract

A series of novel 7-hydroxycoumarin-3-carboxamides was synthesized by the reaction of 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylic acid with various substituted aromatic amines. The newly synthesized compounds were evaluated for their inhibitory activity against the four physiologically relevant human carbonic anhydrase (hCA, EC 4.2.1.1) isoforms CA I, CA II, CA IX and CA XII. The CA inhibition results show that the newly synthesized 7-hydroxycoumarin-3-carboxamides (**4a-n**) exhibited selective inhibition of the tumor associated isoforms, CA IX and CA XII over CA I and II isoforms. The inhibition constants ranged from sub micromolar to low micromolar. Amongst all the compounds tested, compound **4m** was the most effective inhibitor exhibiting sub micromolar potency against both hCA IX and hCA XII, with a K_i of 0.2 µM. Therefore, it can be anticipated that compound **4m** can serve as a lead for development of anticancer therapy by exhibiting a novel mechanism of action. The binding modes of the most potent compounds within hCA IX and XII catalytic clefts were investigated by docking studies.

Keywords: Cancer, Carbonic anhydrase, 7-hydroxycoumarin-3-carboxamides, Isoforms IX and XII, Hypoxic tumors

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1. Introduction

Cancer can be defined as a condition arising due to the uncontrolled growth and malignancy, which in turn arise due to aberrations in normal tissue progenitor and stem cells [1]. The mechanisms underlying cancer have been deeply studied till the genetic level, according to which, cancer can be caused due to dysfunctional oncogenes, tumor suppressor genes or microRNA genes [2]. According to Hanahan and Weinberg, cancer is characterized by several signs or "hallmarks" which include sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, induction of replicative immortality, induction of angiogenesis and activation of invasion and metastasis [3]. According to GLOBOCAN, as of 2018, about 18.1 million new cases were diagnosed and about 9.6 million people have succumbed to the deadly disease [4].

Carbonic anhydrases (CAs, EC 4.2.1.1) are a superfamily of ubiquitous metalloenzymes, which play a catalytic role in the reversible hydration of CO_2 to bicarbonate and proton. Their presence is omnipotent across all biological species, ranging from plants to animals. CAs are distributed into seven families, which are genetically distinct, on their own. These seven families are named as: α, β, γ, δ, ζ, η and θ. CAs are considered to be one of the most efficient physiological catalysts present in the living species, eliciting a wide array of functions [5-7].

The human carbonic anhydrases (hCAs) primarily belong to the α-family of carbonic anhydrases. This family of CA is, in turn, divided into 16 isoforms, which exhibit distinct molecular attributes, protein structure, kinetics, localization and catalytic behavior. The isoforms, with their localizations, can be listed as follows: CA I, CA II, CA III, CA VII and CA XIII are cytosolic, CA IV, CA IX, CA XII, CA XIV and CA XV are transmembrane bound, CA Va and Vb mitochondrial and CA VI secreted in body fluids like saliva and colostrum. The remaining three isoforms, namely CA VIII, CA X and CA XI are catalytically inactive isoforms, also called as CA-related proteins (CARPs). In recent years, various carbonic anhydrase inhibitors have been reported [8,9].

A plethora of research is ongoing currently in order to facilitate the treatment of cancer. As mentioned above, hCAs IX and XII are transmembrane bound, tumor-associated enzymes. In

particular, hCA IX is strongly overexpressed in a variety of solid tumors, mainly hypoxic tumors, with a limited expression in normal cells. It's overexpression contributes in the survival and metastasis of tumor cells due to its property of acidifying the extracellular matrix. In addition, it is also implied in promoting chemoresistance of particular weakly basic anticancer drugs. Its presence in hypoxic tumors makes it an attractive marker and drug target for hypoxic tumors and metastatic hypoxic tumors. CA IX inhibition can mainly be achieved via three ways: (i) Usage of monoclonal antibodies; (ii) Designing novel small molecule inhibitors and (iii) formulating nanoparticles derivatized with small molecule inhibitors. Similarly, hCA XII is also overexpressed primarily in the case of tumors wherein its expression is controlled by hypoxia and estrogen receptors [10-14].

Coumarins are basically 2*H*-chromenes, which means they are actually bicyclic oxygen heterocycles, arising from the fusion of a benzene ring with 5,6 positions of a 2*H*-pyran ring system [15]. Natural as well as synthetic coumarins have been implicated in a variety of biological indications namely, anticancer agents, antimicrobial and antiviral agents, anti-inflammatory and antioxidant agents, anti-Alzheimer agents, aldose reductase inhibitors, antithrombotic agents, in various CNS disorders, alpha-amylase inhibitors and thymidine phosphorylase inhibitors [16]. In the recent years coumarin and its derivatives are reported as inhibitors of CAs by a non-classical mechanism i.e., by occlusion of the entrance leading to the enzyme's active site. This is in contrast to the conventional CA inhibitory mechanism of binding within the active site metal ion. It is reported that umbelliferone (7-hydoxy coumarin) and it's analogues have the capacity of selectively inhibiting hCA(s) IX and/or XII, over CA I and CA II [9,17-20].

