Chromene-Containing Aromatic Sulfonamides with Carbonic Anhydrase Inhibitory Properties

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Abstract: Carbonic anhydrases (CAs, EC 4.2.1.1) catalyze the essential reaction of CO2 hydration in all living organisms, being actively involved in the regulation of a plethora of patho/physiological conditions. A series of chromene-based sulfonamides were synthesized and tested as possible CA inhibitors. Their inhibitory activity was assessed against the cytosolic human isoforms hCA I, hCA II and the transmembrane hCA IX and XII. Several of the investigated derivatives showed interesting inhibition activity towards the tumor associate isoforms hCA IX and hCA XII. Furthermore, computational procedures were used to investigate the binding mode of this class of compounds, within the active site of hCA IX.

Keywords: carbonic anhydrase; inhibitors; metalloenzymes; Chromene

1. Introduction

The carbonic anhydrases (CA’s) are metalloenzymes that catalyze the formation of bicarbonate from carbon dioxide in two steps [1–4]. Sixteen isoforms have been known up to now in humans, five of which CAs are cytosolic (CA I–III, VII, and XIII), two are mitochondrial (CA VA and VB), one is secreted (CA VI), and the others are membrane-bound (CA IV, IX, XII, XIV and XV) [5]. Many CA subtypes are interesting targets for the design of pharmacological agents useful, for example, as antiglaucoma, anticonvulsant, antiurolithic, antidiabetic [28], anti-inflammatory [15,29], and antitubercular [30] activities. Taking into
account all of the above-mentioned, here we report the synthesis of (4-sulfamoylphenyl)-
4H-chromene-2-carboxamide derivatives for its incorporation into one scaffold chromene
and sulfonamide moieties. The experimental studies were developed together with in silico
techniques, aimed to propose a reliable binding disposition for this class of CA Is.

2. Results

2.1. Chemistry

All the target compounds have been synthesized using the well-known strategy depicted
in Scheme 1. It implies the synthesis of diverse 4-oxo-4H-chromene-2-carboxylic acids as
well as amides mini array, which contains a streptocid moiety. The synthesis was started
with commercially available 2'-hydroxyacetophenones 1a–j, applying Fernando Cagide's
optimized synthetic route [31]. The desired carboxylic acids (2a–j) were synthesized using
microwave irradiation in the presence of diethyl oxalate and sodium methoxide with very
high yields (93–97%). To reach our final amides, we used two different methodologies that
have a common characteristic—the formation of acyl imidazoliums. These highly reactive acyl
transfer intermediates have been recognized as important species for amide bond formation.
As a part of the ongoing studies that are focusing on the synthesis of big libraries for biological screening, we discovered that the combination of N,N,N′,N′-tetramethylchloroformamidinium hexafluoro-phosphate (TCFH) and N-methylimidazole (NMI) is a mild method for in situ generation of highly reactive acyl imidazoliums, allowing for the formation of the amides in high yields. This approach is especially efficient for electron-deficient amines. Because of the low nucleophilicity of 4-aminobenzene-sulfonamide, common coupling agents such as CDI, HATU, EDC, and EDCI with TEA or DIPEA, as a base in DMF, are not suitable for amide synthesis. Using the TCFH-NMI method, we have managed to access anilides in very good yields. In order to expand our screening library and to investigate an impact of aliphatic linker on bioactivity, we synthesized a set of amides based on 4-(aminomethyl)benzenesulfonamide and 4-(2-aminoethyl)benzenesulfonamide. In this case, we can use the simple amide coupling agent CDI, considering the high nucleophilic properties of both amines. In this work, we synthesized two new 4-oxo-4H-chromene-2-carboxylic acids using modern microwave methodology, in which a set of amides have been subsequently produced.

2.2. Evaluation of CA Inhibitory Activity

All compounds were evaluated for their inhibitory activity against human CA isoforms, hCA I, hCA II, hCA IX, and hCA XII, and results are presented in Table 1. The result of biological evaluation revealed that all compounds showed activity against all isoforms, here tested, with different ranges of inhibition constants. The Kᵢ values of compounds against hCA I ranged from 213.7 to 5314 nM. The best activity was achieved for compounds 6i (Kᵢ = 213.6 nM) and 6e (Kᵢ = 246.7 nM), higher than that of acetazolamide (AAZ) used as reference drug. On the other hand, the lowest activity was observed for compound 5b with Kᵢ = 5314 nM followed by compound 6j (Kᵢ = 4412 nM). The structure activity relationships (SAR) study revealed that, in general, derivatives of 4-oxo-N-(4-sulfamoylphenethyl)chroman-2-carboxamide are more potent hCA I inhibitors than 4-oxo-N-(4-sulfamoylphenyl)chroman-2-carboxamide and derivatives 4-oxo-N-(4-sulfamoylbenzyl)chroman-2-carboxamide. The presence of methyl groups in positions 7 and 8 of 4H-chromen-4-one moiety of sulfamoylphenethyl derivatives is beneficial for hCA inhibitory activity, while the presence of 7-Me group at 4H-chromen-4-one moiety in case of sulfamoylphenyl derivatives was detrimental. As far as cytosolic hCA II isoform is concerned, the compounds showed, in general, better activity than against hCA I. Two compounds, 5f and 6f, exhibited excellent activity on isoform hCA II, demonstrating Kᵢ values of 9.3 and 7.5 nM, higher than the reference drug AAZ (Kᵢ = 12.1 nM). Compound 6d with Kᵢ value of 16.6 nM was comparable with the reference drug. The SAR revealed that the presence of a methyl group in position 7 of 4H-chromene-2-carboxamide and benzenesulfonamide (6f), is beneficial for CA II inhibitory activity. Removal of a methyl group from position 7 of compound 6f led to compound 6d being twice less active than 6f. On the other hand, the addition of one more methyl group in position 8 of the previous compound and removal of CH₂CH₂ group resulted in compound 5f, with decreased activity compared to 6f, but still more active than AAZ.

