Bioorganic Chemistry 87 (2019) 765–772

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Synthesis and biological evaluation of coumarin-1,3,4-oxadiazole hybrids as selective carbonic anhydrase IX and XII inhibitors

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ARTICLE INFO

Keywords:
Coumarin-1,3,4-oxadiazole hybrids
Cancer
Carbonic anhydrase
hCAs I, II, IX and XII

ABSTRACT

With an aim to develop novel heterocyclic hybrids as potent anticancer agents, we synthesized a series of coumarin-1,3,4-oxadiazole hybrids (**7a-t**) and 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 clearly indicated that the coumarin-1,3,4-oxadiazole derivatives (**7a-t**) exhibited selective inhibition of the tumor associated isoforms, CA IX and CA XII over CA I and II isoforms. Among all, compound **7b**, exhibited significant inhibition in lower micromolar potency against hCA XII, with a K_i of 0.16 μM and compound **7n**, exhibited significant inhibition in lower micromolar potency against hCA IX, with a K_i of 2.34 μM respectively. Therefore, compound **7b** and **7n** could be the potential leads for development of selective anticancer agents by exhibiting a novel mechanism of action through hCA IX and XII inhibition.

1. Introduction

Cancer is one of most life-threatening diseases in the world and characterized by uncontrolled growth and metastasis. The dysfunctional oncogenes, microRNA genes, and tumor suppressor genes are the essential factors to cause different types of cancer [1]. Recent studies from GLOBOCAN, 2018, showed that about 18.1 million new cases were diagnosed and 9.6 million cancer death cases were reported [2]. Most of the cancer cells are recognized by uncontrolled proliferation due to deregulation of key essential enzymes and proteins controlling cell division and growth [3]. Many of chemotherapeutic drugs act on various cancer cells but the drug induced toxicity, poor selectivity and poor tolerance are indicative for the need to develop new anticancer agents with improved cytotoxicity and lower adverse effects.

The human carbonic anhydrases (hCAs) are belong to α-family of carbonic anhydrases and this hCAs, in turn have 16 isoforms. CA I, CA II, CA III, CA VII and CA XIII are cytosolic, CAs, CA IV, CA IX, CA XII, CA XIV and CA XV are transmembrane bound, CA Va and Vb mitochondrial and CA VI secreted from saliva and colostrum. CA VIII, CA X and CA XI are catalytically inactive isoforms, named as CA-related proteins (CARPs) [4,5]. The hCA IX and XII are overexpressed in cancer cells as they are transmembrane bound, tumor-associated enzymes, mainly hypoxic tumors, with a low expression in normal cells. The overexpression further contributes for the tumor growth, proliferation, angiogenesis, and metastasis of variety of cancer cells [6,7]. An anticancer agent, in order to exhibit potent cytotoxicity without adverse effects, should selectively inhibit tumor-associated hCAs IX and XII over cytosolic CAs like hCA I and II. Therefore, development of novel anticancer agents through targeting tumor-associated hCA isozymes (hCA IX and XII) would be the fruitful and effective strategy in cancer therapy [8].

Coumarin (2H-chromen-2-one) is a fused heterocyclic ring system consisting of benzene and 2-pyrone ring. It belongs to the family of neo flavonoids of plant secondary metabolites. Coumarins have ability to make noncovalent interactions viz. hydrogen bonds, hydrophobic, electrostatic, metal coordination, van der Waals force *etc.* with various active sites of the proteins and enzymes [9], and thus display a wide range of pharmacological activities like antimicrobial, anti-inflammatory, anti-tuberculosis, antihyperlipidemic, antiviral, antidepressant, antioxidant and anticancer activities *etc.* [10]. Coumarin heterocycle is present in a number of well-established clinical drugs with diverse therapeutic activities [11]. *Maresca, A* & Supuran *CT. et al,* have reported that coumarins (**1**) are a new class of non-zinc mediated carbonic anhydrases inhibitors and suggested that the coumarin hydrolysis product, a cis-2-hydroxy-cinnamic acid derivative, but not the coumarin moiety bound within the enzyme active site. The same

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https://doi.org/10.1016/j.bioorg.2019.04.004
Received 19 February 2019; Received in revised form 22 March 2019; Accepted 4 April 2019
Available online 05 April 2019
0045-2068/

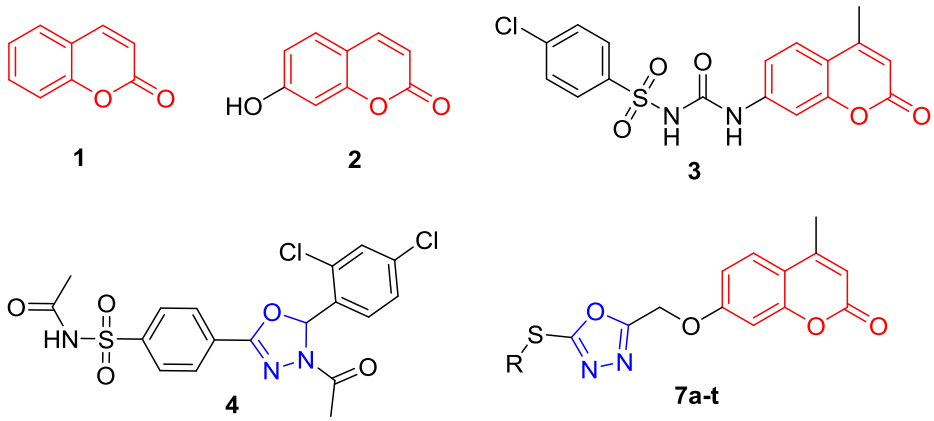


Fig. 1. Representative examples of coumarin & 1,3,4-oxadiazole derivatives as selective tumor-associated hCAs IX & XII inhibitors and rationale for designed molecules (**7a-t**).

research group have also reported umbelliferone (7-hydoxy coumarin, **2**) and its derivatives as selective inhibitors of IX and/or XII, over CA I and CA II [12]. *Aikaterini P & Supuran CT. et al,* have reported a novel 6- and 7-substituted coumarins (**3**) as selective tumor-associated carbonic anhydrases IX and XII inhibitors (Fig. 1) [13].

