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o-Benzenedisulfonimido–sulfonamides are potent inhibitors of the tumor-associated carbonic anhydrase isoforms CA IX and CA XII

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ABSTRACT

By using phthalimido-substituted aromatic sufonamides as lead molecules, a series of new sulfonamides incorporating *ortho*-benzenedisulfonimide moieties have been synthesized and tested against the human (h) cytosolic carbonic anhydrase (CA, EC 4.2.1.1) isozymes hCA I and hCA II and the transmembrane, tumor-associated isozymes hCA IX and hCA XII. All these compounds showed K_i values lower than 100 nM and many of them showed better K_is than the reference compound acetazolamide, a clinically used sulfonamide. The tumor-associated isozymes were better inhibited than the cytosolic ones. A molecular docking within the active site of some CA isoforms, such as hCA I, explained these findings, as the benzenedisulfonimide moiety makes favorable interactions (hydrogen bonds) with amino acid residues involved in binding of inhibitors, such as Gln92, His67, and His64.

1. Introduction

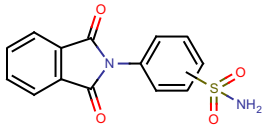
Carbonic anhydrases (CAs, EC 4.2.1.1) are a superfamily of metalloenzymes that mainly catalyze the interconversion between CO_2 and bicarbonate (HCO_3^-).[1–4] There are 16 mammalian CA isoforms that belong to the α-class enzymes which all have a Zn^{2+} ion in the active site. Even though, these CA isozymes share similarity in structure and active site composition, there are differences in the overall structure, expression and their putative role in (patho)physiology.[1–4]

The human CA isoforms I and II (hCA I and hCA II, respectively) are cytosolic enzymes that are widespread throughout the human body. They are drug targets for clinically used diuretics, antiglaucoma drugs and anticonvulsants.[1–4]

In contract, the isoforms IX and XII (hCA IX and hCA XII, respectively) are transmembrane enzymes with the active site on the extracellular side of the cell membrane. These enzymes are mainly found in hypoxic tumors.[1,3] They are validated drug targets for the design anticancer agents specific for solid, hypoxic tumors.[1]

Sulfonamides constitute the main class of CA inhibitors (CAIs)[1–4] and many different families of such compounds have been reported to possess significant inhibitory power against many isoforms.[5–10]

Among the interesting classes of such derivatives investigated earlier[11] were the phthalimido sulfonamides of types **A**, which showed interesting CA I, II and IV inhibitory power and were easily obtained by reaction of amino-containing aromatic/heterocyclic sulfonamides with phthalic anhydride.



In our current work, continuing our interest in sulfonamides as CAIS, and considering compounds A as lead mlecules, we have synthesized six new compounds by reaction of *o*-benzenedisulfonyl chloride with amino-sulfonamides, obtaining the disulfonamido analogs of compounds **A**. We were interested to see whether substituting the phthalimido moiety with the *o*-benzenedisulfonimido one has important consequences for the interaction of these sulfonamides with the various CA isoforms of medicinal chemistry interest, such as CA I, II, IX and XII.

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2. Results and discussion

2.1. Chemistry

The rationale of the present drug design study was to replace the CO moieties from the phthalimido sulfonamides **A** reported earlier[11] by the SO_2 ones. In fact the presence of four oxygen in the disulfonamides reported here compared to the two ones present in the phthalimides reported earlier, may lead to a supplementary stabilization of the enzyme-inhibitor adduct and thus to better CAIs. The simple chemistry outlined below was employed to prepare the new compounds reported here.

