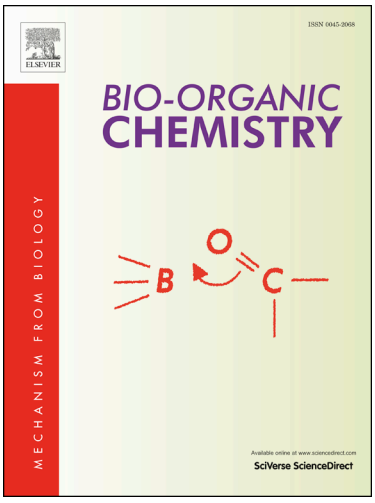

Synthesis of different thio-scaffolds bearing sulfonamide with subnanomolar carbonic anhydrase II and IX inhibitory properties and X-ray investigations for their inhibitory mechanism

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Synthesis of different thio-scaffolds bearing sulfonamide with subnanomolar carbonic anhydrase II and IX inhibitory properties and X-ray investigations for their inhibitory mechanism

Andrea Angeli,[a‡] Damiano Tanini,[b‡] Antonella Capperucci,[b] Gianni Malevolti,[b] Francesca Turco,[b] Marta Ferraroni[b] and Claudiu T. Supuran*[a]

[a] *Department of University of Florence, NEUROFARBA Dept., Sezione di Scienze Farmaceutiche, Via Ugo Schiff 6, 50019 Sesto Fiorentino (Florence), Italy*

[b] *Department of University of Florence, Department of Chemistry "Ugo Schiff", Via della Lastruccia 13, I-50019 Sesto Fiorentino, Italy.*

‡ *These authors contributed equally to this work.*

Abstract. Several new molecules with different thio-scaffolds were designed, synthesised, and evaluated biologically as inhibitors of Carbonic Anhydrases (CAIs). The structure–activity relationship analysis identified thioether derivatives, here reported, as a potent and selective CAIs against hCA II and hCA IX. High resolution X-ray structure of inhibitor bound hCA II revealed extensive interactions with the hydrophobic pocket of active site and provided molecular insight into the binding properties of these new inhibitors

Keywords: carbonic anhydrase; inhibitor, metalloenzymes, sulfur

Corresponding author. Tel./fax:+39-055-4573729; e-mail: claudiu.supuran@unifi.it (CTS)

1. Introduction

The carbonic anhydrases (CAs, EC 4.2.1.1) belong to a superfamily of metalloenzymes present in all life kingdoms and encoded by seven evolutionarily unrelated gene families: the α-, β-, γ-, δ-, ζ- η- and the last recently discover θ- CAs.[1] These enzymes are involved in crucial physiological processes connected with pH and CO_2 homoeostasis/ sensing catalysing the hydration of carbon dioxide to produce bicarbonate and protons.[2] So far, in humans, 15 different α-CAs were described and implicated in several physiological/ pathological processes.[3] Carbonic anhydrase inhibitors (CAIs) like sulphonamide derivatives are in clinical use for the treatment of various diseases such as diuretics[4], glaucoma[5], epilepsy[6] and more recently as antitumor and antimetastatic agents.[7] Owing to the development of new carbonic anhydrase inhibitors, we hypothesized that the poor explored disulphide, thioether and thioester scaffolds, could allow for the development of novel inhibitors that are able to interact with the active site region of carbonic anhydrase. In this study, the synthesis, kinetic analysis, biological evaluation and X-ray structure of one ligand-protein complex were reported.

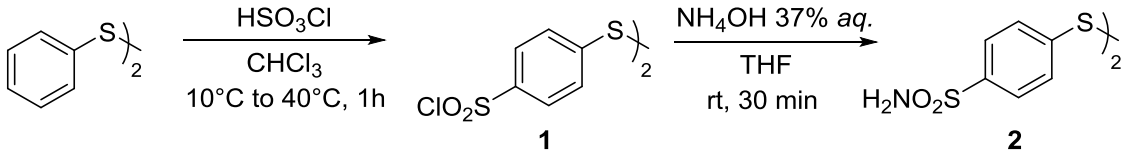
2. Results and discussion

2.1. Chemistry

Our group is involved for a long time in the study of synthetic procedures to access novel class of functionalized organochalcogenides, mainly exploiting the reactivity of a wide range of sulfur-, selenium- and tellurium-containing nucleophiles with different kind of electrophiles.[8-12.] Recently, we became interested in the design and the synthesis of biologically active compounds and we started to apply such procedures to access new chalcogen-containing small molecules as antioxidants[13-15] and CAs inhibitors.[16,17] Very recently, we reported on the synthesis and the evaluation of inhibitory activity for a series of β-functionalized selenides as CA I, II, IV, VII, and IX Inhibitors.[18]

In order to synthesize chemically novel sulfur-containing CAs inhibitors and to investigate the effect of isosteric replacement of selenium with sulfur on such an activity, we explored the reactivity of the disulfide **2**, bearing the benzenesulfonamide moiety, with a *plethora* of electrophiles.

The disulfide **2** was conveniently obtained by treating disulfonylchloride **1** with an acqueous ammonium hydroxide solution. Compound **1** was prepared from diphenyl disulfide and chlorosulfonic acid, following the reported literature procedure (**Scheme 1**).[19]

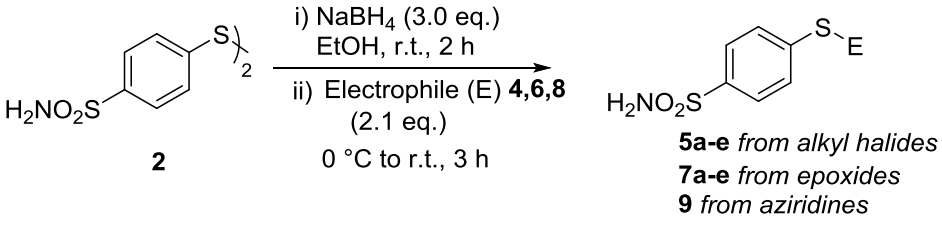


Scheme 1: Synthesis of disulfide 2, bearing the benzenesulfonamide moiety

Thus, in order to synthesize unsymmetrical sulfides incorporating the benzenesulfonamide moiety, **2** was reduced with $NaBH_4$, and the thiolate **3** was treated with the suitable electrophile **4** to achieve the corresponding sulfides **5** in rather good yield, as reported in the **Table 1**.