In view of the selective nature of coumarin towards CA IX and XII, it is decided to synthesize a series of 7-hydroxy coumarin-3- carboxamides (**Fig 1**) derivatives which have not been reported earlier as carbonic anhydrase inhibitors. Coumarin-3-carboxamide derivatives have been previously reported as FXIIa inhibitors and as antiproliferative agents [21] but not for carbonic anhydrase inhibition. Thus, novel 7-hydroxycoumarin-3-carboxamides (**4a-n**) were synthesized. The newly synthesized compounds were evaluated for CA inhibitory activity

against the four major CA isoforms, CA I, II, IX and XII, with the sulphonamide, acetazolamide (AAZ) being utilized as a drug standard.

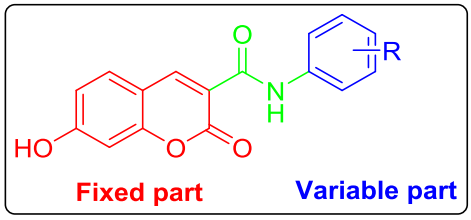


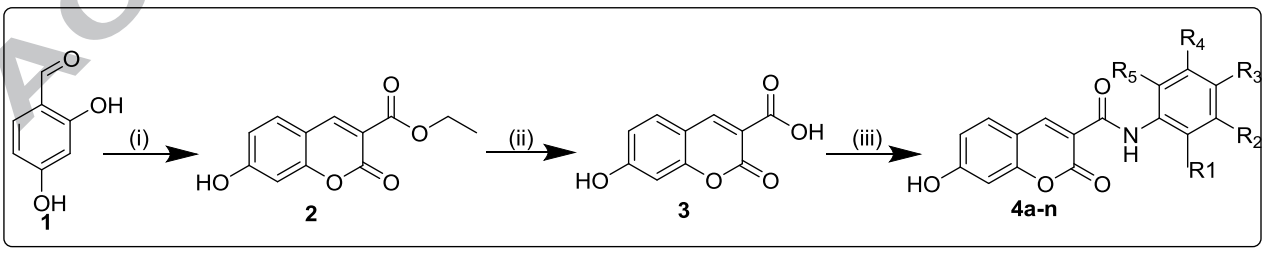
Fig 1 General Structure of the designed compounds

2. Result and Discussion

2.1 Chemistry

The current design of experiment (DOE) was aimed at designing molecules which target tumor-specific carbonic anhydrase isoforms IX and XII. Several coumarin analogues were shown to be specifically targeting these two isoforms as per previously reported literature. Hence, we have designed simple coumarin carboxamides for selectively targeting carbonic anhydrases IX and XII.

The synthesis of the designed 7-hydroxycoumarin-3-carboxamides (**4a-n**) was performed according to the general synthetic scheme as illustrated in Scheme 1. The intermediate 7-hydroxycoumarin-3-carboxylic ester (**2**) and 7-hydroxycoumarin-3-carboxylic acid (**3**) were synthesized according to previously reported literature methods [22,23]. The 7-hydroxycoumarin-3-carboxylic acid (**3**) was subjected to acid-amine coupling using various aromatic amines to afford the corresponding 7-hydroxycoumarin-3-carboxamides (**4a-n**) (**Table 1**).



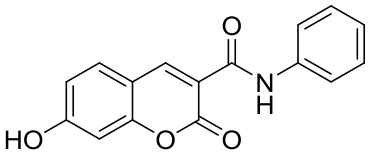
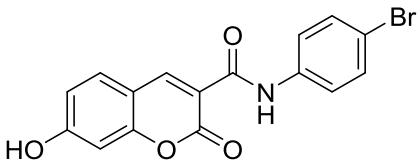
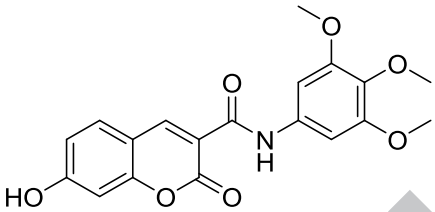
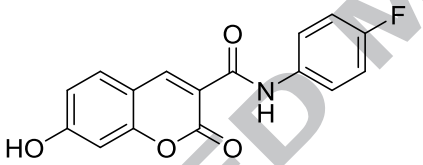
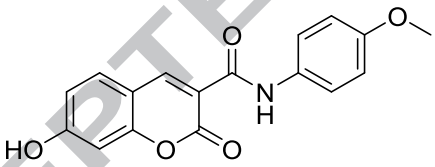
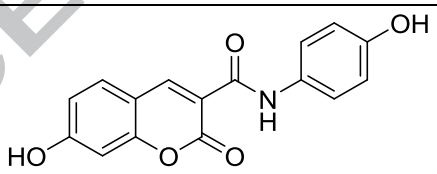
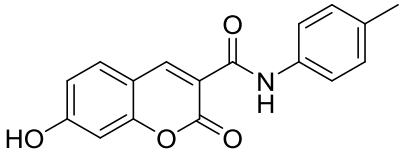
Scheme 1: General synthetic scheme for the synthesis of 7-hydroxy coumarin-3-carboxamides (**4a-n**). Reagents and conditions: (i) Diethyl malonate, piperidine, ethanol, reflux, 12-15 hrs, yield 65% (ii) 2 N NaOH solution, rt, 14 hrs, yield 80% (iii) Substituted aromatic amines, HATU, DIPEA, anhy. DMF, 0°-rt, 18-20 hrs, yield 23-90%.