On the other hand, 1,3,4-oxadiazoles are thermally stable and neutral heteroaromatic compounds containing two nitrogens and oxygen atom, affects the pharmacokinetic and physicochemical properties of the compounds in which it is present. It makes diverse non-covalent interactions with various active sites of enzymes and receptors in biological systems and, thus display versatile pharmacological activities like anti-inflammatory, antibacterial, muscle relaxing, anti-hypertension, and anticancer activities [14] *etc. Giulia Bianco et al*, have reported a series of N-acyl benzene sulfonamide dihydro-1,3,4-oxadiazole hybrids (**4**) selective tumor-associated carbonic anhydrases IX and XII inhibitors and demonstrated that stereochemistry of dihydro-1,3,4-oxadiazole could be considered as key factors to determine activity and selectivity towards hCA isozymes IX and XII [15a]. *Angapelly S et al.,* have reported the sulfocoumarin, Coumarin, 4-Sulfamoyl-phenyl-bearing Indazole-3-carboxamide hybrids as selective tumor associated CA IX and XII inhibitors [15b]. *Adriano Mollica et al.,* have reported the new amide derivatives of Probenecid as selective inhibitors of CA IX and XII [15c]. *Fabrizio Carta et al.,* have reported novel coumarins and 2-thioxo-coumarins as inhibitors of the tumor-associated carbonic anhydrases IX and XII [15d].

In view of the selective nature of coumarin and 1,3,4-oxadiazole towards CA IX and XII, it is decided to synthesize a new series of coumarin-1,3,4-oxadiazole hybrids (Fig. 1), which have not been reported earlier. Thus, novel coumarin-1,3,4-oxadiazole hybrids (**7a-t**) were synthesized and evaluated for CA inhibitory activity against the four major CA isoforms, CA I, II, IX and XII, using acetazolamide (AAZ) as standard drug.

2. Result and discussion

2.1. Chemistry

The target coumarin-1,3,4-oxadiazole hybrids (**7a-t**) were synthesized (Scheme 1) by refluxing various aromatic and/or, aliphatic halides with 7-((5-mercapto-1,3,4-oxadiazol-2-yl) methoxy)-4-methyl-2H-chromen-2-one (**6**). Briefly, synthesis of 7-hydroxy-4-methyl coumarin (**3**) was done by condensation of resorcinol (**1**) with ethyl acetoacetate (**2**) in the presence of concentrated sulphuric acid [16]. In next step, it is converted into ethyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy) acetate (**4**) by reaction with ethyl bromoacetate and anhydrous potassium carbonate in freshly distilled acetone. The 2-((4-methyl-2-oxo-2H-chromen-7-yl) oxy) acetohydrazide (**5**) was synthesized from the compound (**4**) by reacting with hydrazine hydrate in THF as solvent [17]. Finally, the Cyclization of compound (**5**) was achieved by refluxing with carbon disulfide in ethanol in the basic condition thus, 7-((5-mercapto-1,3,4-oxadiazol-2-yl) methoxy)-4-methyl-2H-chromen-2-one (**6**) was obtained [18].

All synthesized compounds (**7a-t**) were characterized by spectral techniques viz. ^{1}H NMR, ^{13}C NMR and HRMS. The ^{1}H NMR spectrum of **7a** showed characteristic protons of 4-methyl group of coumarin ring around δ 2.40 ppm and 7-methyleneoxy group of coumarin ring around δ 5.20 ppm respectively. All the remaining protons appeared in the range of δ 5.0–8.50 ppm. ^{13}C NMR spectrum of **7a** showed the characteristic carbonyl and 4-methyl group of coumarin ring at δ 166.0 and δ 18.6 ppm respectively, and all the remaining carbons appeared in the range of δ 30.0–164.0. The similar pattern was observed for the rest of compounds (**7a-t**).

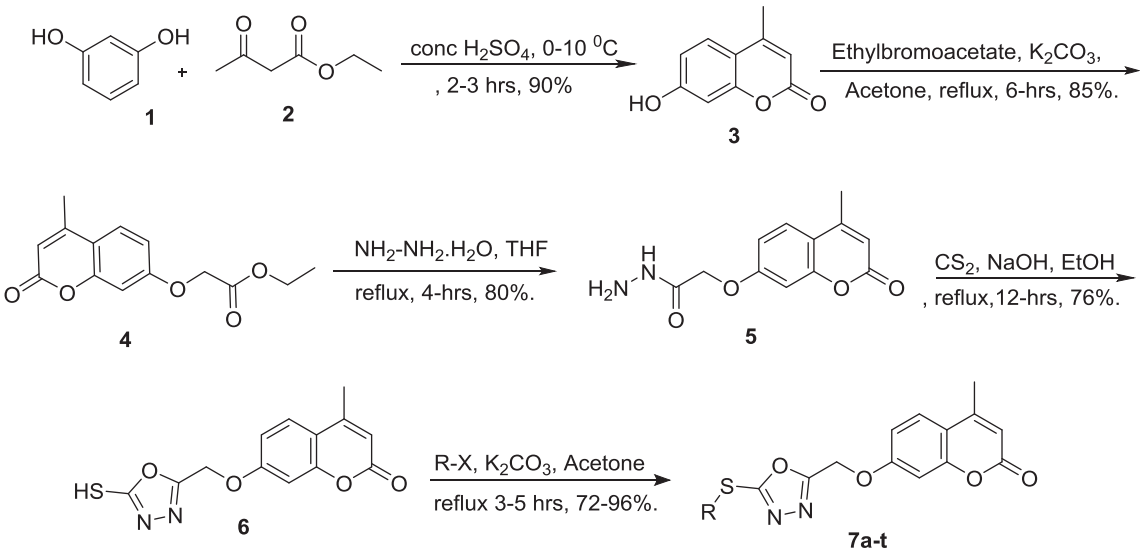
2.2. Carbonic anhydrase inhibition

The target coumarin-1,3,4-oxadiazole hybrids (**7a-t**) were screened for their potentials against the four hCA isoforms, namely, hCA I, II (the cytosolic) and hCA IX, XII (the tumor-associated) by a stopped-flow CO_2 hydrase assay, using acetazolamide (AAZ), as a standard drug. The following Structure-Activity Relationship (SAR) can be inferred from the inhibition data of coumarin-1,3,4-oxadiazole hybrids (**7a-t**) as shown in Table 1:

i. The target compounds **7a-t** had not shown any inhibition towards cytosolic isoforms hCA I and hCA II (Ki > 100 μM).
ii. The compounds **7a-t** have shown significant inhibition against the transmembrane tumor-associated isoform hCA XII. All the target compounds exhibited inhibitory potencies of < 10 μM with compounds **7a**, **7b**, **7c** and **7d** eliciting low micromolar inhibitory potencies (K_is) of **0.28**, **0.16**, **0.41** and **0.52** μM respectively.
iii. The potent inhibition order of coumarin-1,3,4-oxadiazole hybrids (**7a-d**) as follows, hCA XII inhibition: benzoyl (**7b**) > benzyl (**7a**) > Phenacyl (**7c**) > isobutyl group (**7d**).
iv. The compounds **7a-t** have shown variable and diverse inhibition against the transmembrane tumor-associated isoform hCA IX, with K_is below 10 μM. Among all, compound **7n**, bearing a 4-methoxy benzyl moiety on coumarin-1,3,4-oxadiazole hybrid, showed promising inhibitory activity with a K_i value of **2.3** μM.
v. All the others derivatives showed selective hCA IX and XII inhibition over hCA I &II in micromolar range.

3. Conclusion

In conclusion, we have reported the synthesis of coumarin-1,3,4-



Scheme 1. Synthesis of coumarin-1,3,4-oxadiazole hybrids (**7a-t**).

oxadiazole hybrids (**7a-t**), which are designed to target the transmembrane tumor-associated isoforms, hCA IX and XII. The target compounds (**7a-t**) were screened against four hCA 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 have showed significant lower to sub micromolar inhibition for both the isoforms, particularly for CA IX, with K_is in the range of 2.34–9.16 μM for CA IX and 0.16–9.72 for CA XII. The coumarin-1,3,4-oxadiazole hybrids (**7a-t**) proved to be ineffective against both the cytosolic isoforms I and II, thereby validating our hypothesis of selective inhibition of the two tumor-associated enzymes. Therefore, the compound **7b** and **7n** could be the further potential lead candidates for the design of novel small molecules for cancer therapy by a different mechanism.

4. Experimental section

4.1. Chemistry

All reagents and solvents were purchased from commercial suppliers and were used without further purification. Analytical thin layer chromatography (TLC) was performed on MERCK precoated silica gel 60-F_{254} (0.5 mm) aluminum plates. Visualization of the spots on TLC plates was achieved by UV light. ^{1}H and ^{13}C NMR spectra were recorded on bruker 500 MHz spectrometers using tetramethyl silane (TMS) as the internal standard. Chemical shifts for ^{1}H and ^{13}C are reported in parts per million (ppm) downfield from tetra methyl silane. Spin multiplicities are described as s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), and m (multiplet). Coupling constant (*J*) values are reported in hertz (Hz). HRMS were determined with Agilent QTOF mass spectrometer 6540 series instrument. Wherever required, column chromatography was performed using silica gel 60–120.

4.1.1. Synthesis of 7-hydroxy-4-methyl-2H-chromen-2-one (3)

A solution of resorcinol (9.08 mmol) in ethyl acetoacetate (1.3 mL) was added dropwise to an externally cooled conc. H_2SO_4 (10 mL) at 10 °C and the reaction mixture was then stirred at room temperature for 15 min. The mixture was poured into ice water contained in a beaker, the precipitate product (**3**) obtained was collected by suction filtration and washed with cold water and dried. The product was recrystallized from ethanol. Light yellow solid, yield 90%; mp 180–182 °C; ^{1}H NMR (500 MHz, DMSO) δ 10.49 (s, 1H), 7.53 (d, J = 8.7 Hz, 1H), 6.77 (d, J = 8.7, 2.1 Hz, 1H), 6.67 (s, 1H), 6.08 (s, 1H), 2.32 (s, 3H). 13C NMR (125 MHz, DMSO) δ 161.15, 160.28, 154.83, 153.44, 126.51, 112.82, 111.99, 110.25, 102.17, 90.71, 22.35, 18.07; ESI-HRMS (*m/z*) for $C_{10}H_8O_3$, calculated 176.0473, observed 177.0542 $[M + 1]^+$.

4.1.2. Synthesis of ethyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy) acetate (4)

Ethyl bromoacetate (0.7 mL, 6.8 mmol) and anhydrous K_2CO_3 (11.36 mmol) were added to a solution of 7-hydroxy-4-methyl-2H-chromen-2-one (**3**) in freshly distilled acetone (15 mL) and the reaction mixture was stirred under reflux for 6 h. Removal of solvent by evaporation left a pale yellow solid residue that was extracted with ethyl acetate (3x20 ml) and dried over Na_2SO_4. The combined organic layer was concentrated *in vacuo* to give a white color solid purified by recrystallization with ethanol. White solid, yield 85%; mp 167–169 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.52 (d, J = 8.0 Hz, 1H), 6.92 (dd, J = 8.8, 2.6 Hz, 1H), 6.78 (d, J = 2.5 Hz, 1H), 6.16 (s 1H), 4.69 (s, 2H), 4.29 (q, J = 7.1 Hz, 2H), 2.40 (s, 3H), 1.32 (t, J = 7.8 Hz, 3H). ^{13}C NMR (125 MHz, CDCl3) δ 167.93, 161.00, 160.60, 155.02, 152.35, 125.74, 114.37, 112.52, 112.46, 101.66, 65.31, 61.68, 18.63, 14.11; ESI-HRMS (*m/z*) for $C_{14}H_{14}O_5$, calculated 262.0841, observed 263.0920 $[M + 1]^+$.