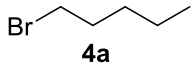
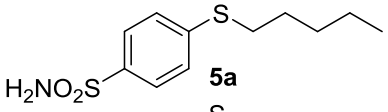
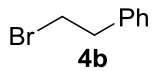
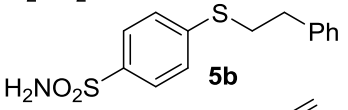
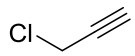
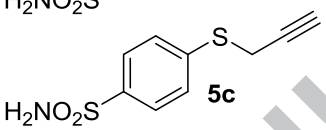
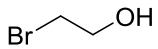
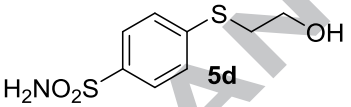
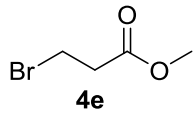
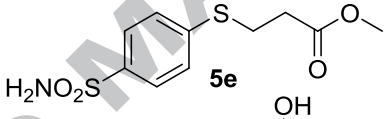
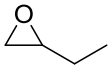
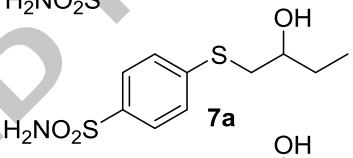
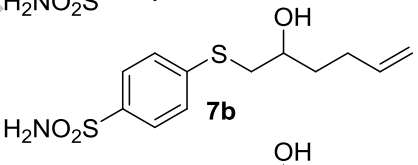
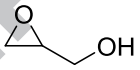
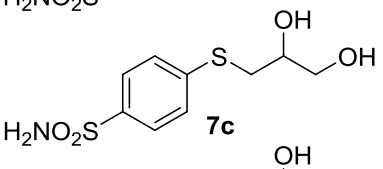
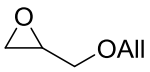
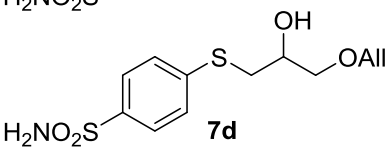
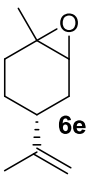
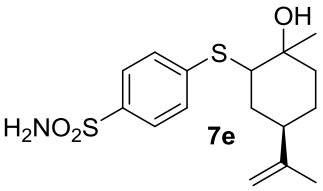
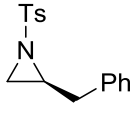
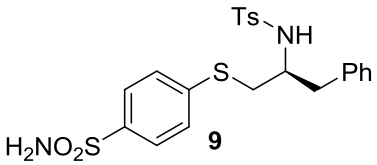
Interestingly, product **5e** (**Table 1**, entry 5) was selectively formed from methyl 3-bromopropionate **4e**, no traces of compounds arising from substitution or reduction at the carbonyl carbon being observed.

Table 1: Synthesis of sulfides bearing benzenesulfonamide moiety



2

5a-e *from alkyl halides*
7a-e *from epoxides*
9 *from aziridines*

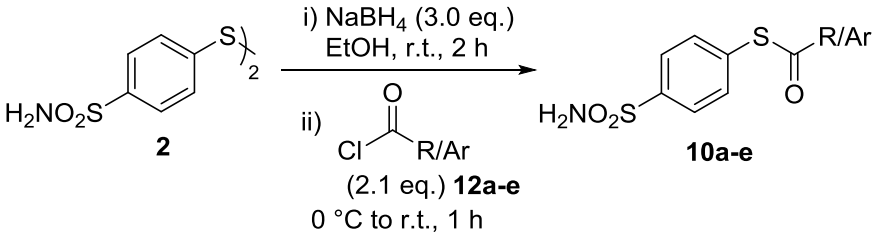
Entry	Electrophile	Product	Yield (%)[a]
1	 **4a**	 **5a**	74
2	 **4b**	 **5b**	61
3	 **4c**	 **5c**	83
4	 **4d**	 **5d**	71
5	 **4e**	 **5e**	86
6	 **6a**	 **7a**	88
7	 **6b**	 **7b**	94
8	 **6c**	 **7c**	76
9	 **6d**	 **7d**	89
10	 **6e**	 **7e**	78
11	 **8**	 **9**	62

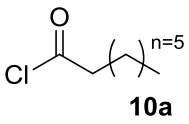
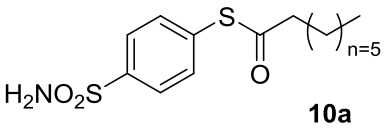
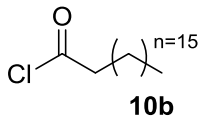
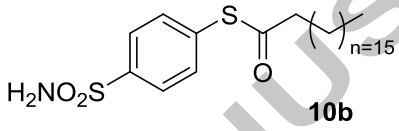
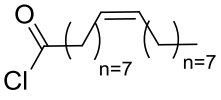
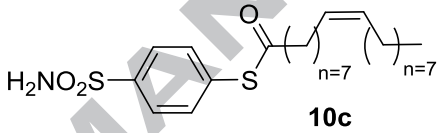
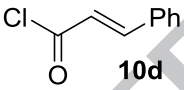
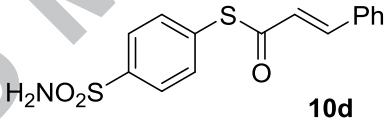
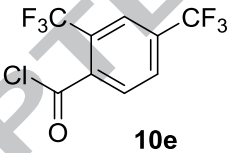
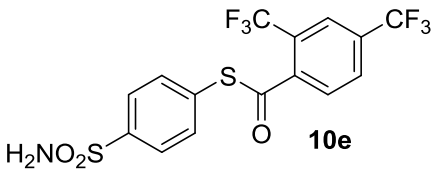
[a] Yields are referred to isolated products

Having explored the reactivity of **3** with alkyl halides, we turned our attention on the ring opening reaction of strained heterocycles. Thus, monosubstituted epoxides **6a-d** were treated with thiolate **3** under the reported conditions affording to β-hydroxysulfides **7a-d** (**Table 1**, entries 6-9), bearing saturated or unsaturated carbon chains, and protected or free hydroxyl groups. Furthermore, the limonene derived sulfide **7e** was synthesised from the disubstituted epoxide **6e**. Finally, with the aim of enlarging the scope of such a procedure to another class of three-membered heterocycles, the enantio-enriched *N*-tosyl aziridine **8**, obtained from L-phenylalanine, afforded the β-amino substituted sulfide **9** (**Table 1**, entry 11). All the ring opening reactions here described were demonstrated to be highly stereo- and regio-selective, as only the isomer arising from the nucleophilic attack at the less hindered carbon of the strained heterocycle was observed.[8,11]