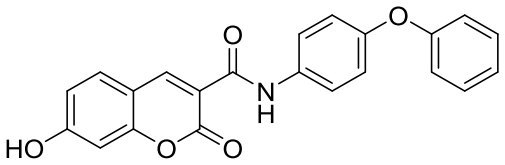
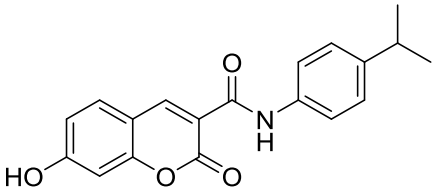
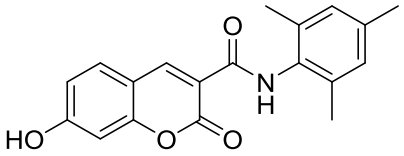
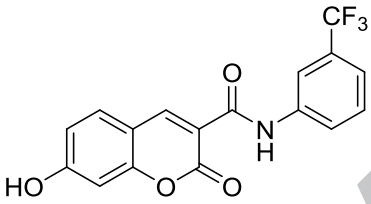
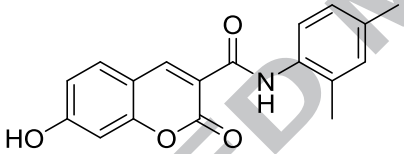
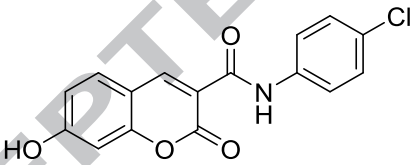
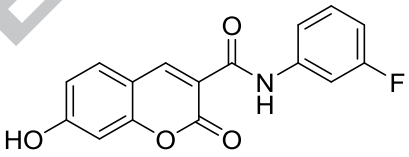
2.2 Carbonic anhydrase inhibition

The newly synthesized novel 7-hydroxycoumarin-3-carboxamides (**4a-n**) were tested for their activity against the four physiologically and pharmacologically relevant isoforms, namely, the cytosolic hCA I and II and the tumor-associated isoforms hCA IX and XII by a stopped-flow CO_2 hydrase assay. The most common clinically used sulphonamide, acetazolamide (AAZ), was employed as a standard drug. The following structure-activity relationship can be inferred from the inhibition data of 7-hydroxycoumarin-3-carboxamides (**4a-n**) as shown in **Table 1**:

i. The cytosolic isoforms hCA I and hCA II were not inhibited by the compounds **4a-4n** (Ki > 100 µM).
ii. The compounds **4a-n** showed diverse inhibitory profiles against the transmembrane tumor-associated isoform hCA IX. All of the compounds elicited inhibitory potencies of < 10µM with compounds **4b**, **4m** and **4n** eliciting sub micromolar inhibitory potencies (K_is) of **0.6**, **0.2** and **0.3** µM respectively. All the three potent compounds incorporate a halogen atom in various positions of the aromatic amine ring (variable part) of the 7-hydroxycoumarin-3-carboxamides.
iii. The other transmembrane tumor-associated isoform, hCA XII, was also inhibited in a variable and diverse manner by the synthesized compounds, **4a-4n**, with K_is below 10µM for all the compounds. Among all the compounds, compound **4m**, bearing a chlorine atom at the para position of the aromatic amine ring (variable part) showed promising inhibitory activity with a K_i value of **0.2** µM.
iv. All the derivatives exhibited selective inhibition of hCA IX and XII as compared to hCA I &II in micromolar range.

Table 1. Inhibition of hCA isoforms I, II, IX and XII with compounds **4a-n** and acetazolamide (AAZ) as standard inhibitor.

Compound	Structure	hCA I	hCAII	hCA IX	hCA XII
4a		>100	>100	2.4	7.9
4b		>100	>100	**0.6**	7.7
4c		>100	>100	7.1	7.5
4d		>100	>100	0.7	9.0
4e		>100	>100	8.1	8.9
4f		>100	>100	9.2	8.4
4g		>100	>100	9.1	8.4

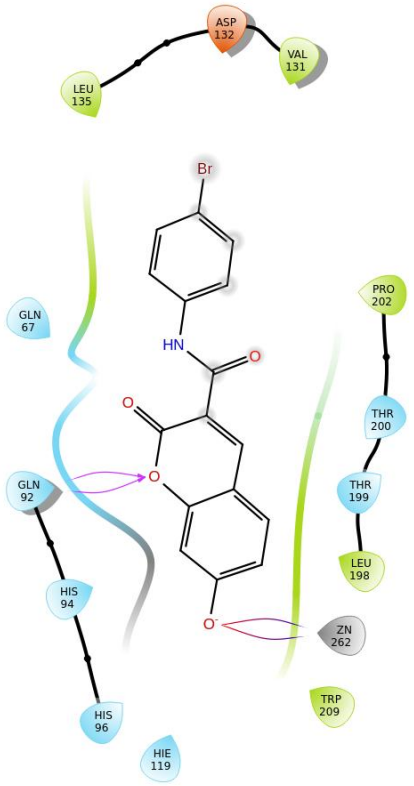
4h		>100	>100	8.4	7.6
4i		>100	>100	9.1	9.7
4j		>100	>100	9.4	8.7
4k		>100	>100	9.1	9.6
4l		>100	>100	9.1	9.1
4m		>100	>100	**0.2**	**0.2**
4n		>100	>100	**0.3**	7.9
AAZ		0.25	0.012	0.026	0.006

* Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5-10 % of the reported values).