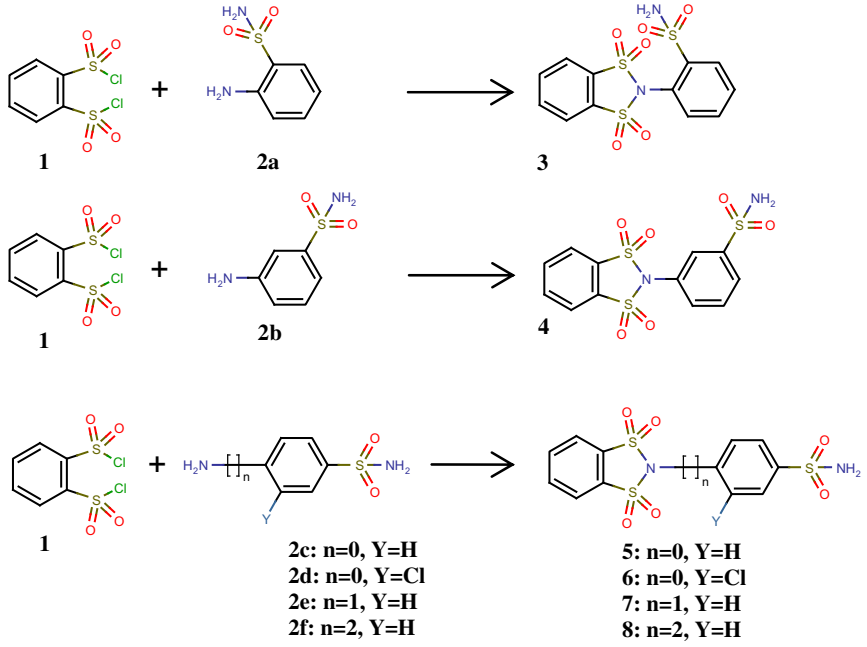
Reaction of *o*-benzenedisulfonyl chloride **1** with aminosulfonamides **2a–f**, led to the formation of o-benzenedisulfonimides **3–8** (Scheme 1). The new compounds **3–8** were characterized by spectroscopic and analytic methods which confirmed their structures (see Section 4).

We have included variously substituted benzenesulfonamides (*ortho, meta* and *para* such derivatives) and different spacers between the benzenesulfonamide head and the *ortho*-benzenedisulfonamide scaffold of compounds **3–8** reported here, as shown in Figure 1.

2.2. Carbonic anhydrase inhibition

The newly synthesized sulfonamides **3–8** as well as the reference compound **AZA** have been tested for their enzyme inhibition activities against human CA isoforms hCA I, hCA II, hCA IX and hCA XII (Table 1). All tested compounds have K_i values in the lower nM region (K_i <100 nM, see Table 1). Interestingly, the overall difference between the lowest and highest K_i values are only 13-fold (i.e., K_i value of compound **8** for hCA I = 7.2 nM; K_i value of compound **3** for hCA II = 95.6 nM; Table 1).

The hCA I enzyme was inhibited well by all tested compounds (Table 1) and the worst inhibition was observed for compounds **3** and **5** (K_i = 51.4 and 48.5 nM, respectively). Compound **4**, which has a sulfonamide group on the *meta* positon (Fig. 1), showed a slight decrease in K_i value compared to compounds **3** and **5**. The



Scheme 1. Preparation of *o*-benzenedisulfonimide derivatives. Reagents and conditions: Et_3N, CH_2Cl_2, reflux.

