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Research paper

# Synthesis of coumarin-sulfonamide derivatives and determination of their cytotoxicity, carbonic anhydrase inhibitory and molecular docking studies

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ABSTRACT

Carbonic anhydrases isoforms CA IX, and XII are known to be highly expressed in various human tissues and malignancies. CA IX is a prominent target for especially colorectal cancers, because it is overexpressed in colorectal cancer and this overexpression leads poor prognosis. Inhibition of CA IX activity by small molecule CA inhibitors like sulfonamides, sulfonamide derivative or coumarins leads to inhibition of tumorigenesis. Novel twenty-seven compounds in three series (sulfonamide-based imines (**6a-6i**), coumarin-based aldehydes (**7a-7i**), and coumarin-sulfonamide-based target molecules (**8a-8i**)) were synthesized and characterized by means of IR, NMR, and mass spectra. All compounds were tested for their ability to inhibit CA I, CA II, CA IX, and CA XII isoforms. 4-((((2-((1-(3-((2-oxo-2H-chromen-7-yl)oxy) propyl)-1H-1,2,3-triazol-4-yl)methoxy)naphthalen-1-yl)-methylene)amino)methyl)benzenesulfonamide (**8i**) exhibited the highest hCA IX inhibition with the *Ki* of 45.5 nM. In addition, **8i** was found to be potent in inhibiting cancer cell proliferation as selective ($IC_{50} = 17.01 \pm 1.35\,\mu M$ for HT-29, $IC_{50} = 118.73 \pm 1.19\,\mu M$ for HEK293T). This novel compound inhibited the CA IX and CA XII protein expression in HT-29 cells. These findings indicate that **8i** can inhibit cellular proliferation in human colon cancer cells by specifically targeting the CA IX and CA XII expression.



## 1. Introduction

The carbonic anhydrases (CAs; EC 4.2.1.1) which include a metallic core of $Zn^{2+}$ ion at their active centre are a superfamily of metalloenzymes that present in all organisms [1,2]. The most important task of carbonic anhydrase for metabolism is to catalyse the reversible conversion of carbon dioxide into bicarbonate [3–6]. Sixteen different α-CA isoforms were isolated from mammals, where they act crucial physiological roles. Some of them are cytosolic (CA I, CA II, CA III, CA VII, CA XIII), others are membrane-bound (CA IV, CA IX, CA XII, CA XIV and CA XV), CA VA and CA VB are mitochondrial, and CA VI is secreted in saliva and milk [7,8]. These CA isoforms have been shown to play an active role in the mechanism of many diseases. In particular, the expression of the CA IX and CA XII isoenzymes in hypoxic tumor cells has led to the selection of these enzymes as the target of the anticancer compound designs by the researchers.

Sulfonamides are one of the most well-known groups of carbonic anhydrase inhibitors. These compounds have a wide range of applications including diuretic, antiglaucoma, antiobesity, antiepileptic and antitumor [9–14]. In addition to sulfonamides, coumarin and its derivatives have been reported to show selective inhibition, particularly on CA IX and XII [15–19]. Coumarin and its bioisosteres (thiocoumarins, 2-thioxocoumarins) act as “prodrugs”, whereas their hydrolysis products (formed through CA-mediated esterase activity) are the actually CA inhibitors [20,21]. Many studies have shown that 7-hydroxycoumarin, 6-nitro-7-hydroxycoumarin,

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0223-5234/

scopoletin and esculetin can be used in the treatment of cancer [22]. Coumarins have a variety of pharmacological effects and they are mostly used in anticancer drug design and discovery [23]. Coumarins demonstrate different mechanisms of antitumor activity at different stages of cancer formation. These mechanisms are; blockage of the cell cycle, apoptosis of the cell, blockage of the estrogen receptor, or inhibition of DNA-related enzymes such as topoisomerase [11].

This study aimed to investigate biological activities of novel synthesized compounds bearing both coumarin and sulfonamide moieties as the effective CA inhibitors. Their effects on CA I, CA II, CA IX, and CA XII isoforms and cytotoxic properties on the HT-29 cell line were evaluated. Furthermore, molecular modelling studies were performed to suggest possible modes of binding of these inhibitors inside the active sites of hCAs.

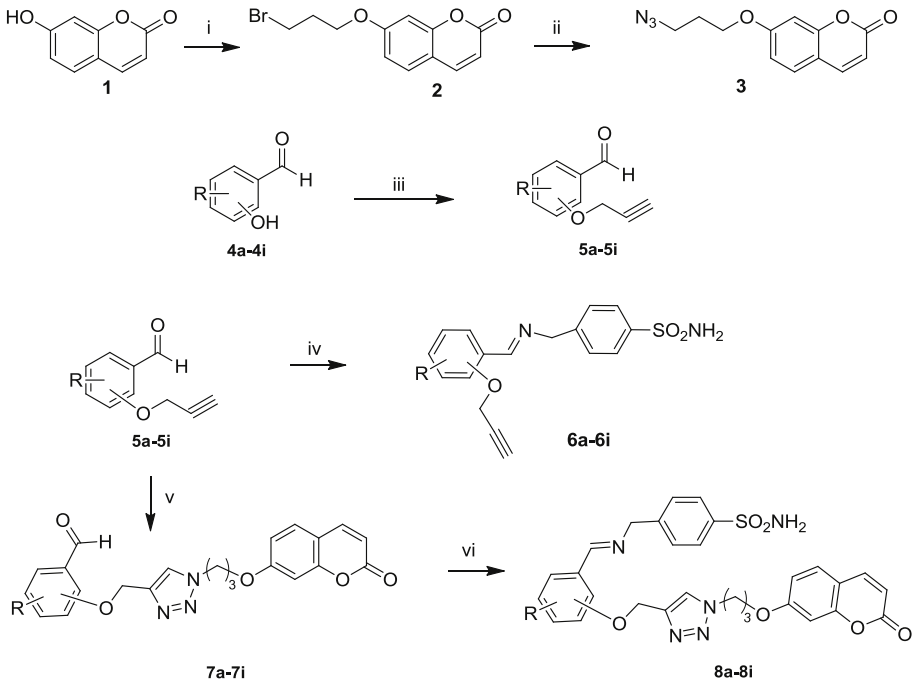
## 2. Result and discussion

### 2.1. Chemistry

The syntheses of the target compounds (**8a-8i)** are depicted in Scheme 1. Firstly, the propargyl group was bonded to hydroxyl groups of various aldehydes, and they were attached to coumarin by the click reactions. Then, the synthesis of target compounds containing both coumarin and sulfonamide moieties were carried out by forming an imine bond between the aldehyde fragment of the coumarin derivatives and the aminomethyl moiety of benzenesulfonamide.

Various hydroxy-aldehydes (**4a**-**i**) were reacted with propargyl bromide in DMF at room temperature and then obtained **5a**-**i** [24] were attached to 4-(aminomethyl)benzene sulfonamide by forming imine group in an alkaline solution of methanol:chloroform at 60 °C, for the synthesis of first series (sulfonamide-based imines (**6a**-**6i**)) [25]. On the other hand, the coumarin derivative (**3**) bearing propylazide were synthesized from 7-hyroxycoumarin (**1**) reacting with 1,3-dibromopropane and sodium azide, respectively [18]. To obtain the second series (coumarin-based aldehydes (**7a-i**)) [26], **5a**-**i** and **3** were bonded through triazole-bridge using $CuSO_4 \cdot 5H_2O$ as a catalyst. The synthesis of the third series (coumarin-sulfonamide-based target molecules) (**8a-i**) was carried out by the reaction of coumarin derivatives (**7a-i**) in the second series with 4-(aminomethyl)benzenesulfonamide.

All of the newly synthesized compounds were characterized by $^1$H NMR, $^{13}$C NMR, IR and MS. According to the IR spectra of the synthesized **6a-i** derivatives, it was possible to observe the absorption at about 3300 $cm^{-1}$ relating to $NH_2$ stretch of sulfonamide groups. The CH stretch of the alkyne group present in the **7a-i** shows an absorbance about 3150 $cm^{-1}$, while the stretch of the coumarin and aldehyde carbonyl moiety shows the absorbance between 1710 and 1740 $cm^{-1}$. Additionally, relating to $NH_2$ stretch of sulfonamide groups in the **8a-i** have observed the absorption at about 3300 $cm^{-1}$ and the stretch of the coumarin carbonyl moiety between 1710 and 1700 $cm^{-1}$. From the $^1$H NMR spectra; for the **6a-i**; the signals for aromatic protons were observed between 7.00 and 7.80 ppm, the signal of CH proton of imine group was detected at about 8.40–82 ppm and $NH_2$ proton signals observed about at 7.30–7.36 ppm. In addition, the signals of aliphatic protons were determined between 3.52 and 4.85 ppm. For the **7a-i**; the signals for aromatic protons were observed between 6.30 and 8.38 ppm, the signal of CHO proton of aldehyde group was detected at about 9.90–10.40 ppm and aliphatic proton signals observed about at 2.20–5.35 ppm. Similarly, for the **8a-i**; the signals for aromatic protons were observed between 6.20 and 8.40 ppm, the signal of CH proton of imine group was detected at about 8.60–8.80 ppm and aliphatic proton signals observed about at 2.20–5.35 ppm. From the $^{13}$C NMR spectra, the signals of the 160.0 and 165.5 ppm can be seen for carbonyl of lactone groups, C atom of imine group and C atom of near the oxygen atom. The signals of the aliphatic and aromatic carbons were observed at 13–66 ppm and 101–156 ppm, respectively. In addition, the C atoms of the aldehyde group were




**Scheme 1.** Synthesis of new coumarin-sulfonamide derivatives. Reaction conditions: (i) 1,3-dibromopropane, $K_2CO_3$, $CH_3CN$, reflux, 2h; (ii) $NaN_3$, DMF, rt, 16h; (iii) Propargyl bromide, DMF, $K_2CO_3$, rt, 16h; (iv) 4-(aminomethyl)benzenesulfonamide, NaOH, MeOH:$CHCl_3$, 60 °C, 18h; (v) **3**, $CuSO_4 \cdot 5H_2O$, Sodium Ascorbate, THF:$H_2O$, 40 °C, 2h; (vi) 4-(aminomethyl)benzenesulfonamide, NaOH, EtOH, 80 °C, 18h.

observed at about 185–192 ppm.

$^{1}$H NMR, $^{13}$C NMR, and MS spectra of the synthesized compounds are given in supplementary materials.