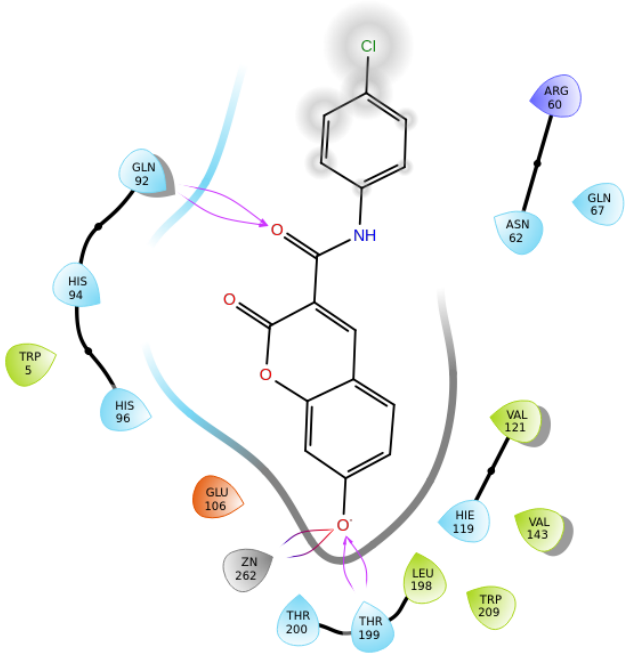
2.3 Docking studies

With a view to shed more light on the enzyme inhibition shown by the compounds and to study their interactions, docking studies of the most active compounds with hCA IX (PDB 3IAI) and hCA XII (PDB 4WW8) binding site has been performed. Compounds **4b**, **4m** and **4n** which had shown sub micromolar inhibitory profiles against hCA IX were docked against hCA IX and compound **4m** which had shown sub micromolar inhibitory profile against hCA XII was docked against hCA XII. Ligand interaction diagram (**Fig 2**) shows that all the three compounds exhibited interaction with Zn262 of hCA IX through the ionized hydroxyl group present in the coumarin moiety. These results are very different from the experimental, X-ray crystallographic results [17,18], which showed that coumarins bound to other CA isoforms, such as CA II for example, at the entrance of the cavity by hydrolysis of the lactone ring and conversion to 2-hydroxy cinnamic acids. Furthermore, there are also differences with the binding of phenols, which anchor to the zinc-coordinated water molecule. Thus, we consider the binding mode reported in Fig. 2 as a hypothetical, not yet experimentally confirmed inhibition mechanism which warrants further studies. Probably the bulky nature of the groups present in position 3 of the coumarin ring interferes with the hydrolysis of the lactone ring affording thus a novel CA inhibitory mechanism for these derivatives.

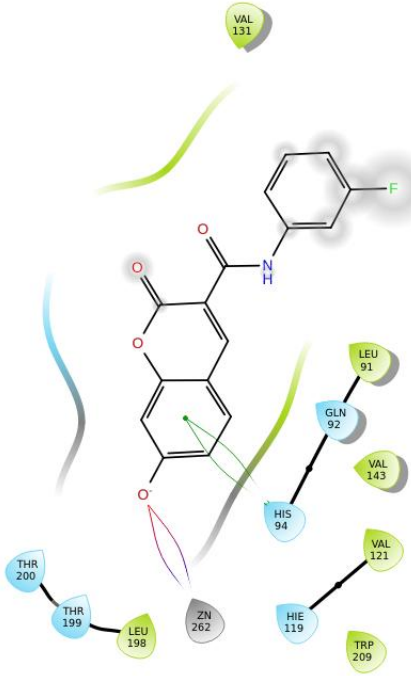
In addition, the coumaryl oxygen of compound **4b** showed hydrogen bond interaction with key active site residue Gln92 and π- π stacking was observed between coumaryl ring system and His94 in case of compound **4n**. The most potent compound **4m** showed interactions with Thr199 and Gln 92, in addition to Zn262. Also, other major active site residues like Thr198, Pro200 and His94 are seen lying in the periphery of the compound. These features allow compound **4m** to exhibit potent sub micromolar inhibition in comparison to other analogues. In case of hCA XII, compound **4m** is shown to interact with Zn301, Thr198 and Asn64, which contributes to its potency against hCA XII. We stress again, these docking results are not confirmed experimentally for the moment.



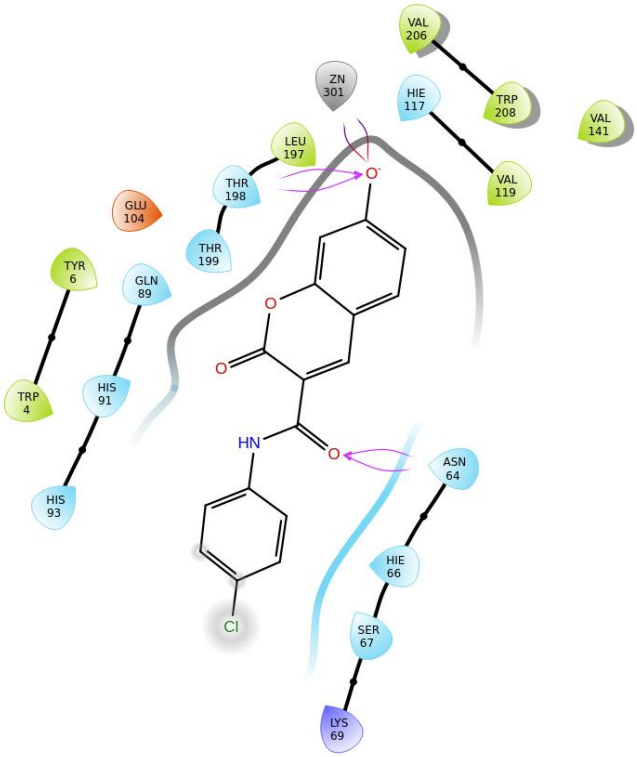
(I)