Having obtained a number of sulfides through the reaction of **2** with different electrophiles, we extended this procedure to the synthesis of thiol esters bearing the benzenesulfonamide moiety. Thus, the thiolate **3** was *in situ* treated with acyl chlorides (**12a-e**) to obtain thiol esters **10a-e**. The reaction proved to be efficient, allowing the synthesis of saturated and unsaturated fatty acid derived thiol esters **10a-c**, obtained from caprylic, stearic, and oleic acid, respectively. Furthermore, α,β-unsaturated thiol ester **10d** and aromatic thiol esters **10e** were achieved from cinnamoyl chloride and 2,4-bis(trifluoromethyl)benzoyl chloride as reported in **Table 2.**

Table 2: Synthesis of thiol esters bearing the benzenesulfonamide moiety

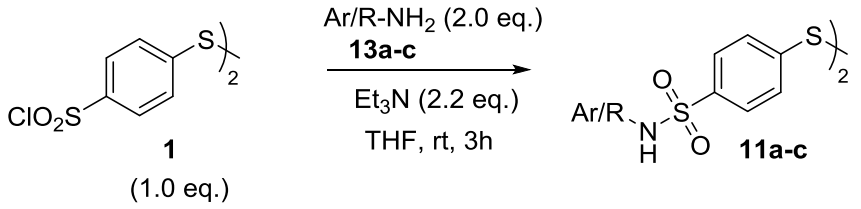


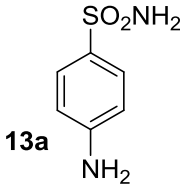
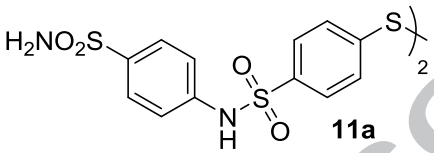
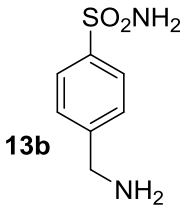
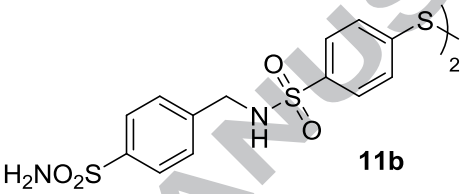
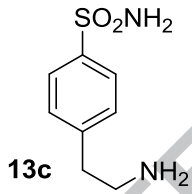
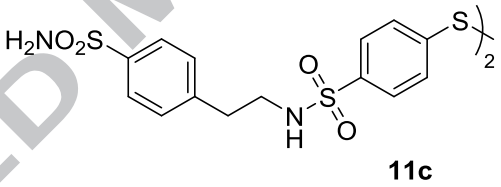
Entry	Electrophile	Product	Yield (%)[a]
1	 **10a**	 **10a**	42
2	 **10b**	 **10b**	54
3	 **10c**	 **10c**	61
4	 **10d**	 **10d**	58
5	 **10e**	 **10e**	46

[a] Yields are referred to isolated products

Having synthesised a representative series of functionalized sulfides and thiol esters encorporating the benzenesulfonamide moiety, we also prepared disulfides **11a-c**, bearing two primary and two secondary sulfonamide groups. Compounds **11a-c** could be easily achieved by reacting the disulphonylchloride **1** with the suitable amine (**13a-c)** in the presence of trimethylamine as outlined in **Table 3**.

Table 3: Synthesis of disulfides **11a-c**



Entry	Electrophile	Product	Yield (%)[a]
1	 13a	 11a	36
2	 13b	 11b	53
3	 13c	 11c	32

[a] Yields are referred to isolated products

2.2. Carbonic anhydrase inhibition

All compounds **2**, **5a-e**, **7a-e**, **9**, **10a-e** and **11a-c** were tested in vitro for their inhibitory activity against the physiologically relevant hCA isoforms I, II, IV and IX by means of the stopped-flow carbon dioxide hydration assay[20] and their activities were compared to the standard CAI acetazolamide (**AAZ**) (**Table 4**).

Table 4. Inhibition data of human CA isoforms I, II, IV and IX with compounds **2**, **5a-e**, **7a-e**, **9**, **10a-e**, **11a-c** and **AAZ** by a stopped flow CO_2 hydrase assay.[20]

	K_I (nM)*			
Cmp	**hCA I**	**hCAII**	**hCA IV**	**hCA IX**
2	412.7	0.9	368.3	0.5
5a	773.6	0.4	436.5	0.6
5b	227.5	0.4	80.9	0.9
5c	30.2	29.5	838.9	7.6
5d	135.6	37.2	878.0	36.7
5e	41.7	0.6	808.4	6.6
7a	138.1	0.8	683.6	2.0
7b	139.9	4.0	505.6	6.8
7c	74.8	31.0	552.6	41.7
7d	50.4	3.7	709.6	6.0
7e	9.8	1.4	87.7	0.6
9	685.2	197.2	2045.7	969.0
10a	76.7	47.0	5357.8	166.3
10b	513.3	663.5	9500.0	4687.0
10c	519.3	879.0	9647.4	37.3
10d	89.3	4.9	6888.2	268.9
10e	32.3	4.9	4933.2	5.2
11a	88.1	53.1	430.9	5.6
11b	542.5	69.0	752.8	55.4
11c	244.4	8.3	1985.8	139.3
AAZ	250	12.1	74.0	25.8

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

The following structure–activity relationship (SAR) can be drawn from the data obtained and reported in **Table 4**:

i) The cytosolic isoform, hCA I, was inhibited by disulfide **2** with inhibition constant in the high nanomolar range (K_i 412.7 nM). Thioether scaffolds **5a-e** play a crucial role for the potency of inhibition. Compounds **5c** and **5e** showed an efficacy over 4 fold than the similar derivatives. On the other hand, β-hydroxysulfide from natural product limonene **7e** results the most potent inhibitor against this isoform. Like as compounds mentioned above, thioester tails lead to significant changes in the inhibition potency. Compounds **10b-c** with high hydrophobic moieties showed a significant decrease of the inhibitory potency in high nanomolar range (K_i 513.3 and 519.3 nM respectively), instead, the efficacy increased when the moiety was changed with short aliphatic chain (**10a** and **10e**) or cinnamic scaffold (**10d**). The potency of inhibition for disulfides **11a-c** can be modulate changing the methylene chain length between primary and secondary sulphonamide. Indeed, compound **11a** showed a potency over 2 times better than compound with an ethylene chain (**11c)** and over 6 times for compound with a methylene chain (**11b**).