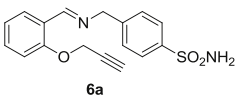
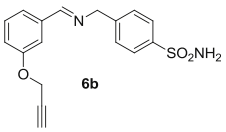
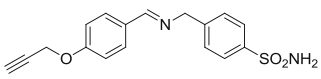
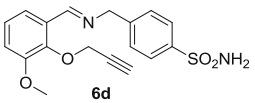
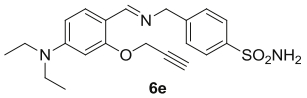
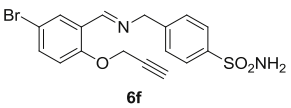
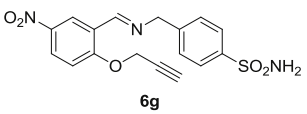
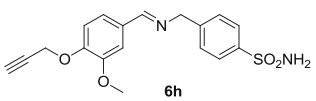
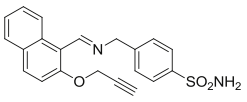
### 2.2. CA inhibition

The CA inhibitory profiles of the synthesized compounds were evaluated by applying a stopped flow carbon dioxide hydrase assay [27], in comparison to acetazolamide (AAZ) as a standard CAI against four physiologically significant isoforms CA I, II, IX, and XII.

a) From Table 1, the $K_i$ values of the sulfonamide-based compounds **6a-i**, the first subseries, were obtained in the range of 15.9–7608 nM, 81.2–2028 nM and 659.3–7345 nM against hCA II, hCA IX and hCA XII, respectively. This subseries weakly inhibited the cytosolic isoform hCA I (with $K_i > 10000$ nM). Among them, **6e** strongly inhibited the cytosolic isoform hCA II (associated with glaucoma) with $K_i$ of 15.9 nM, which is close to that of AAZ ($K_i$ of 12.1 nM). **6h** had the best inhibitory activity against hCA IX (tumor associated isoform) with $K_i$ of 81.2 nM, whereas **6d** had the strongest inhibition against isoform hCA XII (associated with tumor) with $K_i$ of 659.3 nM.

b) In terms of structure-activity relationship, **6a** ($K_i = 826.3$ nM), containing the *O*-propyne group at 2- position of the phenyl ring, has shown almost 8-9-fold higher inhibition of hCA II as compared to **6b** ($K_i = 7608$ nM) and **6c** ($K_i = 6493$ nM) (*O*-propyne group at 3- and 4- positions, respectively). Furthermore, **6b** (the *O*-propyne group at 3-position) exhibited the weakest inhibitory activity against hCA II, hCA IX and hCA XII with $K_i$ of 7608 nM, 2028 nM and 2899 nM, respectively (comparing with **6a** and **6c**). The presence of the methoxy group at 3- position (**6d**), the diethylamine group at 4- position (**6e**) and the bromine at 5-position (**6f**) of the phenyl ring of **6a** increased dramatically the inhibitory activity against hCA II isoform (with $K_i$ of 23.7 nM, 15.9 nM and 35.4 nM for **6d**, **6e** and **6f**, respectively), whereas the nitro group at 5- position (**6g**) of the phenyl ring

**Table 1**
*In vitro* inhibition $K_i$ values (nM) of **6a-i** for the hCA I, II, IX and XII.

| Compound | $K_i$ (nM)[a] | | | | | |
|---|---|---|---|---|---|---|
| | hCA I | hCA II | hCA IX | hCA XII | S·I.* (hCA IX/II) | S.I. (hCA XII/II) |
|  **6a** | >10000 | 826.3 | 1068 | 900.8 | 1.29 | 1.09 |
|  **6b** | >10000 | 7608 | 2028 | 2899 | 0.266 | 0.38 |
|  **6c** | >10000 | 6493 | 230.5 | 888.3 | 0.035 | 0.136 |
|  **6d** | >10000 | 23.7 | 1186 | 659.3 | 50.04 | 27.81 |
|  **6e** | >10000 | 15.9 | 197.9 | 921.9 | 12.44 | 59.98 |
|  **6f** | >10000 | 35.4 | 189.8 | 7345 | 5.36 | 207.48 |
|  **6g** | >10000 | 2154 | 211.8 | 5752 | 0.098 | 2.67 |
|  **6h** | >10000 | 438.7 | 81.2 | 2942 | 0.185 | 6.70 |
|  **6i** | >10000 | 307.7 | 1088 | 860.7 | 3.54 | 2.79 |
| **AAZ** | 250.0 | 12.1 | 25.8 | 5.7 | 2.13 | 0.47 |

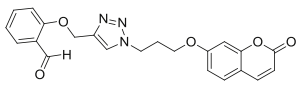
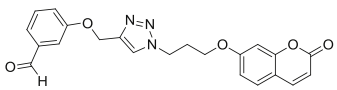
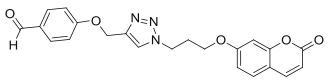
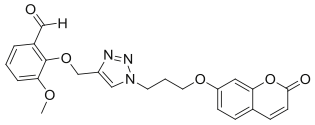
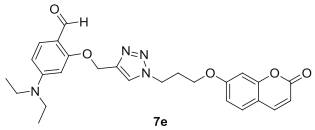
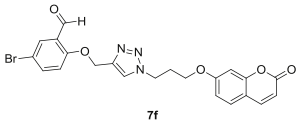
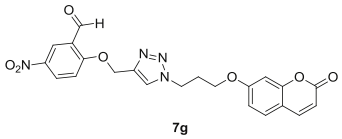
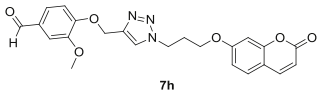
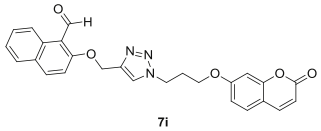
[a] Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5–10% of the reported values). S.I.: Selectivity Index.

of **6a** decreased almost 3-fold the hCA II inhibition (with $K_i$ of 2154 nM for **6g**). Similarly, the presence of the methoxy group at 3- position (**6h**) of the phenyl ring of **6c** enhanced the inhibitory activity against hCA II and hCA IX isoforms (with $K_i$ of 438.7 nM and 81.2 nM, against hCA II and hCA IX, respectively, for **6h**). On the other hand, the presence of diethylamine group at 4- position (**6e**), the bromine at 5- position (**6f**) and the nitro group at 5- position (**6g**) of the phenyl ring of **6a** rised strongly the inhibitory activity against hCA IX isoform (with $K_i$ of 197.9 nM, 189.8 nM and 211.8 nM for **6e**, **6f** and **6g**, respectively), whereas they reduced the hCA XII inhibition (with $K_i$ of 921.9 nM, 7345 nM and 5752 nM for **6e**, **6f** and **6g**, respectively). Additionally, the presence of naphthyl (**6i**) instead of phenyl ring of **6a** didn't have significant effect on hCA IX and hCA XII inhibition, while it increased 2.5-fold the hCA II inhibition.

c) From Table 2, the $K_i$ values of the coumarin-based compounds, containing triazole as a linker, **7a**-**i**, the second subseries, were obtained in the range of 127.5−1166 nM, and 60.9->10000 nM against hCA IX and hCA XII, respectively. This subseries weakly inhibited the cytosolic isoforms hCA I and hCA II (with $K_i > 10000$ nM). Among them, **7a** strongly inhibited the tumor-associated isoforms hCA IX and hCA XII with $K_i$ of 127.5 nM and 60.9 nM, respectively.

d) Regarding with structure-activity relationship, **7a**, containing the aldehyde group at 2- position of the phenyl ring, have shown higher inhibition of hCA IX as compared to **7b** ($K_i = 192$ nM) and **7c** ($K_i = 171.1$ nM) (aldehyde group at 3- and 4- positions, respectively). Furthermore, the moving

**Table 2**
*In vitro* inhibition $K_i$ values (nM) of **7a-i** for the hCA I, II, IX and XII.

| Compound | $K_i$ (nM)[a] | | | |
|---|---|---|---|---|
| | hCA I | hCAII | hCA IX | hCA XII |
|  7a | >10000 | >10000 | 127.5 | 60.9 |
|  7b | >10000 | >10000 | 192.0 | 8965 |
|  7c | >10000 | >10000 | 171.1 | >10000 |
|  7d | >10000 | >10000 | 1166 | >10000 |
|  7e | >10000 | >10000 | 157.9 | >10000 |
|  7f | >10000 | >10000 | 195.9 | >10000 |
|  7g | >10000 | >10000 | 136.6 | >10000 |
|  7h | >10000 | >10000 | 132.7 | >10000 |
|  7i | >10000 | >10000 | 189.5 | >10000 |
| **AAZ** | 250.0 | 12.1 | 25.8 | 5.7 |

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

aldehyde group from 2- position to 3- and 4- positions of the phenyl ring significantly decreased the inhibitory activity against hCA XII (comparing **7a** ($K_i = 60.9$ nM) with **7b** ($K_i = 8965$ nM) and **7c** ($K_i > 10000$ nM)). The presence of the methoxy group at 6- position (**7d**) of the phenyl ring of **7a** strongly reduced the inhibitions of both hCA IX and hCA XII (with $K_i$ of 1166 nM and >10000 nM, respectively, for **7d**). Additionally, the presence of diethylamine group at 5- position (**7e**), the bromine at 4- position (**7f**) and the nitro group at 4- position (**7g**) of the phenyl ring of **7a** decreased dramatically the inhibitory activity against hCA XII isoform (with $K_i$ of >10000 nM), whereas they didn't have significant effect on hCA IX inhibition. Similarly, the presence of naphthyl (**7i**) instead of phenyl ring of **7a** weakly reduced the inhibitory activity against hCA IX (with $K_i$ of 189.5 nM, for **7i**), while it extremely decreased the inhibitory activity against hCA XII (with $K_i$ of >10000 nM, for **7i**).

e) From Table 3, the $K_i$ values of the both coumarin and sulfonamide-based compounds, containing triazole and phenyl rings as the linker, **8a**-**i**, the target series, were obtained in the range of 230–790.6 nM, 45.5–2184 nM and 596.6–6538 nM against hCA II, hCA IX and hCA XII, respectively. The cytosolic isoform hCA I was weakly inhibited by this target compounds (with $K_i > 10000$ nM). Among them, **8e** strongly inhibited the cytosolic isoform hCA II with Ki of 267.8 nM, while **8i** had the best inhibitory activity against tumor associated isoforms hCA IX and hCA XII with $K_i$ of 45.5 nM and 596.6 nM, respectively.

f) With regard to structure-activity relationship, the moving benzylideneamino-sulfonamide moiety from 3- position to 2- or 4- positions of the phenyl ring decreased the inhibitory activity against hCA II (comparing **8b** ($K_i = 383.9$ nM) with **8a** ($K_i = 503.4$ nM) and **8c** ($K_i = 651.4$ nM)) and hCA XII (comparing **8b** ($K_i = 879.2$ nM) with **8a** ($K_i = 932.7$ nM) and **8c** ($K_i = 2725$ nM)). The hCA IX isoform was the weakest inhibited by **8b** (benzylideneamino-sulfonamide moiety at 3- position of the phenyl ring) with $K_i$ of 955.3 nM as compared to **8a** ($K_i = 187.5$ nM) and **8c** ($K_i = 169.2$ nM) (benzylideneamino-sulfonamide moiety at 2- and 4- positions, respectively). The presence of the methoxy group at 6- position (**8d**) of the phenyl ring of **8a** strongly decreased the inhibitions of both hCA IX and hCA XII (with $K_i$ of 1479 nM and 6538 nM, respectively, for **8d**). Additionally, the presence of diethylamine group at 5- position (**8e**) and the nitro group at 4- position (**8g**) of the phenyl ring of **8a** decreased dramatically the inhibitory activity against hCA IX isoform (with $K_i$ of 2184 nM and 856.7 nM, respectively), whereas they didn't have significant effects on hCA II and hCA XII inhibitions. Similarly, the presence of bromine group at 4- position (**8f**) of the phenyl ring didn't have major effects on hCA II, hCA IX and hCA XII inhibitions. The presence of naphthyl (**8i**) instead of phenyl ring of **8a** increased 2- or 3- fold the inhibitory activity against hCA II, hCA IX and hCA XII inhibitions (with $K_i$ of 230 nM, 45.5 nM and 569.6 nM, respectively, for **8i**).

## 2.3. Molecular modelling studies

Compounds **6a** – **8i** have been tested in enzyme inhibition assays against hCA I, II, IX and XII (Tables 1–3). None of the compounds showed $K_i$ values in the 0–100 nM range for hCA I. For hCA II, hCA IX and hCA XII, the number of compounds with $K_i$ values in the 0–100 nM range are 3 (compounds **6d**, **6e** and **6f**), 2 (compounds **6h** and **8i**) and 1 (compound **7a**), respectively. Interestingly, these compounds inhibited hCA II, IX or XII selectively (Tables 1–3).