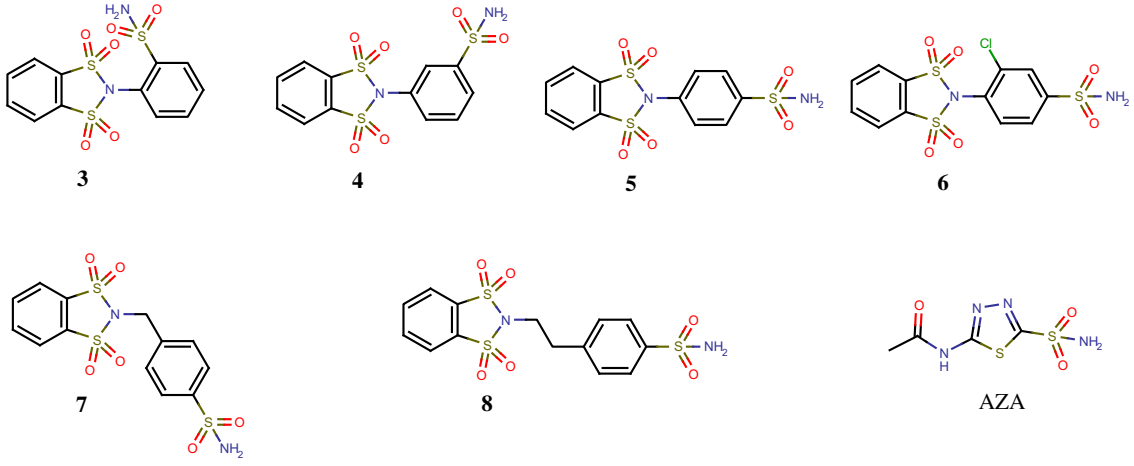


Figure 1. The chemical structures of sulfonamides **3–8** and the reference compound acetazolamide (AZA).

Table 1
Inhibition of human CA isoforms hCA I, hCA II, hCA IX and hCA XII with sulfonamides **3–8**

Compounds	K_i[a] (nM)			
	hCA I[b]	hCA II[b]	hCA IX[b]	hCA XII[b]
3	51.4 ± 4	95.6 ± 8	66.9 ± 7	50.5 ± 4
4	31.2 ± 3	61.7 ± 5	32.2 ± 2	71.5 ± 5
5	48.5 ± 5	54.8 ± 6	82.5 ± 6	19.0 ± 2
6	35.8 ± 4	61.7 ± 5	17.1 ± 2	34.9 ± 3
7	26.5 ± 3	28.2 ± 3	49.8 ± 4	31.4 ± 1
8	7.2 ± 0.6	27.1 ± 1	86.0 ± 7	32.0 ± 3
AZA	250.0 ± 15	12.1 ± 1	25.4 ± 3	5.6 ± 0.2

AZA has been included as a reference compound.
[a] Mean ± standard error, from three different assays, by a CO_2 hydration stopped-flow assay.
[b] Human, recombinant isozymes, pH 7.5, 20 mM Tris·HCl buffer.

latter two compounds have the sulfonamide group on the *ortho* and *para* positions respectively. The addition of a chlorine atom to the *ortho* position of compound **5** (to yield compound **6**) again decreased the K_i value (Table 1). Even though the differences in K_i value between compounds **3–6** were rather small, the data might suggest that either a sulfonamide group or a chlorine group on the meta position is well tolerated.

Compounds **7** and **8** having methylene and ethylene groups, respectively, as spacers between the phenyl and benzenedisulfonamide groups (Fig. 1). In contrast, compound **5** had a direct bond between the two moieties (Fig. 1). Increasing the size of this spacer led to a decrease in the K_i value of the compounds (compound **5**: K_i = 48.5 nM; compound **7**: K_i = 26.5 nM; compound **8**: K_i = 7.2 nM). This difference in inhibition is almost 7-fold between compounds **5** and **8** (Table 1).

The human hCA II enzyme showed a different inhibition profile for the tested compounds. The K_i values for compounds **3–6** show that the sulfonamide and chlorine substituents in the *ortho* position (compound **3** and **6**) and a sulfonamide substituent in the *meta* position (compound **4**) were less well tolerated compared to a sulfonamide substituent in the *para* position (compound **5**; Table 1). The addition of either a methyl spacer (compound 7) or an ethylene spacer (compound **8**) between the phenyl and benzenedisulfonamide moieties of compound **3** decreased the K_i value (Table 1). However, the K_i values of compounds **7** and **8** were similar to each other and no difference was observed related to the spacer length (compound **7**: K_i = 28.2 nM; compound **8**: K_i = 27.1 nM).

Some of the new compounds reported here, tested on the tumor-associated hCA IX showed similar enzyme inhibition than the reference compound **AZA** (Table 1). This enzyme showed a similar inhibition profile for compounds **3–6** as hCA I, but with larger changes in the K_i values (Table 1). The sulfonamide group was best tolerated on the *meta* position (compound **4**, K_i = 32.2 nM) compared to the *ortho* and *para* positions (compounds **3**, K_i = 66.9 nM and **5**, K_i = 82.5 nM). However, adding a chlorine group to compound **5** (to yield compound **6**) decreased the K_i value approximately 5-fold (Table 1). This difference was only approximately 1.4-fold for the human hCA I enzyme.