ii) The dominant cytosolic isoform, hCA II, is strong inhibited by disulfide 2 and most thioether compounds **5a-e** in sub-nanomolar range (K_i 0.4 to 0.9 nM). An interesting case is constituted by thioesther compounds **10a-e**, for which the long hydrophobic tails led to a decrease of potency over 100 fold compared to the other similar products (K_i 663.5 and 879.0 nM for **10b**, **c** than 4.9 nM **10d, e**). Unlike the other cytosolic isoform, the introduction of a methylene linker in compounds **11a-c** had diverse effects on the inhibition profile. A decrease of the potency was observed for a methylene linker with one or without a carbon

atom (**11b**, a K_i of 69.0 nM and **11a** a K_i of 53.1 nM), whereas, a further chain elongation, as in **11c**, led to an increase of the potency, with a K_I of 8.2 nM.

iii) The membrane-bound hCA IV was inhibited by most of the compounds investigated here in the high nanomolar range or in the micromolar range. Thioethers **5a-e** and β-hydroxysulfides **7a-e** compounds showed an efficacy against this isoform, near ten times better than thioesters derivatives **10a-e** (K_i 0.4 to 37.2 nM for **5a-e** and **7a-e** than 4.9 to 879.0 nM for **10a-e**). However, the affinity of these inhibitors for CA IV is lower than to CA II. The side chain of amino acids in the active site pocket of CA IV might be responsible for reduced affinity of compounds here reported.

iv) hCA IX, the tumor-associated isoform, was effectively inhibited by many of the compounds reported here, especially thioether derivatives **5a-b**, **7e** and disulphide **2** in sub-nanomolar range (K_i 0.5 to 09 nM). On the other hand, compounds **10a-e** showed an over 100 times decrease in potency against this isoform. Unlike the other isoforms, the introduction of methylene chain in compounds **11a-c** had an almost linear effect on efficacy of inhibition. Indeed, increasing the linker led to loss of potency near ten fold with one atom of carbon (**11b** K_i 55.4 nM) and over 20 fold with two atoms of carbon (**11c** K_i 139.3 nM).

2.3 Structure of hCA II/ Thioether Ligand Complex.

In order to identify the key interactions involved into protein-inhibitor interactions at the atomic level, we solved the X-ray complex of hCA II/**5b** inhibitor at 1.5 Å resolution. Data collection and refinement of adduct was performed as described in the Experimental Section (see Supporting Information for statistics). Active site of hCA II showed the classic binding mode of sulfonamide inhibitors, where, the deprotonated nitrogen atom displaces the hydroxyl ion/water molecule present in the native enzyme and coordinates the zinc ion with a tetrahedral geometry by His94, His96 and His119. In addition, is present

another hydrogen bond interaction with residue Thr199 further contribute to stabilize the binding (**Figure 1**).

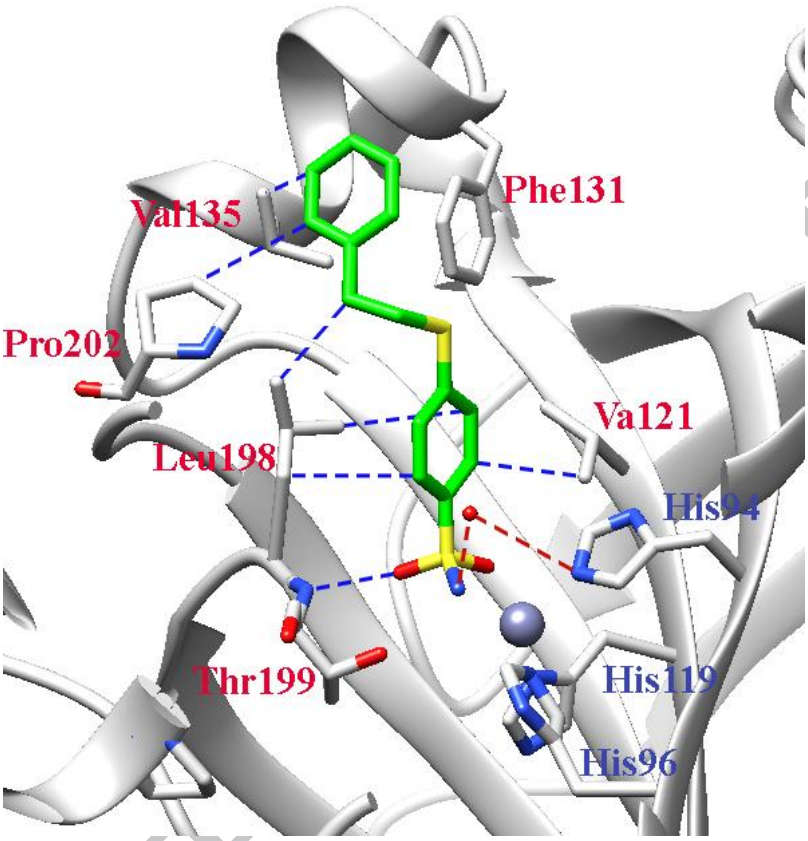


Figure 1: Ligand **5b** in the active site of hCA II (PDB: 6GOT) is shown in green. The zinc ion is the gray sphere with its protein ligands (His94, 96 and 119 in blue) shown as stick model, in CPK colors. Residues involved in the binding of inhibitors are also shown. Hydrophobic interactions and H-bonding are shown as blue dashed lines, π-Stacking as magenta and water bridges as red.

The lipophilic tail of inhibitor **5b** is located in the hydrophobic region of the hCA II active site in a small pocket delimited by residues Phe131, Val135, Leu198, and Pro202 (**Figure 1**). and forms strong hydrophobic interactions with these residues. Finally, we also evidenced a π-stacking interaction between Phe131 and the aromatic ring scaffold of inhibitor.

3. Conclusions

Based on the well-known tail approach, we designed a new chemotype with CA inhibitory properties. Many compounds here reported, showed a strong inhibition against hCA II (a well- established cytosolic glaucoma target) and hCA IX (a transmembrane isoform believed to be involved in many hypoxic tumors). However, the selectivity established for some compounds, such as **2**, **5a, b, e** and **7a, e** for inhibiting selectively hCA II over hCA I and hCA IV, constitutes a very significant finding in light of isoform-selective CA targeting. Indeed, the X-ray structure of the hCA II complex with compound **5b** showed the effectiveness of building contacts with the lipophilic side of the hCA II active site, which strongly correlates with the inhibition potency against this isoform.