Removal of both methyl groups from compound 5f led to compound 5a (Kᵢ = 35.1 nM), which is almost four times less active than compound 5f, while removal of Me group from position 8 of compound 5f, decreased more the activity (Kᵢ = 39.5 nM). The presence of 6-Cl, 7-Me substitution on 4H-chromen-4-one moiety of sulfamoylphenyl derivatives was found to be very negative for hCA II inhibitory activity. In the case of hCA IX, compounds showed activity with Kᵢ ranging from 16.6 to 3285 nM. Three compounds, 4-oxo-N-(4-sulfamoylphenyl)chroman-2-carboxamide 5a (Kᵢ = 16.6 nM); 7,8-dimethyl-4-oxo-N-(4-sulfamoylphenyl)chroman-2-carboxamide 5f (Kᵢ = 19.5 nM); and 5,8-dimethyl-4-oxo-N-(4-sulfamoylphenyl)chroman-2-carboxamide 5h (Kᵢ = 22.5 nM) displayed excellent activity superior compared to AAZ (Kᵢ = 25.7 nM).
### Table 1. Inhibition data of human CA isoforms hCA I, II, IX, and XII with compounds 1–14 and the standard sulfonamide inhibitor acetazolamide (AAZ).

|        | Kᵢ (nM) * |        |        |        | Kᵢ (nM) * |        |        |        |        |        |
|--------|-----------|--------|--------|--------|-----------|--------|--------|--------|--------|--------|
| N      | hCA I     | hCA II | hCA IX | hCA XII| N         | hCA I  | hCA II | hCA IX | hCA XII|
| 5a     | 525.2 ± 44.4 | 35.1 ± 3.3 | 16.6 ± 1.5 | 20.1 ± 1.7 | 6b        | 612.3 ± 49.5 | 81.7 ± 4.2 | 2039.0 ± 189.0 | 320.4 ± 23.2 |
| 5b     | 5314 ± 272 | 95.9 ± 8.0 | 77 ± 6.5 | 64.8 ± 6.1 | 6c        | 2646 ± 227 | 678.8 ± 45.0 | 3285 ± 302 | 59.4 ± 5.0 |
| 5c     | 3563 ± 228 | 39.5 ± 3.2 | 63.4 ± 6.3 | 50.8 ± 4.3 | 6d        | 321.9 ± 27.9 | 16.6 ± 1.2 | 81.0 ± 7.7 | 211.6 ± 13.7 |
| 5e     | 2891 ± 161 | 311.4 ± 28.3 | 298.6 ± 18.4 | 619.1 ± 36.7 | 6e        | 246.7 ± 24.3 | 36.4 ± 2.1 | 72.7 ± 4.3 | 439.3 ± 32.2 |
| 5f     | 341.1 ± 29.6 | 9.3 ± 0.5 | 19.5 ± 1.6 | 76.3 ± 6.0 | 6f        | 333.5 ± 17.5 | 7.5 ± 0.5 | 64.8 ± 3.4 | 337.3 ± 27.4 |
| 5g     | 681.5 ± 40.6 | 61.4 ± 4.4 | 54.7 ± 5.0 | 46.4 ± 4.4 | 6g        | 971.0 ± 82.4 | 279.9 ± 15.5 | 182.6 ± 16.1 | 147.9 ± 8.1 |
| 5h     | 504.8 ± 27.0 | 46.6 ± 2.9 | 22.9 ± 1.9 | 26.8 ± 1.4 | 6h        | 547.3 ± 39.2 | 51.2 ± 3.6 | 51.4 ± 4.5 | 22.6 ± 1.5 |
| 5i     | 2698 ± 265 | 66.2 ± 5.8 | 51.0 ± 4.8 | 424.7 ± 41.0 | 6i        | 213.6 ± 15.9 | 39.2 ± 3.7 | 84.5 ± 4.3 | 132.2 ± 11.0 |
| 5j     | 580.3 ± 50.5 | 900.7 ± 75.4 | 515.5 ± 37.2 | 155.3 ± 10.1 | 6j        | 4412 ± 347 | 143.0 ± 12.8 | 91.1 ± 8.9 | 31.4 ± 2.6 |
| 6a     | 627.7 ± 35.5 | 219.3 ± 11.8 | 855.2 ± 49.2 | 34.0 ± 2.2 | 6k        | 926.9 ± 68.9 | 91.6 ± 7.0 | 1917 ± 146 | 414 ± 3.4 |
| AAZ    | 250 ± 13.0 | 12.1 ± 0.6 | 25.7 ± 2.1 | 5.7 ± 0.4 | 6f        | 250 ± 13.0 | 12.1 ± 0.6 | 25.7 ± 2.1 | 5.7 ± 0.4 |

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

The structure–activity relationships revealed that unsubstituted 4-oxo-N-(4-sulfamoylphenyl)chroman-2-carboxamide (5a) was beneficial for hCA IX inhibition. The introduction of two methyl group at position 7 and 8 of the previous compound revealed a decrease in activity, leading to compound 5f still being more active than AAZ. Shifting the 7-Me group to position 6 (5h) decreased the activity even more, but the compound remained still more active than AAZ. Introduction to unsubstituted compound (5a) chlorine at position 6 resulted in three times lesser active compound (5i). The presence of 6-Cl substituent at 4H-chromen-4-one moiety and methyl group between 4-oxo-4H-chromene-2-carboxamide and benzensulfonamide (6e) appeared to be detrimental for hCA IX inhibitory activity. For hCA XII, the inhibition constant ranged from 20.1 to 619.1 nM. The best activity against this isoform was exhibited by compounds 5a, 6h, and 5h with Kᵢ values of 20.1, 22.6, and 26.8 nM, respectively, compared to AAZ (Kᵢ = 5.7 nM). None of compounds did not exert the activity of AAZ against hCA XII. Nevertheless, the unsubstituted 4-oxo-N-(4-sulfamoylphenyl)chroman-2-carboxamide was positive for hCA XII inhibitory activity, while the substitution by a 7-Me group of 4H-chromen-4-one moiety of -sulfamoylphenethyl derivatives is unfavorable for this kind of activity. Finally, it should be mentioned that compound 5a was the most active one hCA IX with a selectivity index (SI) of 31.67 compared to hCA I, while compound 6f was the most active hCA II with SI 44.47 compared to hCA I and 44.97 to hCA XII.

#### 2.3. Molecular Modeling Studies

In order to explain the inhibition mechanism of the tested compounds, molecular docking studies were performed. For the in-silico experiments, ligands 5a, 5h, 6h, 6f, 6d, and 6i were selected as representatives of the whole set. Docking studies showed that all tested compounds bind in a deprotonated form, as anions (negative nitrogen of the sulfonamide group), chelating the Zn(II) ion of the active site of the enzymes [32]. It is known that all the active isoforms of human CAs have a similar active site architecture, consisting of three conserved His residues (His94, His96 and His119) acting as zinc ligands and another two conserved residues Thr199 and Glu105, acting as “gate keepers” [33–36]. Nevertheless, these isoforms differ in the amino acids mostly in the middle and to the exit of the active site cavity.