Growing the spacer length of compound **5** with a methylene between the phenyl and benzenedisulfonamide moieties increased the K_i value (Table 1). In contrast, addition of an ethylene group to compound **3** did not increase the K_i value (Table 1).

Finally, the compounds have been tested for the inhibition of the second the human, tumor-associated enzyme hCA XII (Table 1). The inhibition data of compounds **3–6** show that the *para* position (compound **5**) is the best location for the sulfonamide substituent compared to the *ortho* and *meta* positions (Table 1). Adding a chlorine substituent on compound **5** to yield compound **6** did not decrease the K_i value. In addition, increasing the size of the spacer between the phenyl and benzenedisulfonamide groups increased the K_i value (Table 1).

In summary, all four human CA enzymes respond differently to compounds **3–8** in enzyme inhibition assays. The K_i values of all compounds are in the lower nanomolar range. The lowest K_i value is observed for compound **8**, which inhibits hCA I with a K_i value of 7.2 nM. This compound shows a 3.8-fold higher K_i value for hCA II, a 12-fold higher K_i value for hCA IX and a 4.4-fold higher K_i value for hCA-XII compared to hCA I.

2.3. Docking studies

The difference between the lowest and highest measured K_i values per enzyme for compounds **3–8** are relatively small, that is, approximately 7-fold for hCA I, 3.5-fold for hCA II, 5-fold for hCA IX and 3.7-fold for hCA XII (Table 1). Nevertheless, docking studies have been performed to rationalize the enzyme inhibition data of compounds **3–8**. To this end, the GOLD Suite docking programme[12] was used with the GoldScore scoring function. The sulfonamide substituent on the phenyl moiety was assigned a negative charge ($-SO_2NH^-$) to facilitate its interaction with the Zn^{2+} of the enzyme active site.

The crystal structure of hCA I in complex with topiramate (PDB: 3LXE; 1.90 Å) has been used as a template in our docking studies for hCA I. The docking results suggests that compound **4** can relatively easy interact with the Zn^{2+} ion through its meta-sulfonamide substitution on the phenyl moiety. This phenyl group of the ligand also forms hydrophobic interactions with His200. In addition, the benzodithiazole group of compound **4** forms hydrophobic interactions with Trp5 and His64. Compounds **3** and **5** have slightly more difficulties in forming these interactions with the hCA I active site compared to compound **4**, but the difference in docking score is small.

Adding a methyl group between the benzodithiazole group and the phenyl moiety (compound **7**), positions one of the sulfoxide functionalities of the benzodithiazole group near Gln92 and hydro-

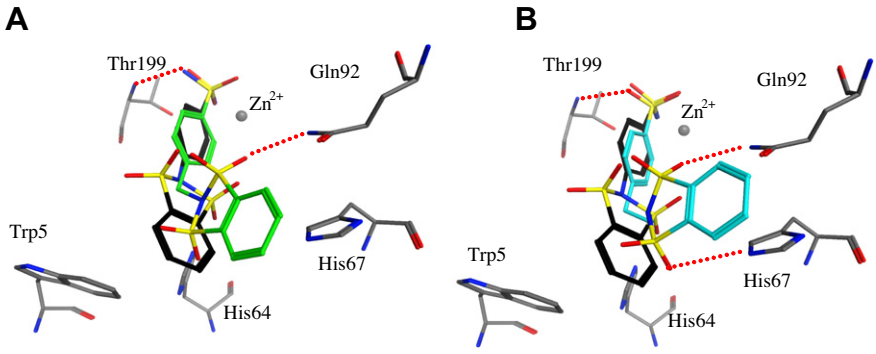


Figure 2. The docked poses of compounds **7** (panel A, green) and **8** (panel B, light blue) in the active site of hCA I. The docked pose of compound **5** (shown in black) is shown in both panels. Hydrogen bonds are depicted in red dashed lines.

gen bonds are formed (Fig. 2A). An ethyl group as a spacer (compound **8**) places the sulfoxide groups near His67 and Gln92 and two hydrogen bonds are formed (Fig. 2B). This is consistent with the fact that the lowest K_i value is observed for compound **8** on hCA I (Table 1).