(II)



(III)



(IV)

Fig 2: - (I) Docking pose of compound **4b** with hCA IX **(II)** Docking pose of compound **4m** with hCA IX **(III)** Docking pose of compound **4n** with hCA IX **(IV)** Docking pose of compound **4m** with hCA XII. The pink arrows in the figure indicate the hydrogen bonding interactions and the green lines indicate π - π stacking. The compounds coordinate to the Zn ion, although this binding mode was not confirmed experimentally for other phenols.

3. Conclusion

In conclusion, we have reported the synthesis of a new series of 7-hydroxycoumarin-3-carboxamide derivatives (**4a-n**), which have been specifically designed to target the transmembrane tumor-associated isoforms, hCA IX and XII. The novel 7-hydroxycoumarin-3-carboxamides (**4a-n**) were screened against the four physiologically and pharmacologically relevant isoforms i.e. the cytosolic isoforms hCA I and II as well as the transmembrane tumor-associated isoforms, hCA IX and XII. All of the compounds exhibited low micromolar to sub micromolar inhibitory profiles for both the isoforms, particularly for CA IX, with K_is in the range of 0.2-9.2 μM for CA IX and 0.2-9.7 for CA XII. The newly synthesized 7-hydroxycoumarin-3-carboxamides (**4a-n**) proved to be ineffective against both the cytosolic isoforms, thereby validating our hypothesis of selective inhibition of the two tumor-associated enzymes. Also, as CA IX has been validated as a target for metastatic hypoxic tumors, the compound **4m** can be considered as a potential lead candidate for the design of novel molecules which target cancer by a different mechanism.

4. Experimental Section

4.1. General

All the chemicals and solvents were procured and utilized as such from the suppliers. Wherever necessary, anhydrous solvents were used. Thin Layer Chromatography (TLC) analysis was done by utilizing Merck silica gel 60 F_{254} aluminum plates. Stuart Digital Melting Point Apparatus (SMP 30) was used in determining the melting points of the compounds, which are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded using Bruker Avance 500MHz and 125MHz respectively using DMSO-d_6 as the solvent. Chemical Shift values are recorded in ppm using TMS as the internal standard. HRMS were determined by Agilent QTOF mass spectrometer 6540 series instrument and were performed using ESI techniques at 70eV.

4.1.1 *Synthesis of ethyl 7-hydroxy-2-oxo-2H-chromene-3-carboxylate* ***(2)***

To a stirred solution of 2,4-dihydroxybenzaldehyde (2g, 14.47 mmol) in anhydrous ethanol (20 ml) was added diethyl malonate (2.3g, 14.47 mmol) and piperidine (1.5 ml). The resulting solution was allowed to reflux and the progress of the reaction was monitored by TLC. Upon completion of the reaction on TLC, the reaction solvent was removed under reduced pressure and the crude residue was subjected to column chromatography using silica gel 60-120 mesh as stationary phase and EtOAc in Hexane (0 percent to 20 percent) as the mobile phase to afford the title compound as a yellowish fluffy solid (Yield 65%).

4.1.2 *Synthesis of 7-hydroxy-2-oxo-2H-chromene-3-carboxylic acid* ***(3)***

Intermediate **2** (2.37g, 10.12 mmol) was dissolved in 2 N NaOH solution (40 ml). The resulting solution was allowed to stir at rt overnight. On completion of the reaction on TLC, the solution was acidified using 2 N HCl solution. The resulting precipitates were filtered off, subsequently washed with water a few times and dried to afford the title compound as a yellow solid (Yield 90%).

4.1.3 *Synthesis of 7-hydroxy-2-oxo-N-phenyl-2H-chromene-3-carboxamides* ***(4a-4n)***

To a stirred solution of Intermediate **3 (**0.1g, 0.48 mmol**)** in anhydrous DMF at 0° C, under nitrogen, was added HATU (0.276g, 0.72 mmol). The resulting solution was stirred at 0° C for half an hour. Thereafter, the corresponding amine (1 eq.) and DIPEA (0.187g, 1.4 mmol) were added and the resulting solution was allowed to stir at rt for overnight. On completion of the reaction as checked by TLC, the reaction mixture was dumped in ice water and extracted twice with ethyl acetate. The ethyl acetate layers were combined, washed with brine, dried over sodium sulphate and subjected to rotary evaporation under reduced pressure. The crude residue was subjected to column chromatography using silica gel 60-120 mesh to afford the final carboxamides **4a-4n**.