According to docking studies, these differences in the active site of the enzymes are the reason for the selectivity of the compounds against each isoform. The N-C=O linker allow compounds to adopt several conformations and interactions within the enzyme active site. These conformations, depending on the nature of the amino acids of the active
site cavity, can affect the inhibition profile of the ligands. For instance, compound 5a, which has a $K_i$ for hCA II of 35.1 nM and a lower $K_i$ for hCA IX of 16.6 nM, adopts a much different conformation upon binding both hCAs. This is probably due to bulky hydrophobic residue Phe131 in hCA II enzyme, unlike that of the smaller residue Val131 in hCA IX, which allows ligands to freely enter the active site in a conformation that favors interactions with residues in the hydrophobic pocket and increases selectivity (Figure 1). The superposition of the two structures of hCAs bound to compound 5a, revealed that this residue (Phe131) could indeed cause steric hindrance (Figure 1C). Therefore, 5a adopts a different conformation within the active site of hCA II enzyme with less interactions and therefore less stability of the complex and probably explains the experimental lower $K_i$ for hCA II. It is north worthy to mention that the sulfonamide is involved in H-bond formation with the backbone of Thr199 of both isoforms and in an additional H-bond formation with the backbone of His94, which further stabilizes the complex ligand hCA IX and contributes to the high inhibition potency of the compound (Figure 1A,B and Table 2).

On the other hand, compounds 6h, 6f, 6d, and 6i differ from compounds 7 and 11 mainly by the presence of an ethylene linker between the benzimidazole scaffold and the side phenyl ring. This ethyl linker provides flexibility to the compounds and maximizes the favorable interactions in hCA IX and hCA XII, increasing the inhibition potency (compound 6h) but, on the other hand, it also allows a better fit into the hCA I and hCA II isoform structures, affecting the ligand selectivity profile (compounds 6f, 6d, and 6i). In the case of compound 6f, the flexible ethyl linker leads the compound to adopt a fold conformation inside the larger active site cavity of hCA IX, unlike to the extended conformation of the hCA II isoform structure (Figure 2C). In both structures, negative nitrogen of the sulphonamide group chelates the Zn(II) ion and the interaction forms a H-bond with the side chain of conserved residue Thr199, but the extended conformation of the compound into the hCA II isoform structure allows the formation of hydrophobic interactions between the benzene ring of the compound and the residues Phe131 and Ile91 (Figure 2A,B and Table 2). These interactions further stabilize the complex ligand-enzyme and positively impacts the selectivity profile of the compound ($K_i$ for hCA II of 7.5 nM and a lower $K_i$ for hCA IX of 64.8 nM).

![Figure 1](image-url)

**Figure 1.** (A) 2D interaction diagram of compound 7 docking pose interactions with the key amino acids in hCA II, (B) in hCA IX, and (C) Superposition of compound 7 bound to hCA IX (grey) in comparison to hCA II (blue), with specific residues labeled. Active site zinc is shown as a blue sphere, red dotted and green arrows indicate H-bond, and yellow spheres indicate hydrophobic interactions. Brown double-headed arrow indicates the direction of conformational change of the compound bound to hCA IX- in comparison to hCA II.
Table 2. Molecular docking free binding energies (kcal/mol) and interactions of tested compounds on hCA I, II, IX, and XII isoforms.

| No. | hCA Isoform | Estimated Free Binding Energy (Kcal/mol) | Chelating the Zn(II) Ion | Residues Involved in H-Bond Interactions | Residues Involved in Hydrophobic Interactions |
|-----|-------------|----------------------------------------|--------------------------|------------------------------------------|---------------------------------------------|
| 5a  | hCA I       | −6.49                                   | Yes                      | Thr199                                   | Ala121, Leu198                             |
|     | hCA II      | −7.42                                   | Yes                      | Thr199                                   | Ile91, Val121, Leu198, Thr200              |
|     | hCA IX      | −9.25                                   | Yes                      | His94, Thr199                            | Val121, Val195, Leu198                     |
|     | hCA XII     | −9.61                                   | Yes                      | His94, His96, Thr199, Thr200             | Val121, Leu198                             |
| 5h  | hCA I       | −5.17                                   | No                       | -                                        | Ala121, Ala135                             |
|     | hCA II      | −7.11                                   | Yes                      | Thr199                                   | Ile91, Leu198, Thr200                      |
|     | hCA IX      | −8.74                                   | Yes                      | His94, Thr199                            | Val121, Leu198                             |
|     | hCA XII     | −9.02                                   | Yes                      | His94, Thr200                            | Val121, Leu198                             |
| 6d  | hCA I       | −6.77                                   | Yes                      | Thr199                                   | Ala121, Ala135, Leu18                  |
|     | hCA II      | −9.14                                   | Yes                      | His94, Thr199                            | Ile91, Val121, Phe131, Thr200              |
|     | hCA IX      | −6.44                                   | Yes                      | Thr199                                   | Val121, Leu198, Thr200                     |
|     | hCA XII     | −4.81                                   | No                       | -                                        | Val121                                    |
| 6f  | hCA I       | −6.62                                   | Yes                      | Thr199                                   | Ala135, Leu198                            |
|     | hCA II      | −9.34                                   | Yes                      | His94, Thr199                            | Ile91, Val121, Phe131, Leu198, Thr200      |
|     | hCA IX      | −6.81                                   | Yes                      | Thr199                                   | Val121, Leu198, Thr200                     |
|     | hCA XII     | −4.72                                   | No                       | -                                        | Val121                                    |
| 6h  | hCA I       | −5.29                                   | Yes                      | -                                        | Ala135, Leu198                            |
|     | hCA II      | −7.87                                   | Yes                      | His94                                    | Val121, Phe131, Leu198                     |
|     | hCA IX      | −6.53                                   | Yes                      | Thr199                                   | Val121, Thr200                            |
|     | hCA XII     | −9.52                                   | Yes                      | Ala131, Ser132, Thr200                   | Trp5, Val121, Leu198                       |
| 6i  | hCA I       | −8.15                                   | Yes                      | Gln92, Thr199                            | Leu131, Ala135, Ala132, Thr202, Leu198      |
|     | hCA II      | −7.14                                   | Yes                      | Thr200                                   | Val121, Phe131, Thr200                     |
|     | hCA IX      | −6.27                                   | Yes                      | Thr199                                   | Val121, Thr200                            |
|     | hCA XII     | −4.35                                   | No                       | -                                        | Val121, Leu198                            |
| AAZ | hCA I       | Yes                                     | Gln92                    | Leu198, Thr199, His200, Pro201, Trp209    |
|     | hCA II      | Yes                                     | Thr199, Thr200           | Val121, Phe131, Leu198, Thr209           |
|     | hCA IX      | Yes                                     | Thr199, Thr200           | Val121, Val143, Val131, Leu198, Thr209   |
|     | hCA XII     | Yes                                     | Thr199, Thr200           | Val121, Val143, Leu198, Thr209           |

* Means that there is no residues involved in H–bond formation.
Figure 2. (A) 2D interaction diagram of compound 17 docking pose interactions with the key amino acids in hCA II, (B) in hCA IX, and (C) superposition of compound 17 bound to hCA IX (grey) in comparison to hCA II (blue), with specific residues labeled. Active site zinc is shown as a blue sphere, red dotted and green arrows indicate H-bond, and yellow spheres indicate hydrophobic interactions. Brown circle.