For the hCA II enzyme, the crystal structure of hCA II in complex with 2-(hydrazinocarbonyl)-3-phenyl-1*H*-indole-5-sulfonamide was used (PDB: 3B4F; 1.89 Å). Compounds **4** and **5** form hydrogen bonds with the side chains of Asn62 and Asn67. These interactions are not present in the binding interactions of compound **3** and hence the K_i value is higher (Table 1). Adding a chlorine substituent to compound **5** does not influence the binding mode, because the active site is large enough to accommodate the chlorine atom.

Increasing the spacer size of compound **5** with either a methyl or an ethyl group places the benzodithiazole groups of compounds **7** and **8** close to the side chain of Gln92 and hydrogen bonds are formed with both ligands.

Docking studies for the hCA IX enzyme were performed using the 3IAI crystal structure (2.20 Å). No clear indications have been found for the difference in K_i value between compounds **3**–**8**. Most of the compounds seem to interact with Asn62 and Gln92. Increasing the spacer size allows for additional interactions with Trp5 and His64.

The final docking was performed using the hCA XII structure in complex with acetazolamide (PDB: 1JD0; 1.50 Å). Again, no clear indications have been found for the measured K_i values of compounds **3**–**8**. It seems however that compound **5** could form hydrogen bonding interactions between its sulfoxide group and the side chain of Gln92.

2.4. Structural comparisons of CA binding pockets

In addition to the docking studies, we compared the overall backbone structures and the binding pockets of the four enzymes (Table 2) using the MOE software package (version 2011.10, CCG, Montreal, Canada). The four crystal structures superpose well on their backbone Cα-atoms (RMSD: 1.560 Å for 260 residues), especially near the binding pocket (RMSD: 0.637 Å for 24 residues; pocket is defined as all residues within 4.5 Å of the ligand in 3LXE and their counterparts in the other structures). Nevertheless, some differences in the backbone orientations of the four enzyme structures are present near the conserved residues Trp5 and His64.

Gln92 of hCA I is conserved in all four crystal structures (Table 2). Even though the conformation of the Gln92 side chain is different in the four structures (RMSD: 0.438 Å for all Gln92 heavy atoms), this residue is able to present its side chain nitrogen or oxygen atom to ligands for hydrogen bonding. Therefore, this residue is involved in most docking poses that are obtained for the four enzyme structures.

His67 of hCA I is also involved in hydrogen bonding in the docking poses. This residue is not conserved amongst the four enzyme structures and its counterparts in the other enzymes differ with respect to their shape, size and charge (Table 2).

His200 of hCA I forms hydrophobic interactions with the phenyl group of the ligands. In hCA II, hCA IX and hCA XII this residue is Thr200.

Table 2
Differences and similarities in the active sites of the four enzymes

hCA I	hCA II	hCA IX	hCA XII
Trp5	Trp5	Trp5	Trp5
Ile60	Leu60	Arg60	Thr60
Val62	Asn62	Asn62	Asn62
His64	His64	His64	His64
His67	Asn67	Gln67	Lys67
Asn69	Glu69	Thr69	Asn69
Gln92	Gln92	Gln92	Gln92
His200	Thr200	Thr200	Thr200

The docked poses of compounds **4** and **5** in hCA II form hydrogen bonds with Asn62 and Asn67. Asn62 is also present in hCA IX and hCA XII, but not in hCA I (Val62, Table 2). Asn67 (hCA II) is the counterpart of His67 (hCA I), Gln67 (hCA IX) and Lys67 (hCA XII).