4. Experimental Part

4.1. General

All reactions were carried out in an oven-dried glassware under inert atmosphere (N_2). Ethanol was dried using a solvent purification system (Pure-Solv™). All commercial materials were used as received without further purification. Flash column chromatography purifications were performed with Silica gel 60 (230-400 mesh). Thin layer chromatography was performed with TLC plates Silica gel 60 F_{254}. NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 with Mercury 400, and Bruker 400 Ultrashield spectrometers operating at 400 MHz (for ^1H), 100 MHz (for ^{13}C) and 376 MHz (for ^{19}F). NMR signals were referenced to nondeuterated residual solvent signals (7.26 and 2.50 ppm for ^1H, 77.0 and 40.5 ppm for ^{13}C). ^1H NMR data are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, ap d = apparent doublet, m = multiplet, dd = doublet of doublet, bs = broad singlet, bd = broad doublet, ecc.), coupling constant (J), and assignment.

Synthesis of 4,4'-Disulfanediyldibenzenesulfonyl chloride (1):

Disulfide 1 was synthesised following a reported procedure. A solution of 0.1 mol of diphenyldisulfide in chloroform (100 mL) was added to a solution of 0.3 mol chlorosulfonic acid in chloroform at 10 °C.

Than, the mixture was stirred for one hour at 40 °C. After quenching on ice, the phases were separated, the organic phase washed twice with sodium bicarbonate solution and dried with Na_2SO_4. The solvent was eliminated and the product obtained as yellow powder with a yield of 74%. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.72 (4H, ap d, *J* = 8.7 Hz), 8.01 (4H, ap d, *J* = 8.7 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 127.1, 128.5, 143.6, 145.5.

Synthesis of 4,4'-Disulfanediyldibenzenesulfonamide (2):

4,4'-Disulfanediyldibenzenesulfonyl chloride 1 (831 mg, 2.0 mmol) was solubilized in THF (50 mL) and treated with an excess of NH_4OH (37% *aq*. Solution, 10 eq.). The reaction was stirred at ambient temperature for 30 min; the solid was then filtered, washed with Et_2O (20 mL) and dried under vacuum to afford 4,4'-Disulfanediyldibenzenesulfonamide 2 (707 mg, 94%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.45 (4H, bs), 7.78 (4H, ap d, *J* = 8.0 Hz), 7.88 (4H, ap d, *J* = 8.0 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 127.6, 127.7, 140.5, 144.0.

General Procedure for the preparation of sulfides 5a-e, 7a-e and 9 from disulfide 2:

$NaBH_4$ (23 mg, 0.60 mmol, 3.0 eq.) was portionwise added to a solution of 4,4'-disulfanediyldibenzenesulfonamide 2 (75 mg, 0.20 mmol, 1.0 eq.) in EtOH (2 mL) at ambient temperature under inert atmosphere (N_2). After 2 h, the suitable electrophile (0.42 mmol, 2.1 eq.) was slowly added and the reaction mixture was stirred at room temperature for 3 h, until complete consumption of the starting material was observed by TLC. The reaction was quenched by addition of saturated *aq*. NH_4Cl (2 mL) and diluted with EtOAc (5 mL), The layers were separated and the aqueous layer was extracted with EtOAc (2 x 5 mL), dried over Na_2SO_4, filtered and concentrated under vacuum. The crude material was purified by flash chromatography to yield sulfides 5, 7 and 9 bearing benzenesulfonamide moiety.

4-(Pentylthio)benzenesulfonamide (5a):

Following the general procedure, disulfide 2 (75 mg, 0.20 mmol) and bromopentane 4a (63 mg, 0.42 mmol) gave 5a (76 mg, 74%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 0.88 (3H, t, J = 7.2 Hz), 1.26-1.35 (2H, m), 1.37-1.44 (2H, m), 1.59-1.67 (2H, m), 3.06 (2H, ap t, J = 7.3 Hz, CH2S), 7.31 (2H, bs, NH_2), 7.46 (2H, ap d, J = 8.7 Hz), 7.73 (2H, ap d, J = 8.7 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 14.9, 22.7, 29.0, 31.4, 32.0, 127.3, 127.6, 141.6, 143.2.

4-(Phenethylthio)benzenesulfonamide (5b):

Following the general procedure, disulfide 2 (56 mg, 0.15 mmol) and (2-bromoethyl)benzene 4b (58 mg, 0.32 mmol) gave 5b (54 mg, 61%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.92 (2H, t, J= 7.6 Hz), 3.34 (2H, t, J = 7.6 Hz), 7.21-7.27 (1H, m), 7.28-7.35 (6H, m), 7.50 (2H, ap d, J = 8.5 Hz), 7.74 (2H, ap d, J = 8.5 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 33.5, 35.4, 127.3, 127.5, 127.8, 129.4, 129.7, 140.8, 141.8, 142.8.

4-(Prop-2-yn-1-ylthio)benzenesulfonamide (5c):

Following the general procedure, disulfide 2 (75 mg, 0.20 mmol) and propargyl chloride 4c (31 mg, 0.42 mmol) gave 5c (75 mg, 83%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 3.21 (1H, t, J = 2.6 Hz, C≡CH), . 4.02 (2H, d, J = 2.6 Hz, CH2C≡C), 7.38 (2H, bs, NH2), 7.56 (2H, ap d, J = 8.6 Hz), 7.80 (2H, ap d, J = 8.6 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 20.6, 75.1, 80.8, 127.2, 128.2, 141.3, 142.3.

4-((2-hydroxyethyl)thio)benzenesulfonamide (5d):

Following the general procedure, disulfide 2 (75 mg, 0.20 mmol) and 2-bromoethanol 4d (53 mg, 0.42 mmol) gave 5d (66 mg, 71%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 3.11 (2H, t, J = 6.7 Hz, CH2S), 3.55-3.62 (2H, m, CH2O), 4.98 (1H, bs, OH), 7.29 (2H, bs, NH2), 7.45 (2H, ap d, J = 8.6 Hz), 7.69 (2H, ap d, J = 8.6 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 34.4, 60.0, 126.6, 127.0, 141.0, 142.5. MS (ESI negative) *m/z* (%): 232 $[M-H]^-$, required $[M-H]^-$ 232.02.