3. Materials and Methods

3.1. Chemistry

Reagents, chemicals, starting materials (1a–j, 3, 4a,b), and solvents were obtained from commercial sources and used without further purification. Melting points were determined on an Microquimica MQAPF-302 apparatus (Palhoça, Brazil). NMR spectra were recorded on a Avance III HD Bruker spectrometer (Rheinstetten, Germany). with chemical shifts values (δ) in ppm relative to TMS using the residual DMSO-d6 signal as an internal standard. High-resolution mass spectra (HRMS) were recorded on an LTQ Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). This system combines an LTQ XL linear ion-trap mass spectrometer and an Orbitrap mass analyzer. The analyses were performed by direct infusion of the sample in DMSO (flow rate of 10 mL/min) in a positive-ion mode, using electrospray ionization (ESI). For the elemental composition, the calculations used the specific tool included in the Qual Browser module of Xcalibur (Thermo Fisher Scientific, release 2.0.7) software. Microwave-assisted synthesis was performed in a Biotage®Initiator Microwave Synthesizer (Biotage, Uppsala, Sweden). The progress of reactions was monitored by thin-layer chromatography (TLC) using precoated TLC sheets with Ultraviolet (UV) fluorescent silica gel (Merck, 60F254, Germany). HPLC (Fuji Silysia Chemical Ltd., Aichi-ken, Japan) purification method: mobile phase, system 20–70% 0–5 min H2O/MeOH, flow: 30 mL/min (loading pump 4 mL/min methanol); column: Chromatorex 18 SMB100-5T 100 × 19 mm 5 µm.

3.1.1. General Procedure for Synthesis of Compounds (2a–j)

The appropriate 2′-hydroxyacetophenone (1a–j) (1.16 mmol, 1 eq.) was dissolved in dioxane (2 mL) in a MW vial. Then diethyl oxalate (3.49 mmol, 474 µL, 3 eq.) and a solution of MeONa in MeOH (2.32 mmol, 531 µL, 25% w/w, 2 eq.) were added. The resulting solution was heated to 120 °C for 20 min. Then, a solution of HCl (18 mmol, 3 mL, 6 M) was added and the reaction was heated to 120 °C for 40 min. The reaction mixture was poured into water (50 mL) and the solid formed was filtered and washed with water. The solid was then dried, washed with dichloromethane, and dried again. Carboxylic acids 2a–e, h–j had been synthesized previously by means of different methods and their QC data did not differ from ones we obtained [34–37].
3.1.2. 7,8-Dimethyl-4-oxo-4H-chromene-2-carboxylic Acid (2f)

93% yield. MP = 259–261 °C. 1H NMR (400 MHz, DMSO-d6) δ: 14.20 (bs, 1H, COOH), 7.81 (d, J = 7.7 Hz, 1H, Ar), 7.42 (d, J = 7.6 Hz, 1H, Ar), 6.85 (s, 1H, Ar), 2.47 (s, 3H, CH3), 2.41 (s, 3H, CH3). MS (ESI): m/z (%) = 219.1 (13.2) [M + H]+. Anal. Calcd. for C12H10O4 (%): C, 66.05; H, 4.62. Found (%): C, 66.18; H, 4.59.

3.1.3. 5,7-Dimethyl-4-oxo-4H-chromene-2-carboxylic Acid (2g)

97% yield. MP = 264–266 °C. 1H NMR (400 MHz, DMSO-d6) δ: 14.25 (bs, 1H, COOH), 7.61 (s, 1H, Ar), 7.38 (s, 1H, Ar), 6.87 (s, 1H, Ar), 2.55 (s, 3H, CH3), 2.39 (s, 3H, CH3). MS (ESI): m/z (%) = 219.2 (11.4) [M + H]+. Anal. Calcd. for C12H10O4 (%): C, 66.05; H, 4.62. Found (%): C, 66.09; H, 4.65.

3.1.4. General Procedure for Synthesis of Compounds 5a–j

To the 4-oxo-4H-chromene-2-carboxylic acid (2a–j) (1.52 mmol, 1 eq.), 4-aminobenzene-sulfonamide 3 (0.314 g, 1.82 mmol, 1.2 eq.) and N-methylimidazole (0.42 mL, 5.33 mmol, 3.5 eq.) combined and dissolved in MeCN (4 mL) TCFH (0.517 g, 1.83 mmol, 1.2 eq.) was added in a single portion. The reaction was stirred until complete by LCMS (21 h). The reaction mixture was then diluted with ethyl acetate (6 mL) and water (4 mL). The layers were separated, the aqueous layer was extracted with ethyl acetate (4 mL), and the combined organics were washed with water (4 mL), dried with MgSO4, filtered, and concentrated before purification by HPLC to afford final amides with excellent yields.

3.1.5. 4-Oxo-N-(4-sulfamoylphenyl)-4H-chromene-2-carboxamide (5a)

93% yield. MP = 296–298 °C. 1H NMR (400 MHz, DMSO-d6) δ: 10.92 (s, 1H, NH), 8.08 (dd, J = 7.8, 1.5 Hz, 1H, Ar), 7.98 (d, J = 8.7 Hz, 2H, Ar), 7.93–7.81 (m, 4H, Ar), 7.54 (t, J = 7.8 Hz, 1H, Ar), 7.23 (s, 2H, SO2NH2), 6.99 (s, 1H, Ar). MS (ESI): m/z (%) = 345.1 (17.6) [M + H]+. Anal. Calcd. for C16H12N2O3S (%): C, 55.81; H, 3.51; N, 8.14; S, 9.31. Found (%): C, 55.87; H, 3.49; N, 8.18; S, 9.28.

3.1.6. 6-Methyl-4-oxo-N-(4-sulfamoylphenyl)-4H-chromene-2-carboxamide (5b)

89% yield. MP = 307–308 °C. 1H NMR (400 MHz, DMSO-d6) δ: 10.97 (s, 1H, NH), 8.02–7.86 (m, 5H, Ar), 7.74 (s, 2H, Ar), 7.34 (d, J = 8.4 Hz, 1H, Ar), 7.25 (s, 2H, SO2NH2), 6.97 (s, 1H, Ar), 2.45 (s, 3H, CH3). MS (ESI): m/z (%) = 359.1 (20.3) [M + H]+. Anal. Calcd. for C17H14N2O3S (%): C, 56.98; H, 3.94; N, 7.82; S, 8.95. Found (%): C, 57.09; H, 3.99; N, 7.76; S, 9.08.