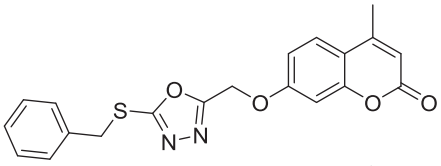
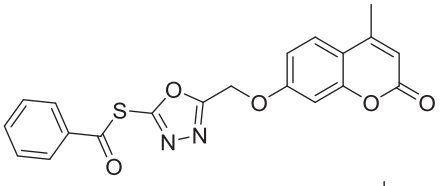
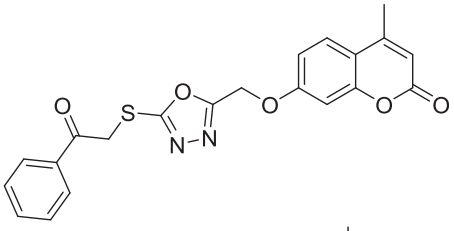
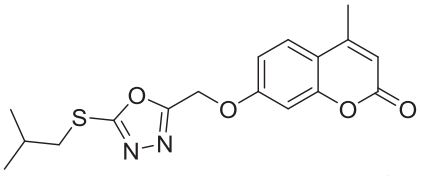
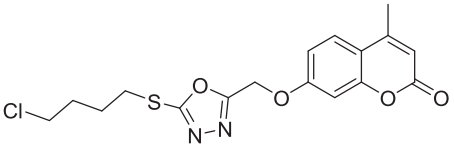
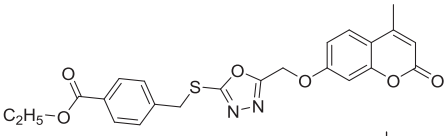
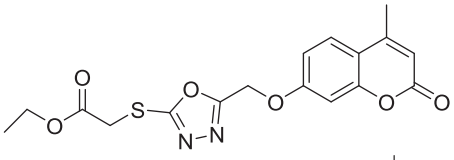
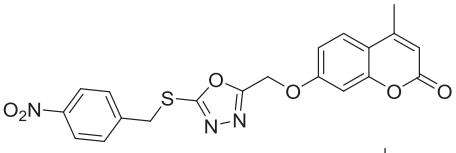
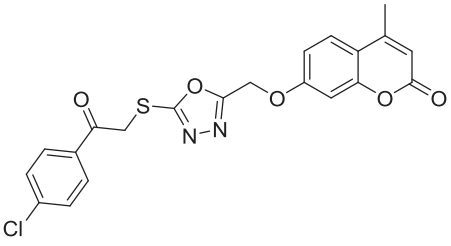
4.1.3. Synthesis of 2-(4-methyl-2-oxo-2H-chromen-7-yloxy) acetohydrazide (5)

Ethyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy) acetate (3.8 mmol) was dissolved into THF (10 mL) and to this hydrazine hydrate (0.28 mL, 5.7 mmol) was added dropwise. The reaction mixture was then stirred under reflux at 65–70 °C for 4 h. On cooling at room temperature 20–30 mL of cold water was added and stirred for 10 min. The white solid separated out was filtered at pump and dried. The product obtained was recrystallized from ethanol. White solid, yield 80%; mp 192–194 °C; ESI-HRMS (*m/z*) for $C_{12}H_{12}N_2O_4$, calculated 248.0797, observed 249.0874 $[M + 1]^+$.

4.1.4. Synthesis of 7-((5-mercapto-1,3,4-oxadiazol-2-yl) methoxy)-4-methyl-2H-chromen-2-one (6)

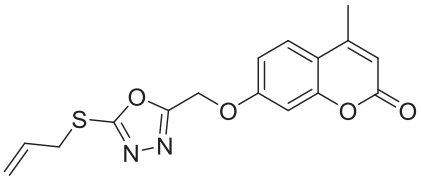
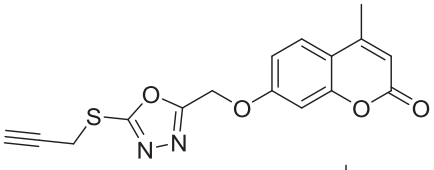
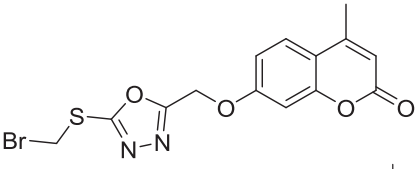
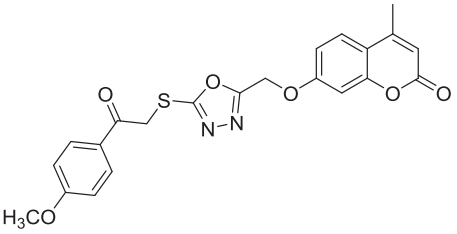
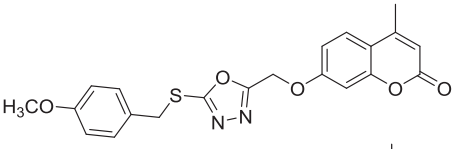
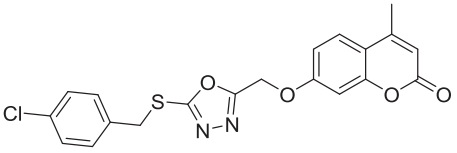
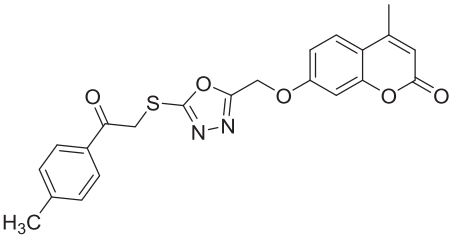
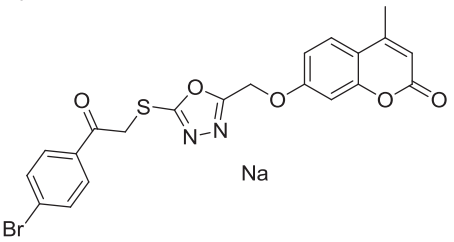
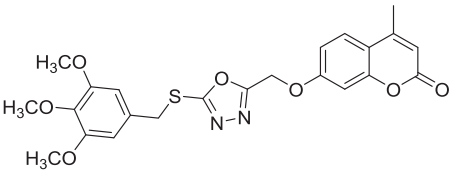
To a solution of NaOH (4.03 mmol) in EtOH (25 mL) were added 3 (2.48 g, 0.01 mol) and CS_2 (0.36 mL, 4.83 mmol), and the mixture was refluxed overnight while stirring. The solvent was removed in vacuo and the residue was dissolved in water and acidified with dil. HCI. The precipitate was washed with water and crystallized from EtOH. Pale yellow solid, yield 76%; mp 202–204 °C; ^{1}H NMR (500 MHz, DMSO) δ 14.43 (s, 1H), 7.62–7.58 (m, 1H), 7.02–6.96 (m, 2H), 6.16 (s, 1H), 5.21

Table 1
Inhibition of hCA isoforms I, II, IX and XII with target compounds (**4a-t**) and acetazolamide (AAZ) as a standard drug (K_i-μM).[a]