Methyl 3-((4-sulfamoylphenyl)thio)propanoate (5e):

Following the general procedure, disulfide 2 (56 mg, 0.15 mmol) and 3-bromopropionate 4e (54 mg, 0.32 mmol) gave 5e (71 mg, 86%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.69 (2H, t, *J* = 7.3 Hz), 3.26 (2H, t, *J* = 7.3 Hz), 3.71 (3H, s), 5.01 (2H, bs, NH2), 7.37 (2H, ap d, *J* = 8.7 Hz), 7.81 (2H, ap d, *J* = 8.7 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 27.3, 33.6, 52.0, 127.0, 127.6, 138.9, 143.0, 171.8. MS (ESI negative) *m/z* (%): 298 $[M+Na]^+$; required $[M+Na]^+$ 298.02.

4-((2-Hydroxybutyl)thio)benzenesulfonamide (7a):

Following the general procedure, disulfide 2 (56 mg, 0.15 mmol) and 1,2-epoxybutane 6a (23 mg, 0.32 mmol) gave 7a (69 mg, 88%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 0.89 (3H, t, *J* = 7.4Hz), 1.36-1.47 (1H, m), 1.52-1.66 (1H, m), 3.04 (1H, dd, *J* = 6.7, 13.1 Hz, CH_aH_bS), 3.10 (1H, dd, *J* = 5.4, 13.1 Hz, CH_aH_bS), 3.53-3.60 (1H, m, CHOH), 4.98 (1H, d, *J* = 5.3 Hz, CHOH), 7.32 (2H, bs, NH2), 7.47 (2H, ap d, *J* = 8.5 Hz), 7.71 (2H, ap d, *J* = 8.5 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 11.0, 30.0, 39.4, 71.1, 127.2, 127.5, 141.4, 143.8. MS (ESI negative) *m/z* (%): 260 $[M-H]^-$, required $[M-H]^-$ 260.05.

4-((2-Hydroxyhex-5-en-1-yl)thio)benzenesulfonamide (7b):

Following the general procedure, disulfide 2 (75 mg, 0.20 mmol) and 1,2-Epoxy-5-hexene 6b (41 mg, 0.42 mmol) gave 7b (108 mg, 94%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.65-1.74 (2H, m), 1.92 (1H, bs, OH), 2.14-2.36 (2H, m), 3.02 (1H, dd, *J* = 8.2, 13.7 Hz, CH_aH_bS), 3.24 (1H, dd, *J* = 3.7, 13.7 Hz, CH_aH_bS), 3.81-3.87 (1H, m, CHOH), 4.92 (2H, bs, NH2), 5.01-5.11 (2H, m), 5.78-5.90 (1H, m), 7.44 (2H, ap d, *J* = 8.4 Hz), 7.83 (2H, ap d, *J* = 8.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 30.5, 36.0, 40.9, 69.9, 116.0, 127.6, 128.4, 138.4, 139.5, 144.0. MS (ESI negative) *m/z* (%): 286 $[M-H]^-$, required $[MH]^-$ 286.06.

4-((2,3-Dihydroxypropyl)thio)benzenesulfonamide (7c):

Following the general procedure, disulfide 2 (75 mg, 0.20 mmol) and glycidol 6c (31 mg, 0.42 mmol) gave 7c (80 mg, 76%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.98 (1H, dd, *J* = 7.2,

13.2 Hz, CH_aH_bS), 3.23 (1H, dd, *J* = 4.6, 13.2 Hz, CH_aH_bS), 3.38 (1H, dd, *J* = 6.0, 12.9 Hz, CH_aH_bO), 3.43 (1H, dd, *J* = 5.3, 10.9 Hz, CH_aH_bO), 3.62-3.69 (1H, m, CHOH), 4.74 (1H, bt, *J* = 5.7 Hz, CH_2OH), 5.07 (1H, bd, *J* = 5.2 Hz, CHOH), 7.30 (2H, bs, NH2), 7.47 (2H, ap d, *J* = 8.6 Hz), 7.71 (2H, ap d, *J* = 8.6 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 36.6, 65.6, 71.3, 127.2, 127.5, 141.4, 143.9. MS (ESI negative) *m/z* (%): 262 $[M-H]^-$, required $[M-H]^-$ 262.03.

(*E*)-4-((2-Hydroxy-3-(prop-1-en-1-yloxy)propyl)thio) benzenesulfonamide (7d):

Following the general procedure, disulfide 2 (37.5 mg, 0.1 mmol) and allyl glycidyl ether 6d (24 mg, 0.21 mmol) gave 7d (54 mg, 89%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.79 (1H, bs, OH), 3.09 (1H, dd, *J* = 6.7, 13.6 Hz, CH_aH_bS), 3.17 (1H, dd, *J* = 5.5, 13.6 Hz, CH_aH_bS), 3.49 (1H, dd, *J* = 5.9, 9.4

Hz, CH_aH_bO), 3.54 (1H, dd, *J* = 3.7, 9.4 Hz, CH_aH_bO), 3.93-3.98 (1H, m, CHOH), 3.98-4.03 (2H, m), 5.17- 5.28 (2H, m, $CH=CH_2$), 4.92 (2H, bs, NH_2), 5.82-5.92 (1H, m, $CH=CH_2$), 7.34-7.37 (2H, m), 7.73-7.75 (2H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 35.7 (CH_2S), 69.0, 72.2, 72.3, 117.7, 126.8, 127.3, 134.1, 138.7, 143.3. MS (ESI negative) *m/z* (%): 302 $[M-H]^-$, required $[M-H]^-$ 302.06.

4-(((5*R*)-2-Hydroxy-2-methyl-5-(prop-1-en-2-yl)cyclohexyl) thio)benzenesulfonamide (7e):

Following the general procedure, disulfide 2 (37.5 mg, 0.1 mmol) and limonene oxide 6e (32 mg, 0.21 mmol) gave 7e (53 mg, 78%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.37 (3H, s), 1.58-1.68 (3H, m), 1.69 (3H, s), 1.73-1.81 (2H, m), 1.84 (1H, bs), 2.16-2.23 (1H, m), 2.28-2.37 (1H, m), 3.48-3.52 (1H, m, CHS), 4.72 (2H, ap d, *J* = 11.5 Hz), 5.12 (2H, bs, NH_2), 7.45 (2H, ap d, *J* = 8.4 Hz), 7.79 (2H, ap d, *J* = 8.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 21.8, 26.7, 29.3, 33.2, 35.7, 39.3, 55.1, 72.9, 110.0, 127.5, 129.7, 139.6, 144.3, 149.2. MS (ESI positive) *m/z* (%): 364 $[M+Na]^+$, required $[M+Na]^+$ 364.10.