3.1.7. 7-Methyl-4-oxo-N-(4-sulfamoylphenyl)-4H-chromene-2-carboxamide (5c)

91% yield. MP = 309–311 °C. 1H NMR (400 MHz, DMSO-d6) δ: 10.87 (s, 1H, NH), 7.99–7.84 (m, 5H, Ar), 7.61 (s, 1H, Ar), 7.34 (d, J = 8.4 Hz, 1H, Ar), 7.25 (s, 2H, SO2NH2), 6.95 (s, 1H, Ar), 2.53 (s, 3H, CH3). MS (ESI): m/z (%) = 359.1 (20.4) [M + H]+. Anal. Calcd. for C17H14N2O3S (%): C, 56.98; H, 3.94; N, 7.82; S, 8.95. Found (%): C, 57.07; H, 3.98; N, 7.78; S, 8.93.

3.1.8. 6-Ethyl-4-oxo-N-(4-sulfamoylphenyl)-4H-chromene-2-carboxamide (5d)

84% yield. MP = 297–299 °C. 1H NMR (400 MHz, DMSO-d6) δ: 10.92 (s, 1H, NH), 7.98 (d, J = 6.9 Hz, 2H, Ar), 7.87–7.83 (m, 4H, Ar), 7.74 (d, J = 1.5 Hz, 1H, Ar), 7.25 (s, 2H, SO2NH2), 6.97 (s, 1H, Ar), 2.78 (q, J = 7.6 Hz, 2H, CH2CH3), 1.29 (t, J = 7.6 Hz, 3H, CH3CH3). 13C NMR (151 MHz, DMSO-d6) δ: 177.68, 158.66, 155.55, 154.00, 142.43, 140.94, 140.41, 135.58, 127.09, 123.96, 123.36, 121.19, 119.34, 111.65, 40.51, 27.98, 15.79. MS (ESI): m/z (%) = 373.1 (21.4) [M + H]+. Anal. Calcd. for C18H16N2O3S (%): C, 58.05; H, 4.33; N, 7.52; S, 8.61. Found (%): C, 58.07; H, 4.37; N, 7.58; S, 8.63.

3.1.9. 6,7-Dimethyl-4-oxo-N-(4-sulfamoylphenyl)-4H-chromene-2-carboxamide (5e)

87% yield. MP = 310–312 °C. 1H NMR (400 MHz, DMSO-d6) δ: 10.95 (s, 1H, NH), 7.98 (d, J = 8.7 Hz, 2H, Ar), 7.87 (d, J = 8.7 Hz, 2H, Ar), 7.80 (s, 1H, Ar), 7.60 (s, 1H, Ar), 7.52 (s, 1H, Ar), 7.42 (d, J = 7.6 Hz, 1H, Ar), 7.38 (s, 1H, Ar), 6.85 (s, 1H, Ar), 2.61 (s, 6H, CH3). MS (ESI): m/z (%) = 381.1 (14.8) [M + H]+. Anal. Calcd. for C19H18N2O3S (%): C, 58.16; H, 4.39; N, 7.51; S, 8.50. Found (%): C, 58.14; H, 4.37; N, 7.53; S, 8.49.
7.35 (s, 2H, SO₂NH₂), 6.93 (s, 1H, Ar), 2.41 (s, 3H, CH₃), 2.34 (s, 3H, CH₃). MS (ESI): m/z (%) = 373.1 (21.4) [M + H]⁺. Anal. Calcd. for C₁₈H₁₆N₂O₅S (%): C, 58.05; H, 4.33; N, 7.52; S, 8.61. Found (%): C, 58.03; H, 4.32; N, 7.51; S, 8.61.

3.1.10. 7,8-Dimethyl-4-oxo-N-(4-sulfamoylphenyl)-4H-chromene-2-carboxamide (5f)

90% yield. MP = 328–330 °C. ¹H NMR (400 MHz, DMSO-d₆): δ: 10.85 (s, 1H, NH), 7.96 (d, J = 8.7 Hz, 2H, Ar), 7.87 (d, J = 8.7 Hz, 2H, Ar), 7.81 (d, J = 8.1 Hz, 1H, Ar), 7.38–7.36 (m, 3H, 1H, Ar + SO₂NH₂), 7.00 (s, 1H, Ar), 2.48 (s, 3H, CH₃), 2.43 (s, 3H, CH₃). ¹³C NMR (151 MHz, DMSO-d₆): δ: 177.86, 162.72, 159.18, 155.19, 155.74, 153.92, 144.79, 144.78, 133.18, 129.04, 129.02, 114.81, 40.86, 40.48, 36.22, 31.21, 20.51, 12.04. MS (ESI): m/z (%) = 373.1 (21.2) [M + H]⁺. Anal. Calcd. for C₁₈H₁₆N₂O₅S (%): C, 58.05; H, 4.33; N, 7.52; S, 8.61. Found (%): C, 58.09; H, 4.35; N, 7.48; S, 8.67.

3.1.11. 5,7-Dimethyl-4-oxo-N-(4-sulfamoylphenyl)-4H-chromene-2-carboxamide (5g)

76% yield. MP = 304–306 °C. ¹H NMR (400 MHz, DMSO-d₆): δ: 10.86 (s, 1H, NH), 7.98 (d, J = 8.7 Hz, 2H, Ar), 7.87 (d, J = 8.7 Hz, 2H, Ar), 7.46 (s, 1H, Ar), 7.31 (s, 2H, SO₂NH₂), 7.12 (s, 1H, Ar), 6.85 (s, 1H, Ar), 2.73 (s, 3H, CH₃), 2.44 (s, 3H, CH₃). ¹³C NMR (126 MHz, DMSO-d₆): δ: 179.43, 158.61, 157.08, 145.14, 140.96, 140.35, 139.94, 134.04, 127.08, 121.12, 120.26, 116.97, 113.00, 22.42, 21.54. MS (ESI): m/z (%) = 373.1 (21.4) [M + H]⁺. Anal. Calcd. for C₁₈H₁₆N₂O₅S (%): C, 58.05; H, 4.33; N, 7.52; S, 8.61. Found (%): C, 58.07; H, 4.37; N, 7.56; S, 8.64.