Compound	Structure	hCA I	hCAII	hCA IX	hCA XII
7a		> 100	> 100	7.04	0.28
7b		> 100	> 100	7.83	0.16
7c		> 100	> 100	8.04	0.41
7d		> 100	> 100	7.70	0.52
7e		> 100	> 100	7.33	0.74
7f		> 100	> 100	8.16	0.60
7g		> 100	> 100	7.03	0.60
7h		> 100	> 100	8.66	0.82
7i		> 100	> 100	7.04	7.96

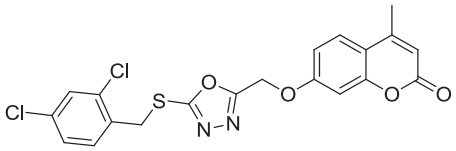
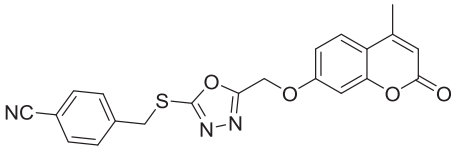
(*continued on next page*)

Table 1 (*continued*)

Compound	Structure	hCA I	hCAII	hCA IX	hCA XII
7j		> 100	> 100	6.94	8.41
7k		> 100	> 100	9.16	8.34
7l		> 100	> 100	7.40	8.67
7m		> 100	> 100	8.94	8.33
7n		> 100	> 100	2.34	9.67
7o		> 100	> 100	7.11	9.19
7p		> 100	> 100	6.21	9.72
7q		> 100	> 100	6.47	7.80
7r		> 100	> 100	5.90	8.78

(*continued on next page*)

Table 1 (*continued*)

Compound	Structure	hCA I	hCAII	hCA IX	hCA XII
7s		> 100	> 100	7.64	8.68
7t		> 100	> 100	7.72	9.16
AAZ		0.25	0.012	0.025	0.0057

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

(s, 2H), 2.44 (s, 3H). ^{13}C NMR (125 MHz, DMSO) δ 178.46, 160.20, 159.45, 157.54, 154.36, 151.97, 125.56, 114.23, 112.07, 111.87, 101.56, 59.37, 18.13; ESI-HRMS (*m/z*) for $C_{13}H_{10}N_2O_4S$, calculated 290.0361, observed 291.0405 $[M + 1]^+$.

4.1.5. General procedure for synthesis of coumarin-1,3,4-oxadiazole hybrids (7a-t)

To a solution of 7-((5-mercapto-1,3,4-oxadiazol-2-yl) methoxy)-4-methyl-2H-chromen-2-one (**6**) in freshly distilled acetone (20 mL), added K_2CO_3 as base and different alkyl halides or aryl halides (3.4 mmol) dropwise and stirred under reflux for 2–3 h. After confirmation was done by TLC, the solvent was evaporated completely and the obtained residue was extracted with ethyl acetate (3x20 ml) and dried over Na_2SO_4. The combined organic layer was concentrated in vacuo and the residues were purified by column chromatography on silica gel.

4.1.5.1. 7-((5-(benzylthio)-1,3,4-oxadiazol-2-yl) methoxy)-4-methyl-2H-chromen-2-one (7a). Yellow solid, yield 81%; mp 282–284 °C; ^{1}H NMR (500 MHz, CDCl3) δ 7.54 (d, J = 7.0 Hz, 1H), 7.45–7.37 (m, 2H), 7.35–7.25 (m, 3H), 7.06–6.81 (m, 2H), 6.18 (s, 1H), 5.27 (s, 2H), 4.49 (s, 2H), 2.41 (s, 3H). ^{13}C NMR (125 MHz, CDCl3) δ 166.02, 162.51, 160.88, 160.12, 155.04, 152.26, 135.18, 129.14, 128.85, 128.22, 125.97, 114.85, 112.86, 112.16, 102.36, 59.90, 36.78, 18.68; HRMS (ESI): *m/z* calc. for $C_{20}H_{16}N_2O_4S$ 380.0831; found 381.0905 $[M + H]^+$.

4.1.5.2. S-(5-(((4-methyl-2-oxo-2H-chromen-7-yl)oxy) methyl)-1,3,4-oxadiazol-2-yl) benzothioate (7b). Light yellow solid, yield 72%; mp 303–305 °C; ^{1}H NMR (500 MHz, DMSO) δ 7.95 (d, J = 8.3, 1.3 Hz, 2H), 7.73 (d, J = 8.6 Hz, 1H), 7.65 (t, J = 7.4 Hz, 1H), 7.51 (t, J = 7.7 Hz, 2H), 7.19–7.04 (m, 2H), 6.25 (s, 1H), 5.43 (s, 2H), 2.40 (s, 3H). ^{13}C NMR (125 MHz, DMSO) δ 167.78, 160.42, 160.39, 154.95, 153.73, 133.33, 131.21, 129.72, 129.03, 127.20, 114.66, 112.88, 112.32, 102.43, 60.41, 18.60; HRMS (ESI): *m/z* calc. for $C_{20}H_{14}N_2O_5S$ 394.0623; found 395.0685 $[M + H]^+$.

4.1.5.3. 4-methyl-7-((5-((2-oxo-2-phenylethyl) thio)-1,3,4-oxadiazol-2-yl) methoxy)-2H-chromen-2-one (7c). White solid, yield 80%; mp 321–323 °C; ^{1}H NMR (500 MHz, CDCl3) δ 8.03 (d, J = 7.5 Hz, 2H), 7.65 (t, J = 7.4 Hz, 1H), 7.56–7.50 (m, 3H), 7.08–6.79 (m, 2H), 6.18 (s, 1H), 5.29 (s, 2H), 4.95 (s, 2H), 2.41 (s, 3H). ^{13}C NMR (126 MHz, CDCl3) δ 191.74, 165.89, 162.68, 160.87, 160.09, 155.05, 152.24, 134.79, 134.35, 129.01, 128.52, 125.97, 114.87, 112.88, 112.24, 102.32, 59.87, 41.65, 18.68; HRMS (ESI): *m/z* calc. for $C_{21}H_{16}N_2O_5S$ 408.0780; found 409.0850 $[M + H]^+$.