7-hydroxy-2-oxo-N-phenyl-2H-chromene-3-carboxamide (**4a**) Yield: 55%; Color: Yellow solid; mp: 276-278°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.14 (s, 1H), δ 10.65 (s, 1H), δ 8.88 (s, 1H), δ 7.86 (d, J = 8.6 Hz, 1H), 7.71 (d, J = 7.9 Hz, 2H), 7.38 (t, J = 7.8 Hz, 2H), 7.14 (t, J = 7.3 Hz, 1H), 6.91 (dd, J = 8.6, 2.0 Hz, 1H), 6.85 (d, J = 1.7 Hz, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.44, 161.79, 160.65, 156.87, 148.85, 138.53, 132.62, 129.46, 124.58, 120.33, 115.03, 114.60, 111.72, 102.39; HRMS (ESI): *m/z* calcd for $[M+H]^+$ $C_{16}H_{12}NO_4$ 282.0766; found 282.0733.

N-(4-bromophenyl)-7-hydroxy-2-oxo-2H-chromene-3-carboxamide (**4b**) Yield: 23%; Color: Yellow solid; mp: 327-329°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.27 (s, 1H), δ 10.70 (s, 1H), δ 8.86 (s, 1H), δ 7.86 (d, *J* = 8.6 Hz, 1H), 7.70 (d, *J* = 8.5 Hz, 2H), 7.55 (d, *J* = 8.5 Hz, 2H), 6.91 (d, *J* = 8.5 Hz, 1H), δ 6.84 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.66, 161.65, 160.86, 156.92, 148.94, 137.89, 132.65, 132.22, 122.33, 116.21, 115.10, 114.33, 111.62, 102.41; HRMS (ESI): *m/z* calcd for $[M+H]^+C_{16}H_{11}BrNO_4$ 359.9871; found 359.9826.

7-hydroxy-2-oxo-N-(3,4,5-trimethoxyphenyl)-2H-chromene-3-carboxamide (**4c**) Yield: 49%; Color: Yellow solid; mp: 238-240°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.17 (s, 1H), δ 10.55 (s, 1H), δ 8.85 (s, 1H), δ 7.83 (d, *J* = 8.6 Hz, 1H),δ 7.11 (s, 2H), 6.91 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.84 (d, *J* = 2.0 Hz, 1H), δ 3.79 (s, 6H), δ 3.65 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.69, 161.75, 160.53, 156.90, 153.36, 148.80, 134.59, 132.58, 115.12, 114.29, 111.63, 102.42, 98.34, 92.12, 60.59, 56.35; HRMS (ESI): *m/z* calcd for $[M+H]^+$ $C_{19}H_{18}NO_7$ 372.1083; found 372.1050.

N-(4-fluorophenyl)-7-hydroxy-2-oxo-2H-chromene-3-carboxamide (**4d**) Yield: 90%; Color: Yellow solid; mp: 293-295°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.14 (s, 1H), δ 10.64 (s, 1H), δ 8.86 (s, 1H), δ 7.85 (d, *J* = 8.6 Hz, 1H), 7.75 (dd, *J* = 8.8, 5.0 Hz, 2H), 7.21 (t, *J* = 8.8 Hz, 2H), 6.91 (dd, *J* = 8.5, 2.0 Hz, 1H), 6.84 (d, *J* = 1.8 Hz, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.64, 161.68, 159.92, 158.01, 156.91, 148.75, 134.95, 132.61, 122.30, 115.92, 115.09, 114.37, 111.61, 102.40; HRMS (ESI): *m/z* calcd for $[M+H]^+C_{16}H_{11}FNO_4$ 300.0672; found 300.0638.

7-hydroxy-N-(4-methoxyphenyl)-2-oxo-2H-chromene-3-carboxamide (**4e**) Yield: 56%; Color: Yellow solid; mp: 271-273°C; ^{1}H NMR (500 MHz, DMSO-d_6)) δ 11.13 (s, 1H), δ 10.52 (s, 1H), δ 8.86 (s, 1H), δ 7.85 (d, *J* = 8.6 Hz, 1H), 7.64 (d, *J* = 8.9 Hz, 2H), 6.95 (d, *J* = 9.0 Hz, 2H), 6.91 (dd, *J* = 8.6, 2.1 Hz, 1H), 6.84 (d, *J* = 1.9 Hz, 1H), 3.76 (s, 3H) ; ^{13}C NMR (125 MHz, DMSO) δ 164.57, 161.83, 160.24, 156.85, 156.33, 156.30, 148.57, 132.53, 131.67, 121.88, 115.09, 114.59, 111.65, 102.39, 55.71.HRMS (ESI): *m/z* calcd for $[M+H]^+$ $C_{17}H_{14}NO_5$ 312.0872; found 312.0872

7-hydroxy-N-(4-hydroxyphenyl)-2-oxo-2H-chromene-3-carboxamide (**4f**) Yield: 34%; Color: Yellow solid; mp: 333-335°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.02 (s, 1H), δ 10.44 (s, 1H), δ 9.34 (s, 1H), δ 8.85 (s, 1H), δ 7.85 (d, *J* = 8.6 Hz, 1H), 7.50 (d, *J* = 8.9 Hz, 2H), 6.91 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.84 (d, *J* = 2.0 Hz, 1H), 6.78 – 6.75 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ

164.34, 161.86, 159.99, 156.78, 154.52, 148.46, 132.50, 130.19, 122.02, 115.83, 115.00, 114.64, 111.71, 102.36; HRMS (ESI): *m/z* calcd for $[M+Na]^+$ $C_{16}H_{12}NO_5$ 320.0535; found 320.0489.