3.1.12. 6,8-Dimethyl-4-oxo-N-(4-sulfamoylphenyl)-4H-chromene-2-carboxamide (5h)

88% yield. MP = 309–311 °C. ¹H NMR (400 MHz, DMSO-d₆): δ: 10.87 (s, 1H, NH), 7.93 (d, J = 8.7 Hz, 2H, Ar), 7.88 (d, J = 8.7 Hz, 2H, Ar), 7.48 (s, 1H, Ar), 7.42 (s, 2H, SO₂NH₂), 7.23 (s, 1H, Ar), 6.87 (s, 1H, Ar), 2.63 (s, 3H, CH₃), 2.42 (s, 3H, CH₃). MS (ESI): m/z (%) = 373.1 (21.5) [M + H]⁺. Anal. Calcd. for C₁₈H₁₆N₂O₅S (%): C, 58.05; H, 4.33; N, 7.52; S, 8.61. Found (%): C, 58.11; H, 4.30; N, 7.51; S, 8.70.

3.1.13. 6-Chloro-4-oxo-N-(4-sulfamoylphenyl)-4H-chromene-2-carboxamide (5i)

92% yield. MP = 296–298 °C. ¹H NMR (400 MHz, DMSO-d₆): δ: 10.92 (s, 1H, NH), 8.00–7.84 (m, 7H, Ar), 7.23 (s, 2H, SO₂NH₂), 7.02 (s, 1H, Ar). ¹³C NMR (151 MHz, DMSO-d₆): δ: 176.66, 158.30, 155.88, 154.12, 140.84, 140.49, 135.47, 131.09, 127.12, 125.26, 124.40, 121.90, 121.21, 111.72, 40.51. MS (ESI): m/z (%) = 380.0 (39.2) [M + H]⁺. Anal. Calcd. for C₆H₅ClN₂O₂S (%): C, 50.73; H, 2.93; Cl, 9.36; N, 7.40; S, 8.47. Found (%): C, 50.81; H, 2.95; Cl, 9.38; N, 7.45; S, 8.53.

3.1.14. 6-Chloro-7-methyl-4-oxo-N-(4-sulfamoylphenyl)-4H-chromene-2-carboxamide (5j)

92% yield. MP = 305–307 °C. ¹H NMR (400 MHz, DMSO-d₆): δ: 10.97 (s, 1H, NH), 8.00–7.85 (m, 6H, Ar), 7.35 (s, 2H, SO₂NH₂), 7.00 (s, 1H, Ar), 2.52 (s, 3H, CH₃). ¹³C NMR (151 MHz, DMSO-d₆): δ: 176.46, 158.38, 155.69, 153.94, 144.05, 140.89, 140.45, 131.91, 127.13, 124.64, 123.35, 121.53, 121.11, 111.66, 40.51, 20.75. MS (ESI): m/z (%) = 394.0 (39.5) [M + H]⁺. Anal. Calcd. for C₁₇H₁₃ClN₂O₂S (%): C, 51.98; H, 3.34; Cl, 9.03; N, 7.13; S, 8.16. Found (%): C, 52.03; H, 3.27; Cl, 9.08; N, 7.15; S, 8.21.

3.1.15. General Procedure for Synthesis of Compounds 6a–k

The corresponding 4-oxo-4H-chromene-2-carboxylic acid (2a–j) (1.52 mmol, 1 eq.) was dissolved in DMF (10 mL), and 1,1-carbonyldiimidazole (0.320 g, 1.98 mmol, 1.3 eq.) was added in one portion. The reaction mixture was stirred at 80–90 °C for 1.2 h; afterward, the corresponding amine (4a,b) (1.82 mmol, 1.2 eq.) in DMF (7 mL) was added and the mixture was continually heated for another 1 h. After cooling to room temperature, the reaction mixture was treated with water (20 mL) and extracted with ethyl acetate (2 × 30 mL), washed with brine, and dried with Na₂SO₄. The solvent was evaporated under reduced pressure and the residue was purified by HPLC to afford the desired amide (6a–k).
3.1.16. 4-Oxo-N-(4-sulfamoylbenzyl)-4H-chromene-2-carboxamide (6a)

96% yield. MP = 284–286 °C. 1H NMR (400 MHz, CD3COCD): δ: 8.31 (d, J = 6.7 Hz, 1H, Ar), 8.08–7.95 (m, 3H, Ar), 7.89 (m, 4H, Ar), 7.56 (s, 1H, Ar), 4.88 (s, 2H, CH2). 13C NMR (126 MHz, DMSO-d6): δ: 183.40, 176.72, 159.49, 156.09, 154.12, 143.36, 142.87, 135.38, 128.23, 126.23, 125.39, 124.18, 119.19, 110.89, 40.90, 34.86. MS (ESI): m/z (%) = 389.1 (23.0) [M + H]+. Anal. Calcd. for C17H14N2O3S (%): C, 57.02; H, 3.87; N, 7.79; S, 8.94.

3.1.17. 6-Methyl-4-oxo-N-(4-sulfamoylbenzyl)-4H-chromene-2-carboxamide (6b)

91% yield. MP = 293–295 °C. 1H NMR (400 MHz, DMSO-d6): δ: 9.75 (t, J = 8.0 Hz, 1H, NH), 7.60 (m, 4H, Ar), 7.35 (s, 1H, Ar), 4.58 (d, J = 8.0 Hz, 2H, Ar), 2.47 (s, 3H, CH3). 13C NMR (151 MHz, DMSO-d6): δ: 186.41, 177.67, 159.41, 155.75, 153.81, 143.97, 136.50, 136.15, 128.22, 126.23, 125.39, 119.19, 110.89, 40.90, 34.86. MS (ESI): m/z (%) = 373.1 (21.5) [M + H]+. Anal. Calcd. for C16H16N2O3S (%): C, 58.05; H, 3.43; Cl, 9.03; N, 7.13; S, 8.16. Found (%): C, 58.10; H, 4.29; N, 7.50; S, 8.71.

3.1.18. 6-Chloro-4-oxo-N-(4-sulfamoylbenzyl)-4H-chromene-2-carboxamide (6c)

84% yield. MP = 299–301 °C. 1H NMR (400 MHz, DMSO-d6): δ: 9.74 (t, J = 7.9 Hz, 1H, NH), 7.15–7.45 (m, 6H, Ar), 7.31 (s, 1H, Ar), 4.79 (d, J = 6.0 Hz, 2H, Ar), 2.43 (s, 3H, CH3). 13C NMR (126 MHz, DMSO-d6): δ: 177.67, 159.45, 155.75, 153.81, 143.97, 136.50, 136.15, 128.22, 126.23, 125.39, 124.18, 119.19, 110.89, 42.83, 40.51, 20.91. MS (ESI): m/z (%) = 394.0 (39.5) [M + H]+. Anal. Calcd. for C16H16ClN2O3S (%): C, 51.98; H, 3.34; Cl, 9.03; N, 7.13; S, 8.16. Found (%): C, 52.04; H, 3.28; Cl, 9.09; N, 7.16; S, 8.22.