It should be noted that differences in ligand recognition and binding are not only dependent on the residues that directly line the binding pocket but also on more distant residues. In addition, charged residues that are not in direct contact with the ligand, such as Arg60 (hCA IX), Lys67 (hCA XII) and Glu69 (hCA II), can also influence binding processes.

3. Conclusions

In summary, six structurally new sulfonamides have been synthesized and tested against the cytosolic hCA I and hCA II isozymes and the tumor-associated hCA IX and hCA XII isozymes. All measured K_i values were lower than 100 nM for all four hCA isozymes. Several of the new sulfonamides showed better enzyme inhibition than the reference sulfonamide in clinical use acetazolamide.

4. Materials and methods

4.1. Chemistry

Melting points were estimated with a Buchi 540 melting point apparatus in open capillaries and are uncorrected. Elemental analyses were performed on a Thermo Finnigan Flash EA 1112 elemental analyzer. IR spectra were recorded on KBr discs, using a Perkin–Elmer Model 1600 FT-IR spectrometer. ^{1}H NMR and HSQC-2D spectra were obtained on Bruker Avance DPX 400 and Varian UNITY INOVA 500 spectrophotometers using DMSO-d_6. Mass spectra were determined on a Mass-AGILENT 1100 MSD instruments. All chemicals and solvents were purchased from Merck–Schuchardt.

4.2. General procedure for the preparation of *o*-benzenedisulfonimide derivatives (3–8)[13]

To a refluxing solution containing 10 mmol of *o*-benzenedisulfonyl chloride **1**, in 20 mL of dry CH_2Cl_2 was added a solution of 11 mmol of substituted benzenesulfonamide **2** and 1.0 mL of Et_3N in 25 mL of CH_2Cl_2 over a period of 60 min. The resulting mixture was stirred overnight at room temperature. The reaction mixture was then washed with 0.1 M aqueous HCl, 5% $NaHCO_3$, water and finally dried over anhydrous $MgSO_4$. Filtration and evaporation of the solvent under reduced pressure gave the crude product as a solid material. Recrystallization from ethanol.

4.2.1. 2-(1,1,3,3-Tetraoxido-1,3,2-benzodithiazol-2-yl)benzenesulfonamide (3)

Mp 251–252 °C; IR(KBr) (ν, cm^{-1}), 3236, 3352 (NH), 1172, 1354 (SO_2); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 7.40 (2H, s, SO_2NH_2), 7.44 (1H, dd, J = 8.79, 1.46 Hz, phenyl C_3–H), 7.75 (1H, td, J = 7.80, 1.95 Hz, phenyl C_5–H), 7.82 (1H, td, J = 8.05, 1.46 Hz, phenyl C_4–H), 8.12 (1H, dd, J = 7.56, 1.46 Hz, phenyl C_6–H), 8.14 (2H, dd, J = 5.86, 2.93 Hz, benzothiazole $C_{5,6}$–H), 8.47 (2H, dd, J = 5.37, 2.93 Hz, benzothiazole $C_{4,7}$–H). Anal. Calcd for $C_{12}H_{10}N_2O_6S_3$ (374.413): C, 38.49; H, 2.69; N, 7.48; S, 25.69. Found: C, 38.37; H, 2.68; N, 7.50; S, 25.42.

4.2.2. 3-(1,1,3,3-Tetraoxido-1,3,2-benzodithiazol-2-yl)benzenesulfonamide (4)

Mp 212–214 °C; IR(KBr) (ν, cm^{-1}), 3286, 3371 (NH), 1165, 1352 (SO_2); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 7.69 (2H, s, SO_2NH_2), 7.83 (1H, dd, J = 7.56, 1.47 Hz, phenyl C_4–H), 7.92 (1H, t, J = 7.81 Hz,

phenyl C_5–H), 8.00 (1H, t, J = 1.95 Hz, phenyl C_2–H), 8.17 (1H, d, J = 7.81 Hz, phenyl C_6–H), 8.22 (2H, dd, J = 5.86, 3.42 Hz, benzothiazole $C_{5,6}$–H), 8.61 (2H, dd, J = 5.86, 3.42 Hz, benzothiazole $C_{4,7}$–H). Anal. Calcd for $C_{12}H_{10}N_2O_6S_3$ (374.413): C, 38.49; H, 2.69; N, 7.48; S, 25.69. Found: C, 38.83; H, 2.79; N, 7.40; S, 25.91.