Docking studies were performed to investigate the possible binding interactions between the selective inhibitors and the hCA active site. As not all compounds contain a sulfonamide group, we assumed that besides direct interactions between the ligand and the zinc ion the ligands could also interact with a zinc-bound water molecule. Therefore, the docking studies were performed with either a zinc-bound water molecule or a free $Zn^{2+}$-ion [28].

### 2.3.1. Binding interactions of compounds 6d, 6e and 6f with hCA II

Compound **6e** shows the lowest measured $K_i$ value for hCA II. The sulfonamide group interacts with the $Zn^{2+}$-ion and Thr199 and Thr200 (Fig. 1). The phenyl group adjacent to the sulfonamide moiety forms hydrophobic interactions with the side chains of His 94 and Leu198. The nitrogen atom between the two phenyl groups of the ligand forms a hydrogen bond with the side chain of Gln92. Hydrophobic interactions are formed with the side chains of Ile 91 and Phe 131. Compounds **6d** and **6f** form similar binding interactions with the hCA II active site. No poses have been identified for these compounds in which the ligand forms an interaction with a zinc-bound water molecule. Many compounds of series **6a** – **6i** show a similar docked pose as described for compound **6e** (Fig. 1). As such, the difference in the measured $K_i$ values for these compounds cannot be explained by docking studies alone.

### 2.3.2. Binding interactions of compounds 6h and 8i with hCA IX

For compound **6h**, docked poses have been obtained with either a direct interaction with the $Zn^{2+}$-ion and with the zinc-bound water molecule. For the first poses, the sulfonamide tail of compound **6h** interacts with the $Zn^{2+}$-ion and Thr199, while its phenyl group forms hydrophobic interactions with the side chain of Leu198 (Fig. 2A). The rest of the molecule is flexible and adopts different conformations and forms hydrophobic interactions with Leu 98, Val131, Leu 135 or Pro202. No hydrogen bonds are formed between ligand and active site residues.

Another docked pose has been obtained for compound **6h** in which the sulfonamide group forms hydrogen bonds with the zinc-bound water molecule, Gln92 and Thr200 (Fig. 2B). In addition, a hydrogen bond is formed with Trp 5. Hydrophobic interactions are formed with Ser 3 and Pro202. Similar docked poses have been obtained for the other members of compound series **6**.

For compound **8i** only one docked pose with a direct interaction with the $Zn^{2+}$-ion has been obtained for the coumarin-closed conformation. The sulfonamide tail is able to form interactions with the $Zn^{2+}$-ion and Thr199 (Fig. 3). Only one weak hydrogen bond may be formed between the backbone Nitrogen atom of Val131 and the ether oxygen of the ligand. Hydrophobic interactions are formed with Leu 91, Val131 and Pro202.

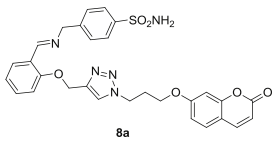
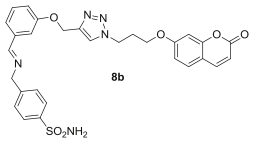
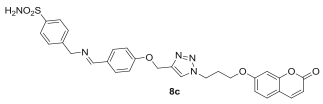
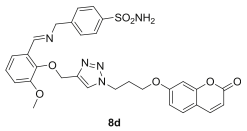
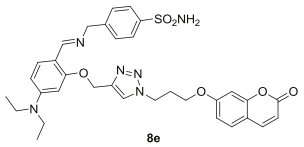
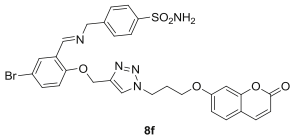
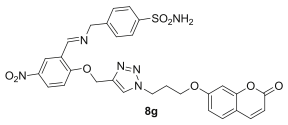
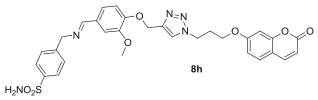
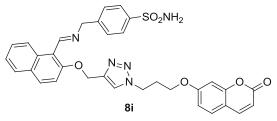
### 2.3.3. Binding interactions of compound 7a with hCA XII

For compound **7a**, only one docked pose has been obtained for the open coumarin form in which the ligand's carboxylic acid moiety forms a direct interaction with the $Zn^{2+}$-ion and Thr199 of the hCA XII active site (Fig. 4). The terminal carbonyl group of the ligand forms hydrogen bonds with Ser 132, while the triazole moiety forms a hydrogen bond with Gln92. The phenyl group of the ligand forms hydrophobic interactions with Leu198.

### 2.3.4. Understanding the low $K_i$ values of the tested ligands

Many of the analogs were able to bind in a similar way as described in Figs. 1–4. As such, the difference in the measured $K_i$ values for these compounds cannot be explained by docking studies alone. The inaccuracy in the calculation of the ligand-protein binding energies with molecular mechanics based force fields as well as induced-fit effects that are not included in these

**Table 3**
*In vitro* inhibition $K_i$ values (nM) of **8a-i** for the hCA I, II, IX and XII.

| Compound | $K_i$ (nM)[a] | | | | | |
|---|---|---|---|---|---|---|
| | hCA I | hCA II | hCA IX | hCA XII | S.I. (hCA IX/II) | S.I. (hCA XII/II) |
|  **8a** | >10000 | 503.4 | 187.5 | 932.7 | 0.375 | 1.85 |
|  **8b** | >10000 | 383.9 | 955.3 | 879.2 | 2.49 | 2.29 |
|  **8c** | >10000 | 651.4 | 169.2 | 2725 | 0.26 | 4.18 |
|  **8d** | >10000 | 388.5 | 1479 | 6538 | 3.81 | 16.82 |
|  **8e** | >10000 | 267.8 | 2184 | 837.0 | 8.16 | 3.125 |
|  **8f** | >10000 | 790.6 | 160.0 | 918.7 | 0.202 | 1.16 |
|  **8g** | >10000 | 466.3 | 856.7 | 891.9 | 1.84 | 1.912 |
|  **8h** | >10000 | 439.8 | 760.3 | 808.9 | 1.73 | 1.84 |
|  **8i** | >10000 | 230.0 | 45.5 | 569.6 | 0.197 | 2.48 |
| **AAZ** | 250.0 | 12.1 | 25.8 | 5.7 | 2.13 | 0.47 |

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

docking protocols are probably amongst the reason for this. In addition, the deprotonation efficiency of the sulfonamide group to yield the R–$SO_2NH^-$ group as well as the efficiency in the opening of the coumarin rings are important as they influence the shape and binding characteristics of the molecule (e.g., R–$SO_2NH^-$ is required for binding to the $Zn^{2+}$-ion). Finally, our docking protocols focussed on inhibitors binding to the active site either directly to the $Zn^{2+}$-ion or to the zinc-bound water molecule. However, inhibitors may also bind to allosteric sites of the enzymes, which were not included in this study. Nevertheless, possible binding interactions between these inhibitors and the hCA active sites have been suggested.

### 2.4. Biological activities

#### 2.4.1. Cell viability against normal and cancer cell lines

Cell viability assay was carried out using human colorectal adenocarcinoma cell line HT-29, HT-29 is CA IX and CA XII positive cell line which they are overexpressed under hypoxic conditions [18]. HT-29 cells were treated with the synthesized compounds for 24 h and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the viability of cells. We have used two reference compounds, namely doxorubicin (**dox**) and acetazolamide (**aaz**). GraphPad Prism software (GraphPad Software, San Diego, CA, USA) was used to calculate the median

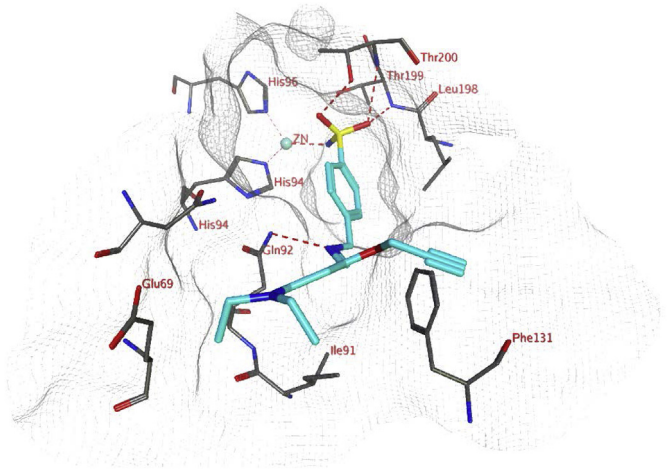



**Fig. 1.** The docked pose of compound **6e** (turquoise) in the active site of hCA II. The $Zn^{2+}$-ion is indicated with a turquoise sphere. Hydrogen bonds and interaction with the $Zn^{2+}$-ion are indicated in dashed red lines. The active site surface is indicated with a gray mesh. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

inhibition concentration ($IC_{50}$) for all compounds. As shown in Table 4, the nine most active compounds **6c**, **7a**, **7d**, **7e**, **7f**, **8a**, **8e**, **8f** and **8i** show potent inhibition of cancer cells growth with $IC_{50}$ ranging from $9.03 \pm 1.33$ to $23.34 \pm 1.51$ μM compared to doxorubicin ($IC_{50}$ value of $5.38 \pm 1.40$ μM). Compound **7e** possessed highest anticancer activity among all new hybrids against cancer cell growth with $IC_{50}$ $9.03 \pm 1.33$ μM for the HT-29 colorectal adenocarcinoma cell line. It is non-selective as it also shows potent cytotoxicity on HEK293T cells. According to these results, especially coumarin-aldehyde (**7a-7i**) and coumarin-sulfonamide (**8a-8i**), based derivatives showed better cytotoxicity. All other compounds showed low activity against the growth of cancer cell line. **7a** and **8i** which showed high activity in enzyme inhibition showed similarly high cytotoxicity. It has also been found that compounds exhibiting enzyme inhibition close to these compounds also exhibit good cytotoxicity. The 11 compounds selected were also tested on healthy cell lines considering both enzyme inhibition values and cell viability values of the compounds. These compounds are **6c**, **6d**, **6h**, **7a**, **7d**, **7e**, **7f**, **8a**, **8e**, **8f** and **8i**. HEK293T (embryonic kidney) cell line was used for this purpose. HEK293T cells were treated with selected compounds for 24 h and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the viability of cells.

The results obtained are given in Table 5. All of the compounds selected according to these results showed lower cytotoxicity than doxorubicin. In particular, it is seen that **8i** does not nearly decrease viability ($IC_{50}$ value of $118.73 \pm 1.19$ μM) of healthy cells. Also, sulfonamide based derivatives (**6c, 6d, 6h**) allowed healthy cells to maintain viability ($IC_{50}$ value ranging from $135.07 \pm 1.29$ to $537.04 \pm 1.31$ μM). **6h** and **8i** show selective cytotoxicity on HT-29, while **7a** displays cytotoxicity both on HT-29 and HEK293T as non-selective. Compounds **6a** and especially **7d** indicate cytotoxicity on HT-29, while they do not seem to potently inhibit hCAs. It is thought that the cytotoxic effect is carried out through different mechanisms. **7a** and **8i** were selected for western blotting studies according to the results of enzyme inhibition, cell viability analysis on healthy and cancer cells and modelling studies.