3.1.19. 4-Oxo-N-(4-sulfamoylphenethyl)-4H-chromene-2-carboxamide (6d)

95% yield. MP = 248–250 °C. 1H NMR (400 MHz, DMSO-d6): δ: 9.21–9.17 (m, 1H, NH), 7.89 (ddd, J = 8.5, 7.1, 1.7 Hz, 1H, Ar), 7.50 (t, J = 8.0 Hz, 2H, Ar), 7.32 (s, 1H, Ar), 4.58 (q, J = 6.9 Hz, 2H, NCH2), 2.98 (t, J = 7.4 Hz, 2H, CH2Ar). MS (ESI): m/z (%) = 361.4 (21.5) [M + H]+. Anal. Calcd. for C18H16N2O3S (%): C, 58.05; H, 4.33; N, 7.52; S, 8.61. Found (%): C, 58.10; H, 4.29; N, 7.50; S, 8.71.

3.1.20. 6-Methyl-4-oxo-N-(4-sulfamoylphenethyl)-4H-chromene-2-carboxamide (6e)

84% yield. MP = 257–259 °C. 1H NMR (400 MHz, DMSO-d6): δ: 9.18–9.14 (m, 1H, NH), 7.82 (s, 1H, Ar), 7.89 (d, J = 8.5 Hz, 2H, Ar), 7.77–7.61 (m, 2H, Ar), 7.42 (t, J = 8.5 Hz, 2H, Ar), 7.19 (s, 2H, SO2NH2), 6.77 (s, 1H, Ar), 3.54 (q, J = 6.9 Hz, 2H, NCH2), 2.96 (t, J = 7.4 Hz, 2H, CH2Ar). MS (ESI): m/z (%) = 371.4 (22.5) [M + H]+. Anal. Calcd. for C19H18N2O3S (%): C, 59.06; H, 4.70; N, 7.25; S, 8.30. Found (%): C, 59.10; H, 4.79; N, 7.23; S, 8.33.

3.1.21. 7-Methyl-4-oxo-N-(4-sulfamoylphenethyl)-4H-chromene-2-carboxamide (6f)

87% yield. MP = 253–255 °C. 1H NMR (400 MHz, DMSO-d6): δ: 9.23 (t, J = 6.9 Hz, 1H, NH), 7.92 (d, J = 8.4 Hz, 1H, Ar), 7.75 (d, J = 8.0 Hz, 2H, Ar), 7.50 (s, 1H, Ar), 7.44 (d, J = 8.0 Hz, 2H, Ar), 7.35 (d, J = 8.4 Hz, 2H, Ar), 7.30 (s, 2H, SO2NH2), 6.76 (s, 1H, Ar), 3.55 (q, J = 6.9 Hz, 2H, NCH2), 2.96 (t, J = 7.4 Hz, 2H, CH2Ar), 2.47 (s, 3H, CH3). MS (ESI): m/z (%) = 381.7 (22.5) [M + H]+. Anal. Calcd. for C19H18N2O3S (%): C, 59.06; H, 4.70; N, 7.25; S, 8.30. Found (%): C, 59.12; H, 4.73; N, 7.24; S, 8.35.

3.1.22. 6-Ethyl-4-oxo-N-(4-sulfamoylphenethyl)-4H-chromene-2-carboxamide (6g)

90% yield. MP = 257–259 °C. 1H NMR (400 MHz, DMSO-d6): δ: 9.21 (s, 1H, NH), 7.85 (d, J = 2.2 Hz, 1H, Ar), 7.80–7.71 (m, 3H, Ar), 7.63 (d, J = 8.6 Hz, 1H, Ar), 7.46 (d,
3.1.23. 6,7-Dimethyl-4-oxo-N-(4-sulfamoylphenethyl)-4H-chromene-2-carboxamide (6h)

82% yield. MP = 280–282 °C. 1H NMR (400 MHz, DMSO-d$_6$) δ: 8.75 (t, J = 6.0 Hz, 1H, NH), 7.77–7.74 (m, 3H, Ar), 7.43 (d, J = 8.1 Hz, 2H, Ar), 7.30 (d, J = 8.4 Hz, 1H, Ar), 7.20 (s, 2H, SO$_2$NH$_2$), 6.75 (d, J = 1.2 Hz, 1H, Ar), 6.30 (q, J = 6.0 Hz, 2H, NCH$_2$), 2.96 (t, J = 7.3 Hz, 2H, CH$_2$Ar), 2.52 (s, 3H, CH$_3$), 2.48 (s, 3H, CH$_3$). MS (ESI): m/z (%) = 387.1 (22.5) [M + H]$^+$. Anal. Calcd. for C$_{20}$H$_{20}$N$_2$O$_5$S (%): C, 59.99; H, 5.03; N, 7.00; S, 8.01. Found (%): C, 59.94; H, 5.05; N, 7.02; S, 8.03.

3.1.24. 7,8-Dimethyl-4-oxo-N-(4-sulfamoylphenethyl)-4H-chromene-2-carboxamide (6i)

82% yield. MP = 280–282 °C. 1H NMR (400 MHz, DMSO-d$_6$) δ: 8.75 (t, J = 6.0 Hz, 1H, NH), 7.79–7.76 (m, 3H, Ar), 7.43 (d, J = 8.1 Hz, 2H, Ar), 7.30 (d, J = 8.4 Hz, 1H, Ar), 7.20 (s, 2H, SO$_2$NH$_2$), 6.75 (d, J = 1.2 Hz, 1H, Ar), 6.30 (q, J = 6.0 Hz, 2H, NCH$_2$), 2.96 (t, J = 7.3 Hz, 2H, CH$_2$Ar), 2.52 (s, 3H, CH$_3$), 2.48 (s, 3H, CH$_3$). MS (ESI): m/z (%) = 387.1 (22.5) [M + H]$^+$. Anal. Calcd. for C$_{20}$H$_{20}$N$_2$O$_5$S (%): C, 59.99; H, 5.03; N, 7.00; S, 8.01. Found (%): C, 59.91; H, 5.08; N, 7.05; S, 8.00.