4.2.3. 4-(1,1,3,3-Tetraoxido-1,3,2-benzodithiazol-2-yl)benzenesulfonamide (5)

Mp 234–236 °C; IR(KBr) (ν, cm^{-1}), 3280, 3373 (NH), 1161, 1363 (SO_2); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 7.60 (2H, s, SO_2NH_2), 7.81 (2H, d, J = 8.78 Hz, phenyl $C_{3,5}$–H), 8.11 (2H, d, J = 8.30 Hz, phenyl $C_{2,6}$–H), 8.22 (2H, dd, J = 5.85, 2.93 Hz, benzothiazole $C_{5,6}$–H), 8.60 (2H, dd, J = 5.86, 3.42 Hz, benzothiazole $C_{4,7}$–H). ESI (−)m/z (%): 373 (MH^-, 19). Anal. Calcd for $C_{12}H_{10}N_2O_6S_3$ (374.413): C, 38.49; H, 2.69; N, 7.48; S, 25.69. Found: C, 38.32; H, 2.78; N, 7.43; S, 25.76.

4.2.4. 3-Chloro-4-(1,1,3,3-tetraoxido-1,3,2-benzodithiazol-2-yl)benzenesulfonamide (6)

Mp 211–212 °C; IR(KBr) (ν, cm^{-1}), 3248, 3325 (NH), 1176, 1359 (SO_2); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 7.71 (2H, s, SO_2NH_2), 7.91 (1H, d, J = 8.30 Hz, phenyl C_5–H), 8.02 (1H, dd, J = 8.30, 2.44 Hz, phenyl C_6–H), 8.18 (1H, d, J = 2.44 Hz, phenyl C_2–H), 8.22 (2H, dd, J = 5.85, 2.93 Hz, benzothiazole $C_{5,6}$–H), 8.61 (2H, dd, J = 5.86, 2.93 Hz, benzothiazole $C_{4,7}$–H). Anal. Calcd for $C_{12}H_9ClN_2O_6S_3$ (408.858): C, 35.25; H, 2.22; N, 6.85; S, 23.53. Found: C, 35.02; H, 2.22; N, 6.47; S, 23.21.

4.2.5. 4-[(1,1,3,3-Tetraoxido-1,3,2-benzodithiazol-2-yl)methyl]benzenesulfonamide (7)

Mp 154–155 °C; IR(KBr) (ν, cm^{-1}), 3271, 3367 (NH), 1176, 1355 (SO_2); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 5.03 (2H, s, N-CH_2–), 7.37 (2H, s, SO_2NH_2), 7.63 (2H, d, J = 7.81 Hz, phenyl $C_{3,5}$–H), 7.85 (2H, d, J = 7.81 Hz, phenyl $C_{2,6}$–H), 8.16 (2H, dd, J = 5.86, 3.42 Hz, benzothiazole $C_{5,6}$–H), 8.48 (2H, dd, J = 5.85, 3.41 Hz, benzothiazole $C_{4,7}$–H). Anal. Calcd for $C_{13}H_{12}N_2O_6S_3$ (388.439): C, 40.20; H, 3.11; N, 7.21; S, 24.76. Found: C, 40.35; H, 2.99; N, 7.24; S, 24.98.