4.1.5.4. 7-((5-(isobutylthio)-1,3,4-oxadiazol-2-yl) methoxy)-4-methyl-2H-chromen-2-one (7d). Pale yellow solid, yield 71%; mp 231–233 °C; ^{1}H NMR (500 MHz, CDCl3) δ 7.54 (d, J = 8.8 Hz, 1H), 7.11–6.85 (m, 2H), 6.18 (s, 1H), 5.28 (s, 2H), 3.18 (s, 2H), 2.41 (d, J = 1.1 Hz, 3H), 2.18–1.96 (m, 1H), 1.06 (d, J = 6.7 Hz, 6H). ^{13}C NMR (126 MHz, CDCl3) δ 166.98, 162.28, 160.87, 160.16, 155.04, 152.25, 125.95, 114.82, 112.84, 112.17, 102.37, 59.92, 41.01, 28.39, 21.63, 18.68; HRMS (ESI): *m/z* calc. for $C_{17}H_{18}N_2O_4S$ 346.0987; found 347.1064 $[M + H]^+$.

4.1.5.5. 7-((5-((4-chlorobutyl) thio)-1,3,4-oxadiazol-2-yl) methoxy)-4-methyl-2H-chromen-2-one (7e). Pale yellow solid, yield 74%; mp 246–248 °C; ^{1}H NMR (500 MHz, CDCl3) δ 7.55 (d, J = 7.2 Hz, 1H), 7.06–6.89 (m, 2H), 6.18 (d, J = 1.0 Hz, 1H), 5.29 (s, 2H), 3.64–3.52 (t, J = 7.1 Hz, 2H), 3.31 (t, J = 7.0 Hz, 2H), 2.41 (d, J = 1.1 Hz, 3H), 2.08–1.88 (m, 4H). ^{13}C NMR (126 MHz, CDCl3) δ 166.32, 162.47, 160.89, 160.12, 155.04, 152.27, 125.98, 114.85, 112.86, 112.20, 102.32, 59.89, 44.07, 31.78, 31.20, 26.54, 18.69; HRMS (ESI): *m/z* calc. for $C_{17}H_{17}ClN_2O_4S$ 380.0598; found 381.0667 $[M + H]^+$.

4.1.5.6. Ethyl4-(((5-(((4-methyl-2-oxo-2H-chromen-7-yl)oxy)methyl)-1,3,4-oxadiazol-2-yl)thio) methyl) benzoate (7f). Bright yellow solid, yield 79%; mp 319–321 °C; ^{1}H NMR (500 MHz, CDCl3) δ 8.07–7.96 (d, 2H), 7.56 (d, J = 8.7 Hz, 1H), 7.52 (d, J = 8.3 Hz, 2H), 7.00–6.92 (m, 2H), 6.20 (d, J = 1.2 Hz, 1H), 5.29 (s, 2H), 4.53 (s, 2H), 4.39 (q, J = 7.1 Hz, 2H), 2.43 (d, J = 1.2 Hz, 3H), 1.40 (t, J = 7.1 Hz, 3H). ^{13}C NMR (125 MHz, CDCl3) δ 166.07, 165.56, 162.69, 160.88, 160.08, 155.03, 152.27, 140.34, 130.33, 130.03, 129.11, 125.98, 114.87, 112.87, 112.17, 102.31, 61.12, 59.87, 36.24, 18.68, 14.32; HRMS (ESI): *m/z* calc. for $C_{23}H_{20}N_2O_6S$ 452.1042; found 453.1119 $[M + H]^+$.

4.1.5.7. Ethyl2-((5-(((4-methyl-2-oxo-2H-chromen-7-yl)oxy)methyl)-1,3,4-oxadiazol-2-yl)thio) acetate (7g). White solid, yield 88%; mp 277–279 °C; 1H NMR (500 MHz, CDCl3) δ 7.56 (d, J = 8.7 Hz, 1H), 7.04–6.87 (m, 2H), 6.20 (d, J = 1.1 Hz, 1H), 5.31 (s, 2H), 4.27 (q, J = 7.1 Hz, 2H), 4.10 (s, 2H), 2.43 (d, J = 1.1 Hz, 3H), 1.31 (t, J = 7.1 Hz, 3H). 13C NMR (125 MHz, CDCl3) δ 167.19, 165.11, 162.80, 160.86, 160.07, 155.04, 152.25, 125.99, 114.88, 112.87, 112.17, 102.34, 62.52, 59.85, 34.33, 18.68, 14.06; HRMS (ESI): *m/z* calc. for $C_{17}H_{16}N_2O_6S$ 376.0729; found 376.0813 $[M + H]^+$.

4.1.5.8. 4-Methyl-7-((5-((4-nitrobenzyl) thio)-1,3,4-oxadiazol-2-yl) methoxy)-2H-chromen-2-one (7h). Yellow solid, yield 91%; mp 296–298 °C; ^{1}H NMR (500 MHz, CDCl3) δ 7.98 (d, J = 9.5 Hz, 2H), 7.54 (d, J = 8.7 Hz, 1H), 7.50 (d, J = 8.6 Hz, 2H), 7.02–6.91 (m, 2H), 6.17 (d, J = 13.3, 4.2 Hz, 1H), 5.29 (s, 2H), 4.98 (s, 2H), 2.41 (d, J = 1.1 Hz, 3H). ^{13}C NMR (125 MHz, CDCl3) δ 190.65, 165.72, 162.76, 160.92, 160.07, 155.04, 152.27, 141.00, 133.13, 129.93, 129.40, 125.99, 114.89, 112.90, 112.29, 102.28, 59.86, 41.34, 18.71; HRMS

(ESI): *m/z* calc. for $C_{20}H_{15}N_3O_6S$ 425.0682; found 426.0742 $[M + H]^+$.

4.1.5.9. 7-((5-((2-(4-chlorophenyl)-2-oxoethyl)thio)-1,3,4-oxadiazol-2-yl)methoxy)-4-methyl-2H-chromen-2-one (7i). Yellow solid, yield 78%; mp 341–343 °C; ^{1}H NMR (500 MHz, CDCl3) δ 8.51–8.29 (m, 2H), 7.84 (d, J = 8.7 Hz, 2H), 7.75 (d, J = 8.7 Hz, 1H), 7.15 (t, J = 5.6, 2.5 Hz, 2H), 6.40 (d, J = 0.9 Hz, 1H), 5.48 (s, 2H), 4.75 (s, 2H), 2.61 (t, J = 9.3 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 190.65, 165.72, 162.76, 160.92, 160.07, 155.04, 152.27, 141.00, 133.13, 129.93, 129.40, 125.99, 114.89, 112.90, 112.29, 102.28, 59.86, 41.34, 18.71; HRMS (ESI): *m/z* calc. for $C_{21}H_{15}ClN_2O_5S$ 442.0390; found 443.0435 $[M + H]^+$.