(*S*)-4-Methyl-*N*-(1-phenyl-3-((4-sulfamoylphenyl)thio)propan-2-yl)benzenesulfonamide (9):

Following the general procedure, disulfide 2 (37.5 mg, 0.1 mmol) and (*S*)-2-benzyl-1-tosylaziridine 8 (60 mg, 0.21 mmol) gave 9 (60 mg, 62%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.30 (3H, s), 2.66 (1H, dd, *J* = 6.7, 13.5 Hz), 2.81 (1H, dd, *J* = 6.5, 13.5 Hz), 2.96 (1H, dd, *J* = 5.8, 13.3 Hz), 3.01 (1H,

dd, *J* = 5.1, 13.3 Hz), 3.28-3.39 (1H, m, CHNH, overlapped with H_2O), 7.03-7.04 (2H, m), 7.10 (2H, ap d, *J* = 8.5 Hz), 7.15-7.19 (5H, m), 7.32 (2H, bs, NH_2), 7.43 (2H, ap d, *J* = 8.2 Hz), 7.60 (2H, ap d, *J* = 8.5 Hz), 7.97 (1H, d, *J* = 7.5 Hz, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 21.0, 35.7, 40.1 (overlapped with DMSO-d_6 signal), 54.2 (CHNH), 126.0, 126.3, 126.4, 126.5, 128.3, 129.2, 129.3, 137.4, 137.9, 140.8, 141.0, 142.4. MS (ESI negative) *m/z* (%): 475 $[M-H]^-$, required $[M-H]^-$ 475.09.

General Procedure for the preparation of thiol esters 10a-e from disulfide 2:

$NaBH_4$ (23 mg, 0.60 mmol, 3.0 eq.) was portionwise added to a solution of 4,4'-disulfanediyldibenzenesulfonamide 2 (75 mg, 0.20 mmol, 1.0 eq.) in EtOH (2 mL) at ambient temperature under inert atmosphere (N_2). After 2 h, the suitable acyl chloride (0.42 mmol, 2.1 eq.) was slowly added and the reaction mixture was stirred at room temperature for 1 h, until complete consumption of the starting material was observed by TLC. The reaction was quenched by addition of saturated *aq*. NH_4Cl (2 mL) and diluted with EtOAc (5 mL). The layers were separated and the aqueous layer was extracted with EtOAc (2 x 5 mL), dried over Na_2SO_4, filtered and concentrated under vacuum. The crude material was purified by flash chromatography to yield thiol esters 10a-e.

***S*-(4-Sulfamoylphenyl) octanethioate (10a):**

According to general procedure, using disulfide 2 (37.5 mg, 0.1 mmol) and capryloyl chloride (34 mg, 0.21 mmol) 10a (27 mg, 42%) was achieved as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.89 (3H, t, *J* = 6.8 Hz), 1.22-1.39 (8H, m), 1.66-1.76 (2H, m), 2.69 (2H, t, *J* = 7.5 Hz, CH_2(CO)), 4.94 (2H, bs, NH_2), 7.56 (2H, ap d, *J* = 8.5 Hz), 7.94 (2H, ap d, *J* = 8.5 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm):

14.0, 22.6, 25.5, 28.9, 31.6, 44.1, 127.0, 134.0, 134.7, 142.4, 196.0. MS (ESI positive) *m/z* (%): 338 $[M+Na]^+$, required $[M+Na]^+$ 338.09.

_S_-(4-Sulfamoylphenyl) octadecanethioate (10b):

According to general procedure, using disulfide 2 (37.5 mg, 0.1 mmol) and stearoyl chloride (64 mg, 0.21 mmol) 10b (49 mg, 54%) was achieved as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.88 (3H, t, *J* = 6.7 Hz), 1.22-1.27 (28H, m), 1.67-1.78 (2H, m), 2.69 (2H, t, *J* = 7.5 Hz, CH_2(CO)), 4.93 (2H, bs, NH_2), 7.56 (2H, ap d, *J* = 8.4 Hz), 7.94 (2H, ap d, *J* = 8.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 14.1, 22.7, 28.9, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 44.1, 127.0, 128.7, 134.0, 134.2, 134.7, 142.4, 196.0. MS (ESI positive) *m/z* (%): 478 $[M+Na]^+$, required $[M+Na]^+$ 478.24.

_S_-(4-Sulfamoylphenyl) (_Z_)-octadec-9-enethioate (10c):

According to general procedure, using disulfide 2 (37.5 mg, 0.1 mmol) and oleoyl chloride (63 mg, 0.21 mmol) 10c (55 mg, 61%) was achieved as a waxy white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.88 (3H, t, *J* = 6.7 Hz), 1.21-1.41 (20H, m), 1.66-1.75 (2H, m), 1.95-2.07 (4H, m), 2.68 (2H, t, *J* = 7.5 Hz, CH_2(CO)), 5.20 (2H, bs, NH_2), 5.30-5.40 (2H, m), 7.53 (2H, ap d, *J* = 8.3 Hz), 7.92 (2H, ap d, *J* = 8.3 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 14.1, 27.1, 27.2, 28.9, 29.0, 29.1, 29.3, 29.5, 29.6, 29.7, 31.9, 44.0, 126.9, 129.6, 130.0, 133.7, 134.7, 142.5, 196.2. MS (ESI positive) *m/z* (%): 476 $[M+Na]^+$, required $[M+Na]^+$ 476.23.

_S_-(4-Sulfamoylphenyl) (_E_)-3-phenylprop-2-enethioate (10d):

According to general procedure, using disulfide 2 (75 mg, 0.2 mmol) and cinnamoyl chloride (70 mg, 0.4 mmol) 10d (74 mg, 58%) was achieved as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.18 (1H, d, *J* = 15.8), 7.44-7.49 (3H, m), 7.51 (2H, bs), 7.70 (2H, ap d, *J* = 8.3 Hz), 7.72 (1H, d, *J* = 15.8), 7.81-7.83 (2H, m), 7.91 (2H, ap d, *J* = 8.3 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 124.1, 126.3, 129.0, 131.2, 131.6, 133.6, 134.8, 142.2, 144.9, 186.2. MS (ESI negative) *m/z* (%): 318 $[M-H]^-$, required $[M-H]^-$ 318.03.