### 2.4.2. *The effects of 7a and 8i on protein levels of CA IX and CA XII*

In order to examine the effects of test substances on protein levels of CA IX and CA XII, human HT-29 cells were treated with compounds **7a** and **8i** and western blotting assay was performed [29,30]. Cells were seeded in DMEM/F12 (normoxic cells) or DMEM/F12 containing 200 μM $CoCl_2$ (hypoxic and treated cells) to create a hypoxic environment. As shown in Fig. 5, both **7a** and **8i** inhibited protein expression of CA IX and CA XII. Low dose of **7a** (1.56 μM) was not enough to statistically inhibited CA IX expression, despite a reduction in the graph. However higher doses of **7a** (6.25 and 25 μM) reduced CA IX protein expression and it was found to be statistically significant ($p < 0.05$) (Fig. 5A). When cells treaded with **8i**, it is seen from the graph that all three doses (1.56, 6.25 and 25 μM) inhibited CA IX expression gradually ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) (Fig. 5A). When looking at Fig. 5B, similarly low dose of **7a** (1.56 μM) was not enough to statistically inhibited CA XII expression, as we saw with CA IX. However again, higher doses of **7a** (6.25 and 25 μM) reduced CA XII protein expression. It was found to be statistically significant at dose 6.25 μM ($p < 0.01$), however because of high standard error it is not significant at dose 25 μM (Fig. 5B). Reduction in protein expression of CA XII when treated with **8i** was seen at all doses with statistically significance at lower doses (1.56 and 6.25 μM) ($p < 0.05$), however not at dose 25 μM because of high standard error (Fig. 5B).

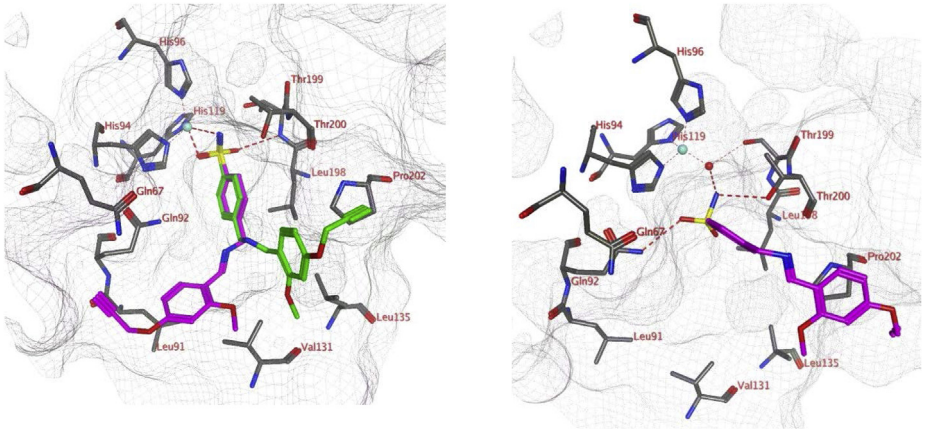



**Fig. 2.** The docked poses of compound **6h** forming direct binding interactions to the $Zn^{2+}$-ion (panel **A**, purple and green) or to the zinc-bound water molecule (panel **B**; purple) in the active site of hCA IX. The $Zn^{2+}$-ion is indicated with a turquoise sphere and the water molecule oxygen atom is indicated with a red sphere. Hydrogen bonds and interaction with the $Zn^{2+}$-ion are indicated in dashed red lines. The active site surface is indicated with a gray mesh. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

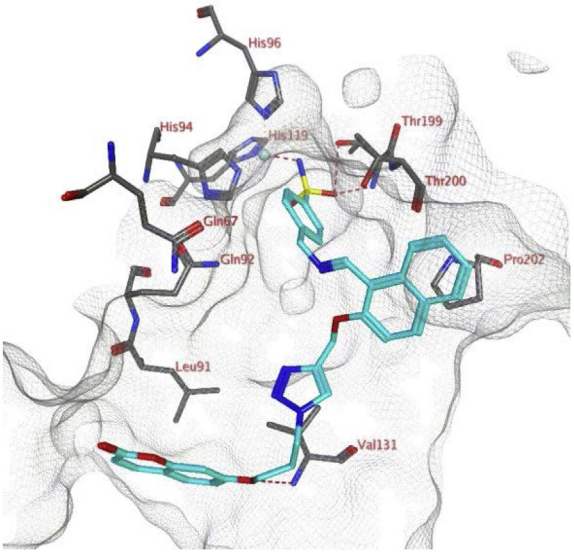



**Fig. 3.** The docked pose of compound **8i** (turquoise) in the closed coumarin form in the active site of hCA IX. The $Zn^{2+}$-ion is indicated with a turquoise sphere. Hydrogen bonds and interaction with the $Zn^{2+}$-ion are indicated in dashed red lines. The active site surface is indicated with a gray mesh. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

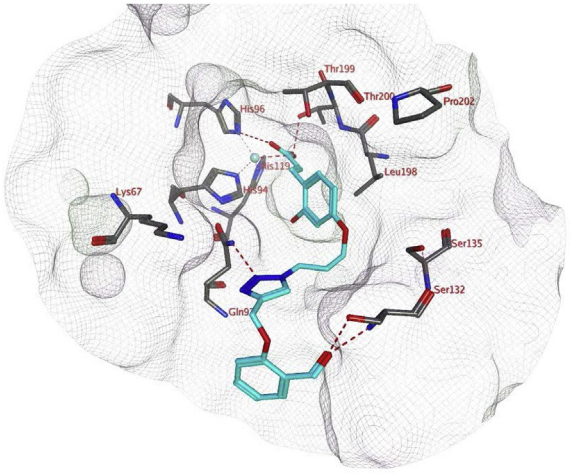



**Fig. 4.** The docked pose of compound **7a** (turquoise) in the open coumarin form in the hCA XII active site. The $Zn^{2+}$-ion is indicated with a turquoise sphere. Hydrogen bonds and interaction with the $Zn^{2+}$-ion are indicated in dashed red lines. The active site surface is indicated with a gray mesh. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

## 3. Conclusion

In tumor cells anaerobic glycolysis is increased to meet the increased metabolic needs. This caused an acidic environment and hypoxic conditions. Carbonic anhydrases (CAs) are a group of zinc-binding enzymes; catalyse the reversible hydration of $CO_2$ to bicarbonate. Tumor cells need CA enzymes to maintain neutral intracellular pH. To be able to tolerate hypoxic conditions; CA IX and CA XII are overexpressed in many cancers, including colorectal cancer. Clinical evidence shows that CA IX expression is associated with the poor survival of patients with colorectal cancer. Because of these concerns, the synthesis of new potential anticancer agents has been realized by targeting CA IX and CA XII enzyme inhibition.

This study demonstrates that this new derivative of coumarin-sulfonamide inhibits cellular proliferation of colorectal cancer cells which is overexpressing CA IX and CA XII in hypoxic conditions as well as the new compounds also shows CA IX and CA XII enzyme inhibition. Three groups of new compound derivatives were synthesized as sulfonamide-based (**6a-6i**), coumarin-aldehyde-based (**7a-7i**) and coumarin-sulfonamide-based derivatives (**8a-8i**); investigated for the inhibition of four physiologically relevant CA isoforms, CA I (cytosolic isoform) and CA II (associated with glaucoma), and CA IX and XII (tumor-associated isoforms). The effect of these compounds on four different carbonic anhydrase enzymes varied according to the isoform of carbonic anhydrases. Compounds that inhibit CA I isoform at more micromolar levels inhibited CA II, CA IX and CA XII isoforms at nanomolar levels. Compounds were evaluated *in vitro* on HT-29 human colorectal adenocarcinoma and healthy HEK293T embryonic kidney cell lines. Among all the molecules, **8i** demonstrated higher anti-proliferation effect on colorectal cancer cell line HT-29. Additionally, **8i** showed lower cytotoxicity on the healthy HEK293T cell line, which is a desirable feature for a drug candidate. Its inhibitory effects on CA IX and CA XII were displayed both enzyme inhibition assays and its possible binding interactions with hCAs were suggested with molecular modelling studies. **8i** caused a dose-dependent decrease in the level of CA IX protein while for CAXII it decreases the protein levels in a non-dose dependent manner. In addition, western blotting studies indicate that the mechanism of action of compound **8i** is not only inhibition of hCA IX, but also by down-regulation of the tumor-associated hCA IX and XII. The data indicated that **8i** may be promising drug candidate.

**Table 4**
$IC_{50}$ values calculated from viability at HT-29 cell line results of synthesized molecules.

| Comp. | $IC_{50}$ (μM) | | $IC_{50}$ (μM) |
|---|---|---|---|
| | HT-29 | Comp. | HT-29 |
| **6a** | 31.04 ± 1.18 | **7g** | 53.63 ± 1.53 |
| **6b** | 103.82 ± 1.54 | **7h** | 65.36 ± 1.90 |
| **6c** | **14.11 ± 1.43** | **7i** | 149.48 ± 2.57 |
| **6d** | 81.28 ± 1.57 | **8a** | **14.29 ± 1.31** |
| **6e** | 62.78 ± 1.45 | **8b** | 106.9 ± 2.82 |
| **6f** | 45.68 ± 1.25 | **8c** | 125.4 ± 1.31 |
| **6g** | 34.83 ± 1.35 | **8d** | 52.46 ± 1.46 |
| **6h** | 136.1 ± 2.10 | **8e** | **23.34 ± 1.51** |
| **6i** | 46.08 ± 1.33 | **8f** | **17.7 ± 1.33** |
| **7a** | **18.89 ± 1.33** | **8g** | 117 ± 4.60 |
| **7b** | 129.3 ± 1.83 | **8h** | 31.55 ± 1.68 |
| **7c** | 100.3 ± 1.56 | **8i** | **17.01 ± 1.35** |
| **7d** | **13.3 ± 1.24** | **AAZ** | 53.78 ± 1.75 |
| **7e** | **9.03 ± 1.33** | **Dox** | 5.38 ± 1.40 |
| **7f** | **7.47 ± 1.43** | | |

The cell viability was represented as a percentage (%) relative to untreated cells as a control.

**Table 5**
$IC_{50}$ values calculated from viability at HEK293T cell line results of selected molecules.

| Comp. | $IC_{50}$ (μM) | | $IC_{50}$ (μM) |
|---|---|---|---|
| | HEK293T | Comp. | HEK293T |
| **6c** | 397.8 ± 1.53 | **7f** | 6.08 ± 1.17 |
| **6d** | 135.07 ± 1.29 | **8a** | 13.91 ± 1.13 |
| **6h** | 537.04 ± 1.31 | **8e** | 29.54 ± 1.20 |
| **7a** | 13.73 ± 1.09 | **8f** | 8.25 ± 1.23 |
| **7d** | 30.4 ± 1.18 | **8i** | 118.73 ± 1.19 |
| **7e** | 19.21 ± 1.11 | **Dox** | 1.051 ± 0.57 |

The cell viability was represented as a percentage (%) relative to untreated cells as a control.

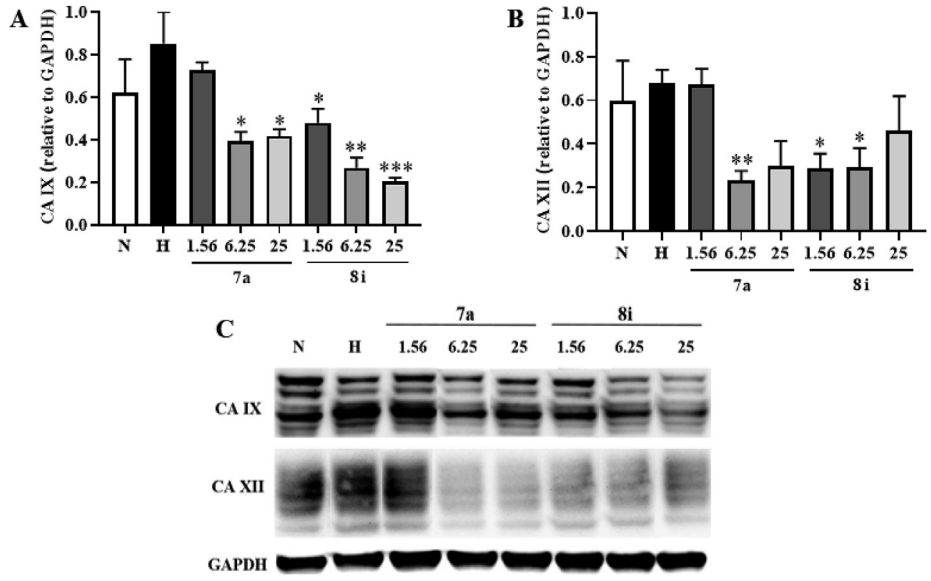



**Fig. 5.** The effects of **7a** and **8i** on protein levels of CA IX and CA XII in HT-29 cells. (**A**) CA IX and (**B**) CA XII protein levels of normoxic (N), hypoxic (H) and treated cells at three doses (1.56, 6.25 and 25 μM). One-way ANOVA with Tukey's post-hoc test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Mean ± SEM. **C.** Representative images of the bands (n = 3).