4.2.6. 4-[2-(1,1,3,3-Tetraoxido-1,3,2-benzodithiazol-2-yl)ethyl]benzenesulfonamide (8)

Mp 193–194 °C; IR(KBr) (ν, cm^{-1}), 3253, 3388 (NH), 1168, 1348 (SO_2); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 3.18 (2H, t, J = 6.83 Hz, CH_2), 3.98 (2H, t, J = 6.83 Hz, N-CH_2), 7.29 (2H, s, SO_2NH_2), 7.51 (2H, d, J = 8.29 Hz, phenyl $C_{3,5}$–H), 7.75 (2H, d, J = 8.29 Hz, phenyl $C_{2,6}$–H), 8.13 (2H, dd, J = 5.86, 2.93 Hz, benzothiazole $C_{5,6}$–H), 8.42 (2H, dd, J = 5.85, 2.93 Hz, benzothiazole $C_{4,7}$–H); ^{13}C NMR (HSQC-2D, DMSO-d_6, 125 MHz) δ (ppm): 34.66 (–CH_2-Ph), 43.47 (N-CH_2–), 123.60 (benzothiazole $C_{4,7}$), 126.43 (phenyl $C_{2,6}$), 130.20 (phenyl $C_{3,5}$), 137.08 (benzothiazole $C_{5,6}$), 134.58 (phenyl C_1), 141.93 (benzothiazole $C_{3a,7a}$), 143.38 (phenyl C_4). ESI (−) m/z (%): 401 (MH–, 100). Anal. Calcd for $C_{14}H_{14}N_2O_6S_3$ (402.46): C, 41.78; H, 3.51; N, 6.96; S, 23.90. Found: C, 41.95; H, 3.42; N, 6.81; S, 23.52.

4.3. CA inhibition assay

An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic/inhibition of various CA isozymes as reported by Khalifah.[14] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nM, with 10 mM Hepes (pH 7.4) as buffer, 0.1 M Na_2SO_4 or $NaClO_4$ (for maintaining constant the ionic strength; these anions are not inhibitory in the used concentration),[15] following the CA-catalyzed CO_2 hydration reaction for a period of 5–10 s. Saturated CO_2 solutions in water at 25 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in DMSO–water 1:1, v/v) and dilutions up to 0.01 nM done with the assay buffer mentioned above. At least seven different inhibitor concentrations have been used for measuring the inhibition constant. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[16,17] and represent the mean from at least three different determinations. Human CA isozymes were prepared in recombinant form as reported earlier by our groups.[18,19]

4.4. Ligand preparation

Compounds **3**–**8** and the reference ligand AZA were built and converted to three-dimensional structures using the MOE software package (version 2011.10, Chemical Computing Group, Montreal, Canada). The sulfonamide substituent of the phenyl group was assigned a negative charge (–SO_2NH^-) because it was expected to interact with the Zn^{2+} ion of the CA active sites. Subsequently, partial atomic charges were calculated and the molecules were energy-minimized according to a steepest-descent protocol using the MMFF94x force field in MOE. The ligands were saved as a multi-mol2 file.

4.5. Template preparation

Crystal structures of hCA I (PDB: 3LXE; 1.90 Å),[20] hCA II (PDB: 3B4F; 1.89 Å),[19] hCA IX (PDB: 3IAI; 2.20 Å)[21] and hCA XII (PDB: 1JD0; 1.50 Å)[22] were obtained from the protein databank. Only 1 protein chain (chain A) and its corresponding Zn^{2+} ion and ligand (CA inhibitor) was retained per PDB file and all other protein chains, ions, buffer molecules and water molecules were deleted. Hydrogen atoms and charges were added to the protein using the protonate 3D tool of the MOE software package (version 2011.10, CCG, Montreal, Canada) and a steepest descent energy minimization was applied (MMFF94x forcefield). All proteins were superposed on the hCA-I structure using their Cα-atoms (RMSD: 1.411 Å) and the proteins were saved as mol2-files.

4.6. Docking studies

Compounds **3**–**8** and the reference ligand AZA were docked into the protein models of hCA I, hCA II, hCA IX and hCA XII using the GOLD Suite docking package (version 5.1, CCDC, Cambridge, UK)[12] and the GoldScore scoring function (25 docking per ligand). The binding pocket was defined as all residues within 12 Å of the central carbon atom of the CA inhibitor topiramate (atom CAL of topiramate in complex with hCA I; PDB: 3LXE). No restrictions were applied for the ligand conformations and binding poses during the docking procedure except for the requirement to form hydrogen bonding with Thr199 (or its counterparts in other CA isozymes).

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