***S*-(4-Sulfamoylphenyl) 2,4-bis(trifluoromethyl)benzothioate (10e):**

According to general procedure, using disulfide 2 (37.5 mg, 0.1 mmol) and 2,4-Bis(trifluoromethyl)benzoyl chloride (58 mg, 0.21 mmol), 10e (40 mg, 46%) was achieved as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.56 (2H, bs NH_2), 7.80 (2H, ap d, J = 8.4 Hz), 7.99 (2H, ap d, J = 8.4 Hz), 8.26 (1H, ap d, J = 8.1 Hz), 8.34 (1H, ap d, J = 8.1 Hz), 8.31 (1H, s). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 123.0 (d, 1JC-F = 273 Hz, CF3), 125.2 (d, $^{1}J_{C\text{-}F}$ = 271 Hz, CF_3), 125.6 (bs), 127.6 (q, $^{2}J_{C\text{-}F}$ = 22 Hz, CCF_3), 127.8, 131.1, 131.2, 131.6 (bs), 133.4 (q, $^{2}J_{C\text{-}F}$ = 33 Hz, CCF_3), 136.1, 140.7, 146.8, 190.3. ^{19}F NMR (376 MHz, DMSO-d_6) δ (ppm): -61.7, -57.5. MS (ESI negative) *m/z* (%): 428 $[M\text{-}H]^-$, required $[M\text{-}H]^-$ 427.99.

Synthesis of 4,4'-disulfanediylbis(N-(4-sulfamoylphenyl) benzenesulfonamide) (11a)

4,4'-Disulfanediyldibenzenesulfonyl chloride 1 (83 mg, 0.2 mmol) was solubilized in THF (5 mL) and treated with sulfanilamide (69 mg, 0.4 mmol). The reaction was stirred at ambient temperature for 30 min; the solid was then filtered, washed with Et_2O (5 mL) and dried under vacuum to afford 11a (49 mg, 36%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.40 (4H, bs, NH_2), 7.53-7.57 (10H, m), 7.85- 7.95 (8H, m). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm):123.7, 127.1, 128.1, 128.5, 130.4, 136.7, 140.3, 143.3.

Synthesis of 4,4'-Disulfanediylbis(*N*-(4-sulfamoylbenzyl) benzenesulfonamide) (11b):

4,4'-Disulfanediyldibenzenesulfonyl chloride 1 (83 mg, 0.2 mmol) was solubilized in THF (5 mL) and treated with 4-aminomethylbenzenesulfonamide hydrochloride (89 mg, 0.4 mmol). The reaction was stirred at ambient temperature for 30 min; the solid was then filtered, washed with Et_2O (5 mL) and dried under vacuum to afford 11b (75 mg, 53%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.07 (4H, d, J = 6.2 Hz, CH_2N), 7.31 (4H, bs, NH_2), 7.43 (4H, d, J = 8.3 Hz), 7.75 (4H, d, J = 8.3 Hz), 7.75 (4H, d, J = 8.6 Hz), 7.83 (4H, d, J = 8.6 Hz), 8.31 (2H, t, J = 6.2 Hz, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 46.6, 126.7, 127.8, 128.7, 128.9, 140.6, 141.6, 142.9, 144.1.

Synthesis of 4,4'-Disulfanediylbis(*N*-(4-sulfamoylphenethyl) benzenesulfonamide) (11c):

4,4'-Disulfanediyldibenzenesulfonyl chloride 1 (42 mg, 0.1 mmol) was solubilized in THF (3 mL) and treated with 4-(2-aminoethyl)benzenesulfonamide (40 mg, 0.2 mmol). The reaction was stirred at ambient temperature for 30 min; the solid was then filtered, washed with Et_2O (5 mL) and dried under vacuum to afford 11c (24 mg, 32%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.76 (4H, t, J = 7.1 Hz), 2.97-3.02 (4H, m), 7.31 (4H, bs), 7.35 (4H, ap d, J = 8.3 Hz), 7.69-7.69 (12H, m), 7.80 (2H, bt, J = 5.6 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 36.0, 44.6, 126.7, 127.8, 128.7, 130.3, 140.3, 141.5, 143.3, 143.9.

4.2 Carbonic anhydrase inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity.[20] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (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 assay, in order to allow for 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,[21-26] and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.[21-26]

4.3 Crystallization and X-ray data collection

Crystals of hCAII were obtained using the hanging drop vapor diffusion method using 24 well Linbro plate. 2 µl of 10 mg/ml solution of hCA II in Tris-HCl 20 mM pH 8.0 were mixed with 2 µl of a solution of 1.5 M sodium citrate, 0.1 M Tris pH 8.0 and were equilibrated against the same solution at 296 K. Crystals of the protein grew in one week. Afterwards hCAII crystals were soaked in 5mM inhibitor solution for 3 days.

The crystals were flash-frozen at 100K using a solution obtained by adding 15% (v/v) glycerol to the mother liquor solution as cryoprotectant. Data on crystals of the complexes were collected using synchrotron radiation at the ID29 beamline at ESRF (Grenoble, France) with a wavelength of 0.827 Å and a PILATUS 6M Dectris CCD detector. Data were integrated and scaled using the program XDS.[27] Data processing statistics are shown in supporting information.

4.4 Structure determination

The crystal structure of hCA II (PDB accession code: 4FIK) without solvent molecules and other heteroatoms was used to obtain initial phases of the structures using Refmac5.[28] 5% of the unique reflections were selected randomly and excluded from the refinement data set for the purpose of Rfree calculations. The initial |Fo - Fc| difference electron density maps unambiguously showed the inhibitor molecules. The inhibitor was introduced in the model with 0.5 occupancy. In the |Fo - Fc| difference electron density map a spheric density was present close to the benezensulphonamide moiety and was interpreted as a solvent molecule at 0.5 occupancy.

Atomic models for inhibitors were calculated and energy minimized using the program JLigand 1.0.40.[29] Refinements proceeded using normal protocols of positional, isotropic atomic displacement parameters alternating with manual building of the models using COOT.[30] Solvent molecules were introduced automatically using the program ARP.[31] The quality of the final models are assessed with COOT and

RAMPAGE.[32] Crystal parameters and refinement data are summarized in Electronic Supplementary Information (†ESI). Atomic coordinates were deposited in the Protein Data Bank (PDB accession code: 6GOT). Graphical representations were generated with Chimera.[33]

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Highlights

1. Synthesis of novel different thio-scaffolds bearing sulfonamide as inhibitors of carbonic anhydrase are reported
2. *In vitro* studies reported potent and selective inhibition activity against hCA II and IX isoforms.
3. Determined X-ray structure of hCA II in complex with one compound in order to obtain ligand−protein interaction.