## 4. Experimental

### 4.1. Material and method

The device STUART SMP40 model was used for the determination of melting points. To measure infrared spectra, Alfa Bruker brand device is used. A Varian Infinity Plus spectrometer was used at 300 and 75 Hz, respectively, to record the $^1$H and $^{13}$C NMR spectra, while the Thermo Fisher Scientific LC-HRMS was used to determine the mass spectra. Other spectrophotometric measurements were performed with BioTek Power Wave XS (BioTek, USA). Cell lines were purchased from American Type Culture Collection (ATCC), Dulbecco's Modified Eagle's Medium-F12, RPMI Medium, fetal calf serum and PBS GIBCO BRL Invitrogen (Carlsbad, CA). Chemicals and solvents were purchased from Merck, Sigma-Aldrich, Across, and Alfa Aesar. The solvents used were used without purification.

### 4.2. General procedures and spectral data

Compounds 2 and 3 were synthesized according to the procedure shown in our previous study [18].

#### 4.2.1. Synthesis of compounds 5a-5i

Propargyl bromide (7.40 mmol) and anhydrous $K_2CO_3$ (1.00 g) were added to a solution of aldehyde derivatives (6.17 mmol) in DMF (15 mL). The mixture was stirred at room temperature for 16h, and then it was poured on crushed ice. The precipitated product was filtered and dissolved in EtOAc (100 mL). The solution was washed with water (50 mL), brine (50 mL), and water (50 mL), respectively. The organic layer was dried over anhydrous $Na_2SO_4$ and concentrated under vacuum to give propargyl aldehyde derivatives. The crude products were crystallized with petroleum ether [24].

2-(prop-2-yn-1-yloxy)benzaldehyde (**5a**): White powder, 90% yield, $^1$H NMR ($CDCl_3$, 300 MHz) δ/ppm: 2.57 (1H, s), 4.83 (2H, s), 7.06–7.13 (2H, m), 7.57 (1H, td, $J = 7.3$, 1.7 Hz), 7.86 (1H, d, $J = 7.6$ Hz), 10.48 (1H, s); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ/ppm: 56.5, 113.3, 121.9, 125.6, 135.9, 159.9, 189.8.

3-(prop-2-yn-1-yloxy)benzaldehyde (**5b**): White powder, 90% yield, $^1$H NMR ($CDCl_3$, 300 MHz) δ/ppm: 2.54 (1H, s), 4.69 (2H, s), 7.17–7.20 (1H, s), 7.37–7.47 (3H, m), 9.91 (1H, s); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ/ppm: 56.1, 113.8, 121.2, 130.3, 137.9, 158.2, 192.1.

4-(prop-2-yn-1-yloxy)benzaldehyde (**5c**): Yellow powder, 85% yield, $^1$H NMR ($CDCl_3$, 300 MHz) δ/ppm: 2.57–2.59 (1H, m), 4.77 (2H, d, $J = 2.3$ Hz), 7.09 (2H, d, $J = 8.7$ Hz), 7.85 (2H, d, $J = 8.4$ Hz), 9.90 (1H, s); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ/ppm: 56.1, 115.3, 130.7, 132.1, 162.5, 191.0.

3-methoxy-2-(prop-2-yn-1-yloxy)benzaldehyde (**5d**): White powder, 80% yield, $^1$H NMR ($CDCl_3$, 300 MHz) δ/ppm: 2.46–2.48 (1H, m), 3.89 (3H, s), 4.87 (2H, d, $J = 2.3$ Hz), 7.13–7.25 (2H, m), 7.42–7.46 (1H, m), 10.48 (1H, s); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ/ppm: 56.2, 61.0, 117.9, 119.1, 125.1, 131.3, 149.7, 153.0, 190.7.

4-(diethylamino)-2-(prop-2-yn-1-yloxy)benzaldehyde (**5e**): Pink powder, 80% yield, $^1$H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.14 (6H, t, $J = 7.3$ Hz), 2.50–2.52 (1H, m), 3.31–3.38 (4H, m), 4.70 (2H, d, $J = 2.3$ Hz), 6.13 (1H, s), 6.23 (1H, dd, $J = 9.0$, 2.3 Hz), 7.63 (1H, d, $J = 8.7$ Hz), 10.04 (1H, s); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ/ppm: 12.8, 45.1, 56.3, 94.5, 105.2, 114.7, 130.8, 153.8, 162.2, 187.0.

5-bromo-2-(prop-2-yn-1-yloxy)benzaldehyde (**5f**): Yellow powder, 85% yield, $^1$H NMR (DMSO-$d_6$, 300 MHz) δ/ppm: 3.66–3.68 (1H, m), 4.99 (2H, d, $J = 3.8$ Hz), 7.26 (1H, d, $J = 8.7$ Hz), 7.4 (1H, s), 7.82 (1H, d, $J = 8.7$ Hz), 10.22 (1H, s); $^{13}$C NMR (DMSO-$d_6$, 75 MHz) δ/ppm: 57.4, 78.9, 80.1, 114.0, 117.6, 126.9, 130.7, 138.9, 159.1, 188.5.

5-nitro-2-(prop-2-yn-1-yloxy)benzaldehyde (**5g**): Yellow powder, 85% yield, $^1$H NMR (DMSO-$d_6$, 300 MHz) δ/ppm: 3.76–3.77 (1H, m), 5.16 (2H, d, $J = 2.0$ Hz), 7.50 (1H, d, $J = 9.0$ Hz), 8.43 (1H, d, $J = 2.3$ Hz), 8.53 (1H, dd, $J = 9.9$, 2.9 Hz), 10.29 (1H, s); $^{13}$C NMR (DMSO-$d_6$, 75 MHz) δ/ppm: 58.0, 78.3, 80.6, 115.9, 124.4, 125.0, 131.4, 142.0, 163.9, 188.4.

3-methoxy-4-(prop-2-yn-1-yloxy)benzaldehyde (**5h**): Yellow powder, 85% yield, $^1$H NMR ($CDCl_3$, 300 MHz) δ/ppm: 2.46–2.5 (1H, m), 3.98 (3H, s), 4.98 (2H, d, $J = 2.3$ Hz), 7.18 (1H, d, $J = 8.7$ Hz), 7.40–7.54 (2H, m), 9.9 (1H, s); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ/ppm: 56.2, 56.7, 109.5, 112.6, 126.5, 131.0, 150.1, 152.3, 191.1.

2-(prop-2-yn-1-yloxy)-1-naphthaldehyde (**5i**): Brown powder, 85% yield, $^1$H NMR ($CDCl_3$, 300 MHz) δ/ppm: 2.56–2.58 (1H, m), 4.90 (2H, d, $J = 2.6$ Hz), 7.33 (1H, d, $J = 9.0$ Hz), 7.42 (1H, t, $J = 7.0$ Hz), 7.61 (1H, t, $J = 7.9$ Hz), 7.75 (1H, d, $J = 8.2$ Hz), 8.02 (1H, d, $J = 9.3$ Hz), 9.26 (1H, d, $J = 8.4$ Hz), 10.87 (1H, s); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ/

ppm: 57.5, 114.1, 118.0, 125.2, 125.4, 128.5, 129.2, 130.1, 131.6, 137.5, 162.1, 192.2.

*4.2.2. Synthesis of compounds 6a-i*

1 mmol of 4-(aminomethyl)benzenesulfonamide and 1.1 mmol of aldehyde derivatives were dissolved in 50 mL of $CHCl_3$:MeOH (5:1, v/v). The solution was refluxed for 18 h. It was cooled and evaporated under vacuum. The pure imine compounds were obtained after washed by ether [25].

4-(((2-(prop-2-yn-1-yloxy)benzylidene)amino)methyl)benzenesulfonamide (**6a**): White powder, 82% yield; mp. 140–141 °C; IR: 3300, 3282, 3040, 2959, 2130, 1625, 1600, 1458, 1338, 1152, 1028, 922, 753 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 3.63 (1H, t, $J = 1.1$ Hz), 4.85 (2H, s), 4.93 (2H, d, $J = 2.3$ Hz), 7.04 (1H, t, $J = 7.6$ Hz), 7.20 (1H, d, $J = 8.4$ Hz), 7.33 (2H, s, $NH_2$), 7.45–7.53 (3H, m), 7.80 (2H, d, $J = 8.2$ Hz), 7.91 (1H, t, $J = 7.6$ Hz), 8.82 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 56.8, 64.2, 79.4, 79.6, 114.2, 122.1, 125.0, 126.4, 127.5, 128.8, 132.8, 143.2, 144.5, 157.2, 158.0; HRMS (ESI): $C_{17}H_{16}N_2O_3S$ $[M+Na]^+$, calculated for 351.078, found 351.0760.

4-(((3-(prop-2-yn-1-yloxy)benzylidene)amino)methyl)benzenesulfonamide (**6b**): White powder, 86% yield; mp. 136–138 °C; IR: 3337, 3291, 3038, 2956, 2128, 1641, 1583, 1492, 1313, 1270, 1151, 1043, 672 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 3.60 (1H, t, $J = 1.1$ Hz), 4.84 (4H, s), 7.09–7.11 (1H, m), 7.34 (2H, s, $NH_2$), 7.39–7.42 (3H, m), 7.52 (2H, d, $J = 7.0$ Hz), 7.80 (2H, d, $J = 8.2$Hz), 8.52 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 56.1, 63.7, 79.1, 79.7, 113.9, 118.5, 122.2, 126.4, 128.8, 130.5, 138.0, 143.3, 144.3, 158.1, 163.0; HRMS (ESI): $C_{17}H_{16}N_2O_3S$ $[M+Na]^+$, calculated for 351.078, found 351.0760.

4-(((4-(prop-2-yn-1-yloxy)benzylidene)amino)methyl)benzenesulfonamide (**6c**): White powder, 76% yield; mp. 188–189 °C; IR: 3322, 3282, 3038, 2900, 2124, 1642, 1604, 1513, 1344, 1151, 1026, 829 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 3.36 (1H, t, $J = 0.5$ Hz), 4.80 (2H, s), 4.86 (2H, d, $J = 0.8$ Hz), 7.06 (2H, d, $J = 8.2$ Hz), 7.32 (2H, s, $NH_2$), 7.50 (2H, d, $J = 8.2$ Hz), 7.74–7.81 (4H, m), 8.46 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 56.2, 63.7, 79.2, 79.6, 115.6, 126.4, 128.7, 130.0, 130.2, 143.2, 144.6, 159.9, 162.3; HRMS (ESI): $C_{17}H_{16}N_2O_3S$ $[M+Na]^+$, calculated for 351.078, found 351.0759.