4.1.5.10. 7-((5-(allylthio)-1,3,4-oxadiazol-2-yl)methoxy)-4-methyl-2H-chromen-2-one (7j). Pale yellow solid, yield 85%; mp 252–254 °C; ^{1}H NMR (500 MHz, CDCl3) δ 7.56 (d, J = 8.8 Hz, 1H), 7.03–6.89 (m, 2H), 6.20 (d, J = 1.2 Hz, 1H), 5.99 (m, J = 17.0, 10.0, 7.0 Hz, 1H), 5.39 (dd, J = 16.9, 2.4, 1.2 Hz, 1H), 5.30 (s, 2H), 5.23 (dd, J = 10.0, 0.9 Hz, 1H), 3.99–3.82 (m, 2H), 2.42 (d, J = 1.2 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.86, 162.57, 160.88, 160.12, 155.03, 152.28, 131.35, 125.98, 120.06, 114.84, 112.84, 112.16, 102.34, 59.91, 35.15, 18.69; HRMS (ESI): *m/z* calc. for $C_{16}H_{14}N_2O_4S$ 330.0674; found 331.0757 $[M + H]^+$.

4.1.5.11. 4-Methyl-7-((5-(prop-2-yn-1-ylthio)-1,3,4-oxadiazol-2-yl) methoxy)-2H-chromen-2-one (7k). Yellow solid, yield 87%; mp 260–262 °C; ^{1}H NMR (500 MHz, CDCl3) δ 7.56 (d, J = 8.8 Hz, 1H), 7.07–6.86 (m, 2H), 6.20 (d, J = 1.0 Hz, 1H), 5.35 (dd, J = 25.1, 17.3 Hz, 2H), 5.19 (t, J = 9.6 Hz, 1H), 4.05 (d, J = 2.6 Hz, 1H), 2.43 (d, J = 0.8 Hz, 3H), 2.35 (t, J = 2.6 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.78, 162.94, 160.87, 160.07, 155.03, 152.26, 125.99, 114.89, 112.89, 112.19, 102.35, 80.89, 79.77, 59.89, 21.07, 18.69; HRMS (ESI): *m/z* calc. for $C_{16}H_{12}N_2O_4S$ 328.0518; found 329.0594 $[M + H]^+$.

4.1.5.12. 7-((5-((bromomethyl)thio)-1,3,4-oxadiazol-2-yl)methoxy)-4-methyl-2H-chromen-2-one (7l). White solid, yield 72%; mp 279–281 °C; 1H NMR (500 MHz, CDCl3) δ 7.54 (d, J = 21.0 Hz, 1H), 6.99 (dd, J = 24.2, 17.9, 13.0 Hz, 2H), 6.20 (d, J = 1.0 Hz, 1H), 5.34 (s, J = 12.3 Hz, 2H), 5.09 (s, J = 11.9 Hz, 1H), 2.43 (d, J = 0.9 Hz, 3H). 13C NMR (125 MHz, CDCl3) δ 164.85, 163.38, 160.88, 160.01, 155.03, 152.29, 126.03, 114.93, 112.90, 112.23, 102.29, 59.86, 29.70, 18.70; HRMS (ESI): *m/z* calc. for $C_{14}H_{11}BrN_2O_4S$ 381.9623; found 380.1758 $[M - H]^+$.

4.1.5.13. 7-((5-((2-(4-methoxyphenyl)-2-oxoethyl)thio)-1,3,4-oxadiazol-2-yl)methoxy)-4-methyl-2H-chromen-2-one (7m). yellow solid, yield 83%; mp 316–318 °C; ^{1}H NMR (500 MHz, CDCl3) δ 8.18–7.73 (d, 2H), 7.52 (d, J = 19.7 Hz, 1H), 7.13–6.83 (m, 4H), 6.18 (d, J = 1.2 Hz, 1H), 5.29 (s, 2H), 4.92 (s, 2H), 3.90 (s, 3H), 2.41 (d, J = 1.2 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 190.19, 166.15, 164.49, 162.59, 160.90, 160.10, 155.05, 152.25, 130.94, 127.78, 125.97, 114.87, 114.19, 112.88, 112.26, 102.32, 59.86, 55.63, 41.57, 18.69; HRMS (ESI): *m/z* calc. for $C_{22}H_{18}N_2O_6S$ 438.0886; found 439.0962 $[M + H]^+$.

4.1.5.14. 7-((5-((4-methoxybenzyl)thio)-1,3,4-oxadiazol-2-yl)methoxy)-4-methyl-2H-chromen-2-one (7n). Pale yellow solid, yield 86%; mp 278–208 °C; ^{1}H NMR (500 MHz, CDCl3) δ 7.56–7.51 (d, 1H), 7.37–7.30 (d, 2H), 6.95 (m, J = 8.3, 4.2 Hz, 2H), 6.89–6.81 (d, 2H), 6.18 (d, J = 1.2 Hz, 1H), 5.27 (s, 2H), 4.45 (s, 2H), 3.79 (s, 3H), 2.41 (d, J = 1.2 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.15, 162.44, 160.88, 160.13, 159.51, 155.05, 152.25, 130.43, 127.04, 125.97, 114.85, 114.22, 112.86, 112.16, 102.36, 59.91, 55.31, 36.47, 18.69; HRMS (ESI): *m/z* calc. for $C_{21}H_{18}N_2O_5S$ 410.0936; found 411.1012 $[M + H]^+$.

4.1.5.15. 7-((5-((4-chlorobenzyl)thio)-1,3,4-oxadiazol-2-yl)methoxy)-4-methyl-2H-chromen-2-one (7o). Pale yellow solid, yield 88%; mp 292–294 °C; ^{1}H NMR (500 MHz, CDCl3) δ 7.52 (d, J = 19.6 Hz, 1H), 7.40–7.34 (d, 2H), 7.32–7.27 (d, 2H), 6.95 (m, J = 6.8, 2.5 Hz, 2H), 6.19 (d, J = 1.1 Hz, 1H), 5.27 (s, 2H), 4.44 (s, 2H), 2.41 (d, J = 1.1 Hz, 3H). ^{13}C NMR (126 MHz, CDCl3) δ 165.59, 162.67, 160.86, 160.08, 155.05, 152.23, 134.14, 133.90, 130.51, 128.99, 125.98, 114.88, 112.90, 112.16, 102.32, 59.88, 35.97, 18.69; HRMS (ESI): *m/z* calc. for $C_{20}H_{15}ClN_2O_4S$ 414.0441; found 415.0519 $[M + H]^+$.