3.1.25. 6,8-Dimethyl-4-oxo-N-(4-sulfamoylphenethyl)-4H-chromene-2-carboxamide (6j)

80% yield. MP = 283–285 °C. 1H NMR (400 MHz, DMSO-d$_6$) δ: 8.81 (t, J = 6.8 Hz, 1H, NH), 7.77 (d, J = 8.0 Hz, 2H, Ar), 7.65 (s, 1H, Ar), 7.53 (s, 1H, Ar), 7.47 (d, J = 8.0 Hz, 2H, Ar), 7.27 (s, 2H, SO$_2$NH$_2$), 6.76 (s, 1H, Ar), 3.58 (q, J = 6.8 Hz, 2H, NCH$_2$), 2.97 (t, J = 7.2 Hz, 2H, CH$_2$Ar), 2.49 (s, 3H, CH$_3$), 2.38 (s, 3H, CH$_3$). 13C NMR (126 MHz, DMSO-d$_6$) δ: 177.84, 159.87, 156.03, 152.33, 145.72, 142.66, 137.18, 135.53, 129.64, 128.33, 126.21, 123.82, 110.72, 94.94, 34.85, 20.85, 15.73. MS (ESI): m/z (%) = 387.1 (22.5) [M + H]$^+$. Anal. Calcd. for C$_{20}$H$_{20}$N$_2$O$_5$S (%): C, 59.99; H, 5.03; N, 7.00; S, 8.01. Found (%): C, 60.03; H, 5.04; N, 7.02; S, 8.06.

3.1.26. 6-Chloro-4-oxo-N-(4-sulfamoylphenethyl)-4H-chromene-2-carboxamide (6k)

73% yield. MP = 285–287 °C. 1H NMR (400 MHz, DMSO-d$_6$) δ: 9.26 (t, J = 5.6 Hz, 1H, NH), 7.99–7.88 (m, 2H, Ar), 7.75 (t, J = 8.9 Hz, 3H, Ar), 7.46 (d, J = 8.1 Hz, 2H, Ar), 7.29 (s, 2H, SO$_2$NH$_2$), 6.83 (s, 1H, Ar), 3.62–3.51 (m, 2H, NCH$_2$), 2.96 (t, J = 7.3 Hz, 2H, CH$_2$Ar). 13C NMR (126 MHz, DMSO-d$_6$) δ: 176.70, 159.21, 156.20, 154.08, 143.66, 142.65, 135.35, 130.90, 129.59, 126.23, 125.19, 124.38, 121.65, 110.81, 40.91, 34.84. MS (ESI): m/z (%) = 408.0 (39.7) [M + H]$^+$. Anal. Calcd. for C$_{18}$H$_{15}$ClN$_2$O$_5$S (%): C, 53.14; H, 3.72; Cl, 8.71; N, 6.89; S, 7.88. Found (%): C, 53.04; H, 3.78; Cl, 8.79; N, 7.16; S, 7.82.

3.2. Carbonic Anhydrase Inhibition

An Applied Photophysics stopped-flow instrument was used for assaying the CA catalyzed CO$_2$ hydration activity [38]. Phenol red (at a concentration of 0.2 mM) was used as an indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.4) as a buffer, and 20 mM Na$_2$SO$_4$ (for maintaining constant ionic strength), following the initial rates of the CA-catalyzed CO$_2$ hydration reaction for a period of 10–100 s. The CO$_2$ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants [39]. The non-catalyzed CO$_2$ hydration was not subtracted from these curves and accounts for the remaining observed activity even at a high concentration of an inhibitor, being in the range of 16–25%. However, the background activity from the
uncatalyzed reaction is always subtracted when IC\textsubscript{50} values are obtained by using the data analysis software for the stopped flow instrument. Enzyme concentrations ranged between 5–12 nM. For each inhibitor, at least six traces of the initial 5–10% of the reaction were used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of the inhibitor (0.1 mM) were prepared in distilled, deionized water, and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to the assay, allowing the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation as reported earlier, which represent the mean from at least three different determinations. All CA isoforms were recombinant proteins obtained in house, as reported earlier [40–42].

3.3. Molecular Modeling Studies (Experimental Part)

Docking calculations were carried out using the AutoDock 4.2 software [43]. The free energy of binding (DG) of the cytosolic isoforms hCA I and II as well as the transmembrane tumor-associated ones IX and XII in complex with the tested compounds was generated using this molecular docking program. The crystal structures of hCA I (PDB code 3W6H), hCA II (PDB code 3HS4), hCA IX (PDB code 3IAI), and hCA XII (PDB code 1JD0) were obtained from the Protein Data Bank [44]. For the enzymes’ preparation, all water molecules were eliminated polar hydrogens, which were added, and the co-crystallized ligands were removed from each enzyme’s active site, while for the preparation of the inhibitors, the charges were added and the rotatable bonds determined. Grid maps have been calculated utilizing Autogrid algorithm and must contain the area to be connected. A set of grids of 60 Å × 50 Å × 50 Å with 0.375 Å spacing was calculated, considering the docking area for all the ligand atom types employing AutoGrid4. Three-dimensional structures of all compounds were constructed using Chem3Dultra 12.0 software (Chemical Structure Drawing Standard; Perkin Elmer Informatics, Waltham, MA, USA). For the present system, the Lamarckian genetic algorithm was applied for minimization using default parameters. The pitch was 1.0 Å, while the quaternion and pivot angle were set to 5 and 0 degrees. For each compound, 200 configurations were produced. The results from the Autodock calculations were grouped using a root mean standard deviation (RMSD) value of 1.5 Å, while the lowest-energy configuration of the largest population group was chosen as the most likely tethering configuration. The LigandScout software program (inte:ligand, Vienna, Austria) was used to display the results and process of the configurations with the highest tie rating. Finally, the docking protocol was verified by re-docking of the co-crystallized ligand acetazolamide (AAZ) in the vicinity of the active sites of each enzyme with RMSD values 0.885, 0.966, 1.034, and 1.176 Å for hCA I, II, IX, and XII, respectively.

4. Conclusions

In conclusion, we synthetized and investigated two novel series of chromene-containing aromatic sulfonamides for their effective inhibition against different and most relevant human carbonic anhydrase isoforms such as the ubiquitous hCA I, hCA II, and the tumor associate isoforms hCA IX and XII, which are involved in a variety of diseases such as glaucoma, retinitis pigmentosa, epilepsy, and tumors. Compound 5a showed a good selectivity index of 31.67 on hCA IX compared to hCA I and over two times compared to hCA II. On the other hand, compound 6f showed to be the most active hCA II with an SI of 44.47 compared to hCA I and 44.97 compared to hCA XII. In addition, the methylene linker among the chromene scaffold and sulfonamide moiety played a crucial role in the modulation of potency and selectivity inhibition against different isoforms. Indeed, compound 5b showed a Ki of 77 nM against hCA IX and the analog 6b, with a methylene linker, and dramatically decreased the potency to 2039 nM, losing the selective inhibition against this isoform. These interesting features make them good candidates for preclinical evaluation in glaucoma or various tumors in which the two enzymes (hCA II and hCA IX)
are involved. Furthermore, computational procedures were used to investigate the binding mode of this class of compounds within the active site of hCA IX.

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**Abbreviations**

CA  | Carbonic Anhydrase  
CAI | Carbonic Anhydrase Inhibitors  
SAR | Structure Activity Relationships  
SI  | Selectivity Index

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