4-(((3-methoxy-2-(prop-2-yn-1-yloxy)benzylidene)amino) methyl)benzenesulfonamide (**6d**): Yellow powder, 80% yield; mp. 143–144 °C; IR: 3292, 3255, 3040, 2956, 2120, 1636, 1583, 1480, 1330, 1154, 1077, 785 $cm^{-1}$;$^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 3.53 (1H, t, $J = 2.3$ Hz), 3.82 (3H, s), 4.77 (2H, d, $J = 2.3$ Hz), 4.82 (2H, s), 7.10–7.18 (2H, m), 7.33 (2H, s, $NH_2$), 7.46–7.50 (3H, m), 7.78 (2H, d, $J = 8.4$ Hz), 8.80 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 56.5, 60.7, 64.3, 79.7, 79.9, 115.6, 118.5, 125.4, 126.4, 128.8, 130.8, 143.3, 144.3, 146.7, 153.2, 159.0; HRMS (ESI): $C_{18}H_{18}N_2O_4S$ $[M+Na]^+$, calculated for 311.088, found 381.0865.

4-(((4-(diethylamino)-2-(prop-2-yn-1-yloxy)benzylidene) amino)methyl)benzenesulfonamide (**6e**): Browne powder, 76% yield; mp. 120–121 °C; IR: 3307, 3287, 3035, 2971, 2121, 1600, 1518, 1402, 1272, 1092, 821 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 1.12 (6H, t, $J = 6.7$ Hz), 3.39–3.43 (4H, m), 3.63 (1H, s), 4.75 (2H, s), 4.91 (2H, s), 6.31 (1H, s), 6.35 (1H, d, $J = 9.0$ Hz), 7.33 (2H, s, $NH_2$), 7.48 (2H, d, $J = 7.0$ Hz), 7.72 (1H, d, $J = 8.7$ Hz), 7.79 (2H, d, $J = 8.2$ Hz), 8.60 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 13.2, 44.6, 56.4, 61.2, 79.2, 80.0, 95.9, 105.6, 118.6, 125.5, 126.3, 128.7, 141.4, 144.5, 146.7, 153.5, 159.0; HRMS(ESI): $C_{21}H_{25}N_3O_3S$ $[M+H]^+$, calculated for 400.162, found 400.1675.

4-(((5-bromo-2-(prop-2-yn-1-yloxy)benzylidene)amino) methyl)benzenesulfonamide (**6f**): White powder, 82% yield; mp. 174–175 °C; IR: 3309, 3284, 3040, 2884, 2125, 1636, 1588, 1475, 1329, 1151, 1016, 822 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 3.68 (1H, t, $J = 8.7$ Hz), 4.87 (2H, s), 4.95 (2H, d, $J = 2.3$ Hz), 7.36 (2H, s, $NH_2$), 7.52(2H, d, $J = 7.9$ Hz), 7.66 (1H, dd, $J = 9.3$, 3.2 Hz), 7.81 (2H, d, $J = 7.9$ Hz), 7.98 (1H, d, $J = 2.6$ Hz), 8.75 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 57.2, 64.0, 79.2, 79.7, 114.0, 116.9, 126.4, 127.1, 128.8, 129.5, 135.1, 143.3, 144.2, 156.3, 156.8; LC-HRMS (*m/z*): 430,9841 for:. HRMS(ESI): $C_{17}H_{15}BrN_2O_3S$ $[M+Na]^+$, calculated for 428.988, found 430.9841.

4-(((5-nitro-2-(prop-2-yn-1-yloxy)benzylidene)amino)methyl) benzenesulfonamide (**6g**): Yellow powder, 68% yield; mp. 149–150 °C; IR: 3341, 3278, 3038, 2891, 2126, 1635, 1608, 1518, 1331, 1150, 999 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 3.77 (1H, s), 4.90 (2H, s), 5.12 (2H, s), 7.31–7.43 (3H, m), 7.51 (2H, d, $J = 8.2$ Hz), 7.80 (2H, d, $J = 7.9$ Hz), 8.35 (1H, dd, $J = 9.3$, 2.9 Hz), 8.62 (1H, d, $J = 2.9$ Hz), 8.79 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 57.7, 64.0, 78.6, 80.4, 114.9, 122.8, 125.3, 126.5, 128.0, 128.9, 142.1, 143.4, 143.9, 156.4, 161.5; HRMS (ESI): $C_{17}H_{15}N_3O_5S$ $[M+Na]^+$, calculated for 396.063, found 396.0610.

4-(((3-methoxy-4-(prop-2-yn-1-yloxy)benzylidene)amino) methyl)benzenesulfonamide (**6h**): White powder, 85% yield; mp. 175–176 °C; IR: 3337, 3305, 3036, 2995, 2120, 1639, 1597, 1510, 1270, 1022, 804 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 3.61 (1H, t, $J = 1.4$ Hz), 3.80 (3H, s), 4.81 (2H, s), 4.85 (2H, d, $J = 1.1$ Hz), 7.10 (1H, d, $J = 8.2$ Hz), 7.30 (1H, d, $J = 8.4$ Hz), 7.31 (2H, s), 7.44 (1H, s), 7.51 (2H, d, $J = 7.6$ Hz), 7.80 (2H, d, $J = 8.2$ Hz), 8.44 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 56.1, 56.6, 70.2, 79.2, 79.6, 113.9, 122.7, 126.4, 127.4, 128.0, 128.9, 130.4, 144.5, 149.9, 156.4, 162.6; HRMS (ESI): $C_{18}H_{18}N_2O_4S$ $[M+Na]^+$, calculated for 381.088, found 381.0864.

4-((((2-(prop-2-yn-1-yloxy)naphthalen-1-yl)methylene) amino)methyl)benzenesulfonamide (**6i**): Yellow powder, 81% yield; mp. 129 °C; IR: 3317, 3300, 2978, 2121, 1637, 1592, 1464, 1334, 1165, 995, 803 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 3.64 (1H, s), 4.94 (2H, s), 5.06 (2H, s), 7.35–7.42 (3H, m), 7.47–7.58 (4H, m), 7.81 (2H, d, $J = 8.2$ Hz), 7.88 (1H, d, $J = 7.9$ Hz), 8.06 (1H, d, $J = 9.0$ Hz), 9.14 (1H, s), 9.23 (1H, d, $J = 8.4$ Hz); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 57.8, 65.9, 79.6, 79.8, 115.7, 117.8, 125.0, 126.5, 128.7, 128.8, 129.0, 129.7, 131.9, 133.5, 143.3, 144.6, 157.1, 160.5; HRMS (ESI): $C_{21}H_{18}N_2O_3S$ $[M+H]^+$, calculated for 379.104, found 379.1097.

*4.2.3. Synthesis of 7a-i derivatives*

The dissolved 0.1 mmol $CuSO_4 \cdot 5H_2O$ and 0.2 mmol sodium ascorbate in water (5 mL) was added to the solution of 7-(3-azidopropoxy)-2H-chromen-2-one (**3**) (1 mmol) and aldehyde derivatives (**5a-i**) (1 mmol) in 20 mL of THF:$H_2O$ (4:1, v/v). The mixture was stirred at 40 °C for 2h. It was concentrated under vacuum; the residue was dissolved in EtOAc (100 mL). The solution was washed with water (50 mL), EDTA (50 mL), and water (50 mL), respectively. The organic layer was concentrated under vacuum. The obtained **7a-i** derivatives were crystallized from ethanol [26].

2-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzaldehyde (**7a**): White powder, 82% yield; mp. 137 °C; IR: 3153, 3069, 2947, 1714, 1686, 1612, 1597, 1394, 1248, 1121, 1050, 834 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 2.29–2.36 (2H, m), 4.09 (2H, t, $J = 5.8$ Hz), 4.58 (2H, t, $J = 7.0$ Hz), 5.35 (2H, s), 6.30 (1H, d, $J = 9.3$ Hz), 6.88–7.03 (2H, m), 7.10 (1H, t, $J = 7.3$ Hz), 7.43 (1H, d, $J = 8.4$ Hz), 7.59–7.70 (3H, m), 7.98 (1H, d, $J = 9.3$ Hz), 8.38 (1H, s), 10.33 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.8, 47.3, 62.9, 66.0, 101.8, 113.1, 113.2, 113.3, 114.8, 121.8, 125.1, 125.4, 128.2, 130.1, 137.0, 143.0, 144.9, 156.0, 160.9, 161.0, 162.1, 189.8; HRMS (ESI): $C_{22}H_{19}N_3O_5$ $[M+Na]^+$, calculated for 428.122, found 428.1205.

3-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzaldehyde (**7b**): Yellow powder, 77% yield; mp. 56 °C; IR: 3140, 3083, 2946, 1720, 1694, 1609, 1394, 1256,

1121, 1016, 832 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz) $\delta$/ppm: 2.45–2.49 (2H, m), 4.04 (2H, t, $J = 5.8$ Hz), 4.63 (2H, t, $J = 7.0$ Hz), 5.25 (2H, s), 6.25 (1H, d, $J = 9.6$ Hz), 6.75 (1H, s), 6.80 (1H, dd, $J = 8.4$, 2.3 Hz), 7.23–7.28 (2H, m), 7.36–7.48 (3H, m), 7.63 (1H, d, $J = 9.3$ Hz), 7.69 (1H, s), 9.95 (1H, s); $^{13}C$ NMR ($CDCl_3$, 75 MHz) $\delta$/ppm: 29.8, 47.3, 62.2, 64.9, 101.7, 112.7, 113.1, 113.6, 122.0, 123.5, 124.0, 129.1, 130.4, 137.9, 143.6, 143.7, 155.9, 158.9, 161.2, 161.6, 192.2; HRMS (ESI): $C_{22}H_{19}N_3O_5$ $[M+Na]^+$, calculated for 428.122, found 428.1202.

4-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzaldehyde (**7c**): White powder, 80% yield; mp. 118 °C; IR: 3150, 3076, 2944, 1708, 1687, 1607, 1392, 1254, 1155, 1122, 1046, 994 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 2.31–2.36 (2H, m), 4.10 (2H, t, $J = 5.8$ Hz), 4.59 (2H, t, $J = 6.7$ Hz), 5.29 (2H, s), 6.30 (1H, d, $J = 9.6$ Hz), 6.90–6.97 (2H, m), 7.25 (2H, d, $J = 8.7$ Hz), 7.62 (1H, d, $J = 8.4$ Hz), 7.87 (2H, d, $J = 8.7$ Hz), 8.0 (1H, d, $J = 9.3$ Hz), 8.35 (1H, s), 9.88 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.8, 47.3, 62.1, 66.0, 101.8, 113.1, 113.2, 113.3, 115.8, 125.7, 130.1, 130.4, 132.4, 142.7, 145.0, 156.0, 160.9, 162.1, 163.6, 192.0; HRMS (ESI): $C_{22}H_{19}N_3O_5$ $[M+Na]^+$, calculated for 428.122, found 428.1203.

3-methoxy-2-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzaldehyde (**7d**): White powder, 86% yield; mp. 144 °C; IR: 3142, 3002, 2954, 1729, 1684, 1627, 1483, 1252, 1140, 953 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 2.24–2.29 (2H, m), 3.92 (3H, s), 4.01 (2H, t, $J = 5.8$ Hz), 4.53 (2H, t, $J = 7.0$ Hz), 5.26 (2H, s), 6.30 (1H, d, $J = 9.6$ Hz), 6.90–6.95 (2H, m), 7.19–7.20(2H, m), 7.39–7.42 (1H, m), 7.63 (1H, d, $J = 8.4$ Hz), 8.0 (1H, d, $J = 9.3$ Hz), 8.24 (1H, s), 10.0 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.9, 47.0, 56.8, 65.7, 66.6, 101.8, 113.1, 113.3, 118.5, 119.3, 125.3, 125.8, 130.1, 130.4, 142.9, 145.0, 150.4, 153.6, 156.0, 160.9, 162.1, 190.6; HRMS (ESI): $C_{23}H_{21}N_3O_6$ $[M+Na]^+$, calculated for 488.133, found 458.1306.