4.1.5.16. 4-Methyl-7-((5-((2-oxo-2-(p-tolyl)ethyl)thio)-1,3,4-oxadiazol-2-yl)methoxy)-2H-chromen-2-one (7p). White solid, yield 72%; mp 332–334 °C; ^{1}H NMR (500 MHz, CDCl3) δ 7.93 (d, J = 8.3 Hz, 2H), 7.54 (d, J = 8.7 Hz, 1H), 7.30 (d, J = 11.9 Hz, 2H), 7.08–6.86 (m, 2H), 6.18 (d, J = 1.2 Hz, 1H), 5.29 (s, 2H), 4.94 (s, 2H), 2.43 (s,3H), 2.41 (d, J = 1.2 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 191.34, 166.04, 162.62, 160.89, 160.10, 155.05, 152.24, 145.51, 132.30, 129.69, 128.64, 125.97, 114.87, 112.88, 112.24, 102.33, 59.86, 41.71, 21.81, 18.69; HRMS (ESI): *m/z* calc. for $C_{22}H_{18}N_2O_5S$ 422.0936; found 423.1001 $[M + H]^+$.

4.1.5.17. 7-((5-((2-(4-bromophenyl)-2-oxoethyl)thio)-1,3,4-oxadiazol-2-yl)methoxy)-4-methyl-2H-chromen-2-one (7q). Yellow solid, yield 75%; mp 357–359 °C;^{1}H NMR (500 MHz, CDCl3) δ 8.00–7.82 (d, 2H), 7.76–7.61 (d, 2H), 7.60–7.50 (d, 1H), 6.98 (m, J = 21.5, 9.8, 6.4 Hz, 2H), 6.19 (d, J = 1.2 Hz, 1H), 5.29 (s, 2H), 4.89 (s, 2H), 2.41 (d, J = 1.2 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 190.86, 165.69, 165.04, 162.76, 160.07, 155.06, 152.22, 133.54, 132.39, 129.96, 129.79, 125.98, 114.90, 112.92, 112.26, 102.29, 59.84, 41.27, 18.69; HRMS (ESI): *m/z* calc. for $C_{21}H_{15}BrN_2O_5S$ 485.9885; found 486.9966 $[M + H]^+$.

4.1.5.18. 4-Methyl-7-((5-((3,4,5-trimethoxybenzyl)thio)-1,3,4-oxadiazol-2-yl)methoxy)-2H-chromen-2-one (7r). Pale yellow solid, yield 80%; mp 310–312 °C; ^{1}H NMR (500 MHz, CDCl3) δ 7.52 (d, J = 19.4 Hz, 1H), 7.08–6.84 (m, 2H), 6.65 (s, 2H), 6.18 (s, 1H), 5.28 (s, 2H), 4.44 (s, 2H), 3.96–3.74 (m, 9H), 2.41 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.06, 162.57, 160.89, 160.10, 155.03, 153.39, 152.30, 137.90, 130.68, 125.99, 114.87, 112.86, 112.23, 106.22, 102.29, 60.85, 59.93, 56.19, 37.30, 18.68; HRMS (ESI): *m/z* calc. for $C_{23}H_{22}N_2O_7S$ 470.1148; found 471.1221 $[M + H]^+$.

4.1.5.19. 7-((5-((2,4-dichlorobenzyl)thio)-1,3,4-oxadiazol-2-yl) methoxy)-4-methyl-2H-chromen-2-one (7s). White solid, yield 77%; mp 317–319 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (t, J = 8.2 Hz, 2H), 7.42 (d, J = 7.5 Hz, 1H), 7.23–7.15 (dd, 1H), 6.95 (m, J = 6.4, 2.5 Hz, 2H), 6.18 (d, J = 1.1 Hz, 1H), 5.27 (s, 2H), 4.54 (s, 2H), 2.41 (d, J = 1.1 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.76, 162.76, 160.90, 160.07, 155.04, 152.26, 135.07, 134.96, 132.34, 132.06, 129.66, 127.40, 125.98, 114.87, 112.89, 112.19, 102.27, 59.85, 33.91, 18.70; HRMS (ESI): *m/z* calc. for $C_{20}H_{14}C_{l2}N_2O_4S$ 448.0051; found 449.0174 $[M + H]^+$.

4.1.5.20. 4-(((5-(((4-methyl-2-oxo-2H-chromen-7-yl)oxy)methyl)-1,3,4-oxadiazol-2-yl)thio) methyl) benzonitrile (7t). Yellow solid, yield 83%; mp 286–288 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77–7.46 (m, 5H), 7.03–6.83 (m, 2H), 6.19 (d, J = 1.1 Hz, 1H), 5.27 (s, 2H), 4.50 (s, 2H), 2.40 (t, J = 4.7 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.18, 162.90, 160.87, 160.04, 155.02, 152.31, 141.04, 132.53, 129.93, 126.03, 118.38, 114.89, 112.89, 112.24, 112.04, 102.23, 59.88, 35.94, 18.70; HRMS (ESI): *m/z* calc. for $C_{21}H_{14}ClN_3O_4S$ 405.0783; found 406.0858 $[M + H]^+$.

4.2. CA inhibition

The inhibition assay of selected CA isozymes was performed using

SX.18V-R Applied Photophysics (Oxford, UK) stopped flow instrument [19]. 10 mM Hepes (pH 7.4) as a buffer, with Phenol Red (at a concentration of 0.2 mM) as an indicator, 0.1 M Na_2SO_4 or $NaClO_4$ (To maintain 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 5–10 s. Saturated CO_2 solutions in water at 25 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in DMSO-water 1:1, v/v) and dilutions up to 0.01 nM 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 [20–22].

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

Thankful to National Institute of Pharmaceutical Education and Research (NIPER), Balanagar, Hyderabad & Department of Pharmaceuticals, Ministry of Chemicals and Fertilizers, Govt. of India.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.04.004.

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