7-hydroxy-2-oxo-N-(p-tolyl)-2H-chromene-3-carboxamide (**4g**) Yield: 66%; Color: Yellow solid; mp: 299-301°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.10 (s, 1H), δ 10.58 (s, 1H), δ 8.86 (s, 1H), δ 7.85 (d, *J* = 8.3 Hz, 1H), 7.59 (d, *J* = 7.5 Hz, 2H), 7.18 (d, *J* = 7.4 Hz, 2H), 6.91 (d, *J* = 8.0 Hz, 1H), 6.84 (s,1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.51, 161.84, 160.41, 156.85, 148.71, 136.04, 133.63, 132.58, 129.84, 120.28, 115.05, 114.50, 111.68, 102.38, 20.94; HRMS (ESI): *m/z* calcd for $[M+H]^+$ $C_{17}H_{14}NO_4$ 296.0923; found 296.0883.

7-hydroxy-2-oxo-N-(4-phenoxyphenyl)-2H-chromene-3-carboxamide (**4h**) Yield: 76%; Color: Yellow solid; mp: 253-255°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.13 (s, 1H), δ 10.64 (s, 1H), δ 8.87 (s, 1H), δ 7.86 (d, *J* = 8.6 Hz, 1H), 7.74 (d, *J* = 9.0 Hz, 2H), 7.42 – 7.37 (m, 2H), 7.13 (t, *J* = 7.4 Hz, 1H), 7.05 (d, *J* = 8.9 Hz, 2H), 7.01 (d, *J* = 7.8 Hz, 2H), 6.91 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.84 (d, *J* = 2.1 Hz, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.57, 161.75, 160.55, 157.59, 156.89, 153.08, 148.74, 134.33, 132.60, 130.47, 123.67, 122.11, 119.90, 118.64, 115.09, 114.47, 111.66, 102.40; HRMS (ESI): *m/z* calcd for $[M+H]^+$ $C_{22}H_{16}NO_5$ 374.1028; found 374.0981.

7-hydroxy-2-oxo-N-(4-phenoxyphenyl)-2H-chromene-3-carboxamide (**4i**) Yield: 62%; Color: Yellow solid; mp: 256-258°C; ^{1}H NMR (500 MHz, DMSO-d_6)δ 11.13 (s, 1H), δ 10.59 (s, 1H), δ 8.88 (s, 1H), δ 7.86 (d, *J* = 8.7 Hz, 1H), 7.62 (d, *J* = 8.5 Hz, 2H), 7.25 (d, *J* = 8.5 Hz, 2H), 6.92 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.85 (d, *J* = 2.1 Hz, 1H), δ 2.88 (septet, 1H), δ 1.21 (d, *J* = 6.9 Hz, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.46, 161.84, 160.44, 156.86, 148.77, 144.76, 136.28, 132.60, 127.18, 120.43, 115.04, 114.55, 111.71, 102.39, 33.37, 24.37; HRMS (ESI): *m/z* calcd for $[M+H]^+$ $C_{19}H_{18}NO_4$ 324.1236; found 324.1177.

7-hydroxy-N-mesityl-2-oxo-2H-chromene-3-carboxamide (**4j**) Yield: 34%; Color: Yellow solid; mp: 307-309°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.06 (s, 1H), δ 9.88 (s, 1H), δ 8.85 (s, 1H), δ 7.84 (d, *J* = 8.6 Hz, 1H), 6.94 – 6.91 (m, 2H), 6.90 (d, *J* = 2.2 Hz, 1H), 6.84 (d, *J* = 2.0 Hz, 1H),)δ 2.25 (s, 3H), δ 2.15 (s, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.26, 161.79, 160.73, 156.88, 148.70, 136.14, 134.95, 132.48, 132.41, 128.82, 114.92, 114.63, 111.63, 102.35, 20.96, 18.59; HRMS (ESI): *m/z* calcd for $[M+H]^+$ $C_{19}H_{18}NO_4$ 324.1236; found 324.1211.

7-hydroxy-2-oxo-N-(3-(trifluoromethyl)phenyl)-2H-chromene-3-carboxamide (**4k**) Yield: 35%; Color: Yellow solid; mp: 294-296°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.18 (s, 1H), δ 10.84 (s, 1H), δ 8.87 (s, 1H), δ 8.25 (s, 1H), δ 7.90 (d, *J* = 8.2 Hz, 1H), 7.86 (d, *J* = 8.6 Hz, 1H), 7.61 (t, *J* = 8.0 Hz, 1H), 7.49 (d, *J* = 7.8 Hz, 1H), 6.92 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.85 (d, *J* = 2.1 Hz, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.66, 161.46, 161.34, 156.98, 149.08, 139.31, 132.71, 130.61, 130.00, 124.18, 123.44, 120.93, 116.63, 115.09, 114.36, 111.61, 102.43; HRMS (ESI): *m/z* calcd for $[M+H]^+$ $C_{17}H_{11}F_3NO_4$ 350.0640; found 350.0624.