4-(diethylamino)-2-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzaldehyde (**7e**): White powder, 72% yield; mp. 125–126 °C; IR: 3149, 3080, 2968, 1726, 1680, 1614, 1587, 1271, 1117, 1030, 846 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm:1.09 (6H, t, $J = 6.7$ Hz), 2.28–2.32 (2H, m), 3.37–3.44 (4H, m), 4.06 (2H, t, $J = 5.5$ Hz), 4.55 (2H, t, $J = 6.7$ Hz), 5.29 (2H, s), 6.24–6.37 (3H, m), 6.85–6.93 (2H, m), 7.47(1H, d, $J = 8.7$ Hz), 7.56 (1H, d, $J = 8.4$ Hz), 7.95 (1H, d, $J = 9.3$ Hz), 8.31 (1H, s), 9.94 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 13.1, 29.8, 44.7, 47.2, 62.5, 66.0, 94.9, 101.8, 105.2, 113.1, 113.2, 113.3, 113.9, 125.2, 130.1, 144.9, 154.2, 156.0, 160.9, 162.1, 163.2, 185.9; HRMS (ESI): $C_{26}H_{28}N_4O_5$ $[M+Na]^+$, calculated for 499.196, found 499.1936.

5-bromo-2-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzaldehyde (**7f**): White powder, 80% yield; mp. 157–158 °C; IR: 3158, 3073, 2970, 1738, 1682, 1663, 1589, 1400, 1273, 1232, 1123, 1047, 846 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 2.31–2.35 (2H, m), 4.08 (2H, t, $J = 5.8$ Hz), 4.58 (2H, t, $J = 7.0$ Hz), 5.36 (2H, s), 6.30 (1H, d, $J = 9.6$ Hz), 6.88–6.95 (2H, m), 7.45(1H, d, $J = 8.4$ Hz), 7.60 (1H, $J = 8.7$ Hz), 7.72 (1H, d, $J = 2.6$ Hz), 7.82 (1H, dd, $J = 7.9$, 2.6 Hz), 7.98 (1H, d, $J = 9.6$ Hz), 8.38 (1H, s), 10.22 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.8, 47.3, 63.3, 66.0, 101.8, 113.1, 113.3, 113.6, 117.7, 125.5, 126.6, 130.1, 130.4, 139.0, 142.7, 144.9, 156.0, 160.0, 160.9, 162.1, 188.7; HRMS (ESI): $C_{22}H_{18}BrN_3O_5$ $[M+Na]^+$, calculated for 506.033, found 506.0305.

5-nitro-2-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzaldehyde (**7g**): Yellow powder, 66% yield; mp. 181 °C; IR: 3160, 3080, 2946, 1723, 1683, 1611, 1588, 1341, 272, 1121, 1034, 838 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 2.32–2.36 (2H, m), 4.09 (2H, t, $J = 5.8$ Hz), 4.59 (2H, t, $J = 7.0$ Hz), 5.54 (2H, s), 6.30 (1H, d, $J = 9.6$ Hz), 6.88–7.03 (2H, m), 7.60(1H, d, $J = 8.4$ Hz), 7.70 (1H, $J = 9.3$ Hz), 7.98 (1H, d, $J = 9.3$ Hz), 8.40 (1H, d, $J = 2.9$ Hz), 8.43 (1H, s), 8.51 (1H, dd, $J = 9.3$, 2.9 Hz), 10.27 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.8, 47.3, 63.9, 66.0, 101.8, 113.1, 113.2, 115.9, 124.1, 124.7, 125.8, 130.1, 131.4, 141.6, 144.9, 155.9, 160.9, 162.0, 164.9, 188.5; HRMS (ESI): $C_{22}H_{18}N_4O_7$ $[M+Na]^+$, calculated for 473.107, found 473.1210.

3-methoxy-4-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzaldehyde (**7h**): White powder, 82% yield; mp. 117–118 °C; IR: 3153, 3083, 2947, 1723, 1674, 1613, 1507, 1279, 1227, 1132, 1048, 835 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz) $\delta$/ppm: 2.44–2.48 (2H, m), 3.90 (3H, s), 4.04 (2H, t, $J = 5.8$ Hz), 4.62 (2H, t, $J = 7.0$ Hz), 5.38 (2H, s), 6.26 (1H, d, $J = 9.6$ Hz), 6.75–6.81 (2H, m), 7.21 (1H, d, $J = 8.2$ Hz), 7.35–7.44 (3H, m), 7.64 (1H, d, $J = 9.6$ Hz), 7.72 (1H, s), 9.84 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.8, 47.4, 56.2, 62.9, 64.8, 101.7, 109.2, 112.5, 112.7, 113.1, 113.6, 127.0, 129.1, 130.7, 143.6, 149.9, 153.1, 155.9, 161.3, 161.6, 191.2; HRMS (ESI): $C_{23}H_{21}N_3O_6$ $[M+Na]^+$, calculated for 458.133, found 458.1304.

2-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)-1-naphthaldehyde (**7i**): Orange powder, 77% yield; mp. 173–174 °C; IR: 3152, 3072, 2958, 1725, 1682, 1610, 508, 1229, 1117, 1043, 829 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz) $\delta$/ppm: 2.44–2.48 (2H, m), 4.01 (2H, t, $J = 5.5$ Hz), 4.63 (2H, t, $J = 6.7$ Hz), 5.47 (2H, s), 6.23 (1H, d, $J = 9.3$ Hz), 6.74–6.77 (2H, m), 7.31 (1H, d, $J = 8.2$ Hz), 7.40–7.58 (2H, m), 7.62–7.64 (2H, m), 7.70 (1H, *s*), 7.76 (1H, d, $J = 7.6$ Hz), 8.04 (1H, d, $J = 9.0$ Hz), 9.20 (1H, d, $J = 8.7$ Hz), 10.82 (1H, s); $^{13}C$ NMR ($CDCl_3$, 75 MHz) $\delta$/ppm: 29.8, 47.4, 63.6, 64.8, 101.7, 112.6, 113.1, 113.6, 114.0, 117.4, 125.1, 125.3, 128.5, 129.0, 129.2, 130.2, 131.6, 137.8, 143.5, 155.9, 161.3, 161.6, 162.7, 192.0; HRMS (ESI): $C_{26}H_{21}N_3O_5$ $[M+Na]^+$, calculated for 478.138, found 478.1356.

*4.2.4. Synthesis of compound 8a-i*

1 mmol of 4-(aminomethyl)benzenesulfonamide, 1 mmol of **7a-i** derivatives and 1 mmol NaOH were dissolved in 50 mL of EtOH. The solution was refluxed for 18 h. It was cooled and evaporated under vacuum. The final compounds were obtained after washing by ether.

4-(((2-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzylidene)amino)methyl)benzenesulfonamide (**8a**): White powder, 76% yield; mp. 107–108 °C; IR: 3338, 3063, 2973, 1722, 1614, 1599, 1336, 1283, 1154, 1125, 996 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 2.31–2.35 (2H, m), 4.09 (2H, t, $J = 6.1$ Hz), 4.58 (2H, t, $J = 6.7$ Hz), 4.80 (2H, s), 5.28 (2H, s), 6.28 (1H, d, $J = 9.3$ Hz), 6.88–7.04 (4H, m), 7.33 (2H, d, $J = 7.9$ Hz), 7.43–7.54 (3H, m), 7.60 (1H, d, $J = 8.7$ Hz), 7.80 (2H, d, $J = 8.2$ Hz), 7.89 (1H, d, $J = 7.6$ Hz), 7.94 (1H, d, $J = 9.3$ Hz), 8.37 (1H, s), 8.78 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.8, 47.2, 62.6, 64.2, 65.6, 66.0, 101.8, 113.1, 113.2, 113.3, 114.1, 121.7, 124.7, 125.4, 126.4, 127.4, 128.7, 130.1, 133.0, 143.2, 144.5, 145.0, 155.9, 158.0, 158.1, 160.9, 162.1; HRMS (ESI): $C_{29}H_{27}N_5O_6S$ $[M+Na]^+$, calculated for 596.188, found 596.1548.

4-(((3-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzylidene)amino)methyl)benzenesulfonamide (**8b**): White powder, 72% yield; mp. 134–135 °C; IR: 3333, 3070, 2957, 1730, 1611, 1475, 1271, 1228, 1158, 1120, 1036, 832 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 2.30–2.34 (2H, m), 4.09 (2H, t, $J = 5.8$ Hz), 4.57 (2H, t, $J = 7.0$ Hz), 4.84 (2H, s), 5.19 (2H, s), 6.28 (1H, d, $J = 9.6$ Hz), 6.89–6.96 (2H, m), 7.13–7.17 (1H, m), 7.35–7.39 (3H, m), 7.46 (1H, s), 7.51–7.63 (4H, m), 7.80 (2H, d, $J = 8.2$ Hz), 7.98 (1H, d, $J = 9.6$ Hz), 8.30 (1H, s), 8.51 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.8, 47.2, 61.8, 63.7, 66.0, 101.8, 113.1, 113.2, 113.3, 114.1, 118.2, 121.7, 125.4, 126.2, 126.4, 128.8, 130.1, 130.5, 138.0, 143.2, 143.3, 144.3, 145.0, 155.9, 158.9, 160.9, 162.1, 163.0; HRMS (ESI): $C_{29}H_{27}N_5O_6S$ $[M+Na]^+$, calculated for 596.188, found 596.1550.

4-(((4-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzylidene)amino)methyl)benzenesulfonamide (**8c**): Yellow powder, 81% yield; mp. 58–59 °C; IR: 3272, 3067,

2951, 1718, 1603, 1508, 1328, 1229, 1156, 1094, 830 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 2.28–2.34 (2H, m), 4.09 (2H, t, $J = 6.1$ Hz), 4.57 (2H, t, $J = 6.7$ Hz), 4.80 (2H, s), 5.20 (2H, s), 6.30 (1H, d, $J = 9.6$ Hz), 6.89–6.96 (2H, m), 7.12 (2H, d, $J = 8.7$ Hz), 7.25–7.30 (1H, m), 7.50 (2H, d, $J = 8.2$ Hz), 7.62 (1H, d, $J = 8.4$ Hz), 7.70–7.88 (5H, m), 7.98 (1H, d, $J = 9.6$ Hz), 8.31 (1H, s), 8.45 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.8, 47.2, 56.6, 61.8, 63.7, 66.0, 101.8, 113.1, 113.2, 113.3, 115.5, 125.5, 126.4, 128.7, 129.7, 130.2, 130.3, 143.0, 143.2, 144.6, 145.0, 156.0, 160.8, 160.9, 162.1, 162.4; HRMS (ESI): $C_{29}H_{27}N_5O_6S$ $[M+Na]^+$, calculated for 596.188, found 596.1550.

4-(((2-methoxy-6-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzylidene)amino)methyl) benzenesulfonamide (**8d**): Yellow powder, 85% yield; mp. 64–65 °C; IR: 3320, 3079, 2941, 1728, 1610, 1476, 1267, 1156, 1124, 1072, 833 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 2.19–2.23 (2H, m), 3.85 (3H, m), 3.96 (2H, t, $J = 5.8$ Hz), 4.47 (2H, t, $J = 6.7$ Hz), 4.69 (2H, s), 5.12 (2H, s), 6.25 (1H, d, $J = 9.3$ Hz), 6.84–6.90 (2H, m), 7.05 (1H, t, $J = 7.9$ Hz), 7.15 (1H, dd, $J = 8.2$, 1.4 Hz), 7.36–7.44 (5H, m), 7.57 (1H, d, $J = 8.4$ Hz), 7.77 (2H, d, $J = 8.2$ Hz), 7.95 (1H, d, $J = 9.6$ Hz), 8.18 (1H, s), 8.53 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.9, 47.0, 56.5, 56.6, 65.8, 66.3, 101.8, 113.1, 113.2, 113.3, 115.6, 118.5, 125.0, 125.6, 126.4, 128.7, 130.1, 130.2, 143.2, 144.3, 147.4, 153.4, 155.9, 158.9, 160.9, 162.0; HRMS (ESI): $C_{30}H_{29}N_5O_7S$ $[M+H]^+$, calculated for 604.179, found 604.1839.

4-(((4-(diethylamino)-2-((1-(3-((2-oxo-2H-chromen-7-yl)oxy) propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzylidene)amino) methyl)benzenesulfonamide (**8e**): Brown powder, 65% yield; mp. 76–77 °C; IR: 3264, 3060, 2969, 1722, 1597, 1509, 1332, 1271, 1155, 1120, 1016, 833 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 1.00–1.10 (6H, m), 2.27–2.31 (2H, m), 3.37–3.42 (4H, m), 4.06 (2H, t, $J = 5.8$ Hz), 4.55 (2H, t, $J = 6.7$ Hz), 4.66 (2H, s), 5.23 (2H, s), 6.24–6.30 (2H, m), 6.39 (1H, s), 6.86 (1H, dd, $J = 8.7$, 2.3 Hz), 6.91 (1H, d, $J = 2.6$ Hz), 7.32 (2H, s), 7.41 (2H, d, $J = 8.2$ Hz), 7.56 (1H, d, $J = 8.7$ Hz), 7.68 (1H, d, $J = 9.0$ Hz), 7.75 (2H, d, $J = 8.2$ Hz), 7.94 (1H, d, $J = 9.3$ Hz), 8.30 (1H, s), 8.55 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm:13.1, 19.2, 29.8, 44.5, 47.2, 56.6, 62.4, 66.0, 101.8, 113.1, 113.2, 113.3, 118.5, 125.2, 125.6, 126.3, 128.6, 130.1, 130.2, 143.0, 143.6, 145.0, 156.0, 159.9, 160.9, 162.1; HRMS (ESI): $C_{33}H_{36}N_6O_6S$ $[M+H]^+$, calculated for 645.242, found 645.2464.

4-(((5-bromo-2-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzylidene)amino)methyl)benzenesulfonamide (**8f**): Yellow powder, 79% yield; mp. 70–71 °C; IR: 3249, 3080, 2942, 1723, 1609, 1555, 1328, 1229, 1156, 1124, 833 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 2.30–2.32 (2H, m), 4.08 (2H, t, $J = 5.8$ Hz), 4.58 (2H, t, $J = 6.7$ Hz), 4.82 (2H, s), 5.30 (2H, s), 6.28 (1H, d, $J = 9.3$ Hz), 6.88–7.02 (2H, m), 7.32–7.35 (3H, m), 7.48 (2H, d, $J = 8.2$ Hz), 7.59–7.65 (2H, m), 7.80 (2H, d, $J = 7.6$ Hz), 7.94–8.01 (2H, m), 8.36 (1H, s), 8.70 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.8, 47.3, 56.6, 64.0, 66.0, 101.8, 113.1, 113.3, 113.6, 116.8, 125.6, 126.4, 126.7, 128.7, 129.5, 130.1, 135.2, 142.8, 143.2, 144.2, 145.0, 155.9, 156.9, 157.1, 160.9, 162.1; HRMS (ESI): $C_{29}H_{26}BrN_5O_6S$ $[M+Na]^+$, calculated for 674.068, found 676.0631.

4-(((5-nitro-2-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzylidene)amino)methyl)benzenesulfonamide (**8g**): Orange powder, 56% yield; mp. 68–69 °C; IR: 3310, 3080, 2950, 1720, 1609, 1510, 1335, 1265, 1156, 1125, 990, 833 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 2.32–2.36 (2H, m), 4.10 (2H, t, $J = 5.8$ Hz), 4.60 (2H, t, $J = 6.7$ Hz), 4.87 (2H, s), 5.48 (2H, s), 6.26–6.31 (1H, m), 6.85–6.97 (3H, m), 7.40–7.66 (4H, m), 7.78–7.83 (2H, m), 7.90–8.04 (3H, m), 8.32–8.44 (1H, m), 8.64 (1H, s), 8.77 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.8, 47.3, 56.6, 64.0, 66.0, 101.8, 113.1, 113.3, 113.6, 116.8, 125.6, 126.4, 126.7, 128.7, 129.5, 130.1, 135.2, 142.8, 143.2, 144.2, 145.0, 155.9, 156.9, 157.1, 160.9, 162.1; HRMS (ESI): $C_{29}H_{26}N_6O_8S$ $[M+Na]^+$, calculated for 641.143, found 641.1398.

4-(((3-methoxy-4-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)benzylidene)amino)methyl) benzenesulfonamide (**8h**): Yellow powder, 74% yield; mp. 86–87 °C; IR: 3304, 3040, 2948, 1724, 1613, 1508, 1268, 1155, 1093, 814 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 2.25–2.32 (2H, m), 3.73 (3H, s), 4.07 (2H, t, $J = 5.8$ Hz), 4.55 (2H, t, $J = 7.0$ Hz), 4.77 (2H, s), 5.16 (2H, s), 6.26 (1H, d, $J = 9.3$ Hz), 6.87–6.93 (2H, m), 7.19–7.27 (2H, m), 7.35–7.39 (3H, m), 7.48 (2H, d, $J = 7.9$ Hz), 7.59 (1H, d, $J = 8.4$ Hz), 7.78 (2H, d, $J = 7.9$ Hz), 7.96 (1H, d, $J = 9.3$ Hz), 8.30 (1H, s), 8.40 (1H, s); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.9, 47.2, 55.9, 56.6, 63.8, 66.0, 101.8, 109.8, 113.1, 113.2, 113.3, 123.3, 125.7, 126.4, 128.9, 129.9, 130.2, 143.0, 143.2, 144.5, 145.0, 149.7, 150.5, 156.0, 161.0, 162.1, 162.6; HRMS (ESI): $C_{30}H_{29}N_5O_7S$ $[M+Na]^+$, calculated for 626.169, found 626.1654.

4-((((2-((1-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)naphthalen-1-yl)methylene)amino)methyl) benzenesulfonamide (**8i**): Orange powder, 75% yield; mp. 93–94 °C; IR: 3236, 3070, 2944, 1728, 1609, 1508, 1328, 1230, 1155, 1124, 932 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) $\delta$/ppm: 2.29–2.33 (2H, m), 4.06 (2H, t, $J = 5.8$ Hz), 4.57 (2H, t, $J = 6.7$ Hz), 4.90 (2H, s), 5.45 (2H, s), 6.27 (1H, d, $J = 9.3$ Hz), 6.86–6.94 (3H, m), 7.20–7.59 (7H, m), 7.70 (1H, d, $J = 9.3$ Hz), 7.81–7.98 (3H, m), 8.06 (1H, d, $J = 9.0$ Hz), 8.36 (1H, s), 9.13 (1H, s), 9.26 (1H, d, $J = 8.4$ Hz); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) $\delta$/ppm: 29.8, 47.2, 55.9, 62.8, 66.0, 101.8, 113.1, 113.2, 113.3, 116.8, 117.5, 123.3, 123.5, 124.3, 125.7, 126.5, 128.6, 129.6, 130.1, 132.6, 133.6, 143.2, 144.6, 145.0, 155.9, 158.0, 160.9, 162.1; HRMS (ESI): $C_{33}H_{29}N_5O_6S$ $[M+Na]^+$, calculated for 646.174, found 646.1702.

### 4.3. CA inhibition assays

In order to determine the carbonic anhydrase inhibition of the compounds, the method mentioned in the previous studies was used and inhibition results were obtained [20–23,27,31].

### 4.4. Molecular modelling studies

#### 4.4.1. Preparation files for docking studies

All necessary hCA crystal structures were obtained from the Brookhaven Protein Data Bank, i.e. hCA I in complex with topiramate (pdb: 3lxe, 1.9 Å), hCA II in complex with 2,5-dihydroxybenzoic acid and a zinc-bound water molecule (pdb: 4e3d, 1.6 Å), hCA IX (pdb: 3iai, 2.2 Å) and hCA XII (pdb: 1jd0, 1.5 Å) both in complex with acetazolamide. The zinc-bound water molecule of the hCA II structure and all ligands (acetazolamide, topiramate and 2,5-dihydroxybenzoic acid) were retained and all other non-protein atoms were deleted from the crystal structures. Only chain A was retained if more than one protein structure was present. Hydrogen atoms were added with the "protonate 3D" [28] tool and subsequently a steepest-descent energy minimization was performed using the AMBER14:EHT force field (MOE software package, version 2018.0101, chemical computing group, inc, Montreal, Canada). The four protein structures were superposed on the backbone atoms of hCA I (Cα atoms, RMSD: 1.395 Å, for 236 residues). The coordinates of the hCA II zinc-bound water molecule were copied into the other hCA structures.

The molecular structures of the ligands were prepared with the MOE software package. If present, the sulfonamide group was either constructed in the neutral ($R-SO_2NH_2$) or negatively charged form ($R-SO_2-NH^-$) and the coumarin moiety was constructed in the open and closed forms. Subsequently, the ligand structures were energy minimized (MMFF94x force field) and the ligands were saved as multi-mol2 files.

#### 4.4.2. Docking studies

Docking calculations were performed with the GOLD software package (v5.6.2, CCDC, Cambridge, UK) using the ChemScore scoring function (25 dockings per ligand) and default settings. The binding pocket was defined as within 14 Å around a centroid (x: −18.899; Y: 36.167; z: 45.640). Dockings into the active site was performed either with or without a zinc-bound water molecule (obtained from hCA II structure 4e3d).

### 4.5. In vitro cytotoxicity assay

The cytotoxicity effect of the test compounds on HT-29 human colorectal adenocarcinoma cell line and HEK293T healthy embryonic kidney cell line (kind gift from Yesim Kesim, Department of Genetics, I.U. Aziz Sancar Institute of Experimental Medicine) were evaluated by MTT (3-(4,5 dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay according to described methods [32].

### 4.6. Western blotting assay

In order to examine the effects of test substances on protein levels of CA IX and CA XII, human HT-29 cells were treated with two of the highly cytotoxic compounds. Cells were seeded in 6-well plate in DMEM/F12 (normoxic cells) or DMEM/F12 containing 200 μM $CoCl_2$ (hypoxic and treated cells) in order to create hypoxia [29]. Cells were incubated for 24 h with compounds at three doses (25, 6.25, 1.56 μM). After the treatment period, cells washed with cold PBS and were homogenized into Ripa cell lysis buffer (Santa Cruz, USA). Lysates were centrifuged at 14,000 rpm for 20 min at 4 °C and the protein concentration of supernatants was measured with Qubit 2.0 Fluorometer. Laemmli sample preparation buffer (Bio-Rad, USA) was added and the protein samples were heated at 95 °C for 5 min. Samples electrophoresed in SDS-polyacrylamide gels and the gels were transferred onto PVDF membranes (Millipore, Germany). The membranes were blocked in 5% fat-free milk (w/v) in Tris-buffered saline with 0.1% Tween 20 (TBST) buffer for 2 h at RT, incubated with the corresponding antibody at 4 °C overnight, then incubated with the horseradish peroxidase (HRP)-labelled secondary antibody for 1 h at RT. The following antibodies were used: anti-CA IX (ab107257, Abcam), anti-CA XII (sc-374314, Santa Cruz), and anti-GAPDH (kindly provided by Dr. Beyza Goncu from Experimental Research Centre Bezmialem Vakif University). Finally, the membranes were stained with ECL reagents (LumiGLO, CST, USA) then imaging was performed with Bio-Rad Chemidoc Imaging System. The bands were calculated with the ImageJ program and analyzed with Graphpad Prism 8.00 (San Diego, CA, USA) [30].

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2019.111702.

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