N-(2,4-dimethylphenyl)-7-hydroxy-2-oxo-2H-chromene-3-carboxamide (**4l**) Yield: 75%; Color: Yellow solid; mp: 311-313°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.15 (s, 1H), δ 10.57 (s, 1H), δ 8.93 (s, 1H), δ 8.05 (d, *J* = 8.2 Hz, 1H), 7.88 (d, *J* = 8.6 Hz, 1H), δ 7.07 (s, 1H), 7.03 (d, *J* = 8.1 Hz, 1H), 6.92 (dd, *J* = 8.5, 1.8 Hz, 1H), δ 6.85 (s, 1H), δ 2.29 (s, 3H), δ 2.26 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.52, 162.38, 160.10, 156.84, 149.11, 134.35, 133.74, 132.68, 131.37, 129.53, 127.28, 121.62, 115.09, 114.14, 111.78, 102.35, 20.91, 17.96; HRMS (ESI): *m/z* calcd for $[M+H]^+$ $C_{18}H_{16}NO_4$ 310.1079; found 310.1060.

N-(4-chlorophenyl)-7-hydroxy-2-oxo-2H-chromene-3-carboxamide (**4m**) Yield: 25%; Color: Yellow solid; mp: 323-325°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.16 (s, 1H), δ 10.71 (s, 1H), δ 8.86 (s, 1H), δ 7.86 (d, *J* = 8.3 Hz, 1H), 7.76 (d, *J* = 8.0 Hz, 2H), 7.43 (d, *J* = 8.0 Hz, 2H), 6.91 (d, *J* = 8.0 Hz, 1H), δ 6.84 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.63, 161.63, 160.88, 156.93, 148.91, 137.49, 132.66, 129.32, 128.18, 121.99, 115.10, 114.40, 111.64, 102.41; HRMS (ESI): *m/z* calcd for $[M+H]^+C_{16}H_{11}ClNO_4$ 316.0377; found 316.0346.

N-(3-fluorophenyl)-7-hydroxy-2-oxo-2H-chromene-3-carboxamide (**4n**) Yield: 78%; Color: Yellow solid; mp: 304-306°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.18 (s, 1H), δ 10.77 (s, 1H), δ 8.88 (s, 1H),δ 7.87 (d, *J* = 8.7 Hz, 1H), 7.78 (d, *J* = 12.6 Hz, 1H),δ 7.43-7.40 (m, 2H), 6.97 (m, 1H), 6.92 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.85 (d, *J* = 2.0 Hz, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.65, 163.64, 161.60, 161.09, 156.96, 149.04, 132.70, 131.03, 124.01, 116.25, 115.11, 114.36, 111.65, 107.37, 107.16, 102.43; ; HRMS (ESI): *m/z* calcd for $[M+H]^+C_{16}H_{11}FNO_4$ 300.0672; found 300.0650.

4.1.4 **CA inhibition**

An SX.18V-R Applied Photophysics (Oxford, UK) stopped flow instrument has been used to assay the catalytic inhibition of various CA isozymes [24]. Phenol Red (at a concentration of 0.2 mM) has been used as an indicator, working at an absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.4) as a buffer, 0.1M Na_2SO_4 or $NaClO_4$ (for maintaining constant the ionic strength; these anions are not inhibitory in the used concentration), following the CA-catalyzed CO_2 hydration reaction for a period of 5-10s. Saturated CO_2 solutions in water at 25°C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10mM (in DMSO-water 1:1, v/v) and dilutions up to 0.01nM done with the assay buffer mentioned above. At least 7 different inhibitor concentrations have been used for measuring the inhibition constant. Inhibitor and enzyme solutions were pre-incubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported in this paper are the mean of such results. The inhibition constants were obtained by non-linear least squares methods using the Cheng-Prusoff equation, as reported earlier, and represent the mean from at least three different determinations. All CA isozymes used here were recombinant proteins obtained as reported earlier by our group [25-27].

4.2 Computational Studies

The 3D crystal coordinates of hCA IX (3IAI) [28] and hCA XII (4WW8) [29] were downloaded from the PDB and were prepared according to protocols specified in Protein Preparation Wizard [30] of Schrödinger suite 2017. Briefly, hydrogens were added, bond orders were assigned, water molecules were removed within 5 Å distance and minimization done using OPLS-2005 force field. Compounds **4b**,**4n**,**4m** were drawn and converted to 3D using LigPrep [31], module of Schrödinger suite 2017. Further Grid has been generated with 10 Å around co-crystal and flexible ligand docking protocol has been performed by Glide [32], module of Schrödinger suite 2017. Ten conformations were generated for each compound and based on docking score and interactions, the final poses were selected. Docking protocol was optimized by calculating the RMSD of crystal and docked pose of co-crystal ligand with <1 Å.

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Conflict of Interest

The authors declare no conflict of interest.

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Highlights

- A series of novel 7-hydroxycoumarin-3-carboxamides was synthesized.
- Thenovel 7-hydroxycoumarin-3-carboxamides were investigated for inhibition against four isoforms hCA I, II, IX and XII.
- The compounds exhibited low micromolar to sub micromolar inhibitory profiles for both the isoforms, particularly for CA IX, with K_is in the range of 0.2-9.2 µM for CA IX and 0.2-9.7 for CA XII.
- The compounds showed exclusive selectivity for hCA IX and XII over hCA I and II.

Graphical abstract