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Carbonic anhydrase inhibitors: Synthesis and inhibition of the human carbonic anhydrase isoforms I, II, IX and XII with benzene sulfonamides incorporating 4- and 3-nitrophthalimide moieties

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ABSTRACT

A series of 4 and 5 nitro-1,3-dioxoisoindolin-2-yl benzenesulfonamide derivatives (compounds **1**–**8**) was synthesized by reaction of benzenesulfonamide derivatives with 4 and 3-nitrophthalic anhydrides. These new sulfonamides were investigated as inhibitors of the zinc metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) and more specifically against the human (h) cytosolic isoforms hCA I and II and the transmembrane, tumor-associated hCA IX and XII. Most of the novel compounds were medium potency-weak hCA I inhibitors (K_is in the range of 295–10,000 nM), but were more effective hCA II inhibitors (K_is of 1.7–887 nM). The tumor-associated hCA IX was also inhibited, with K_is in the micromolar range, whereas against hCA XII the inhibition constants were in the range of 90–3746 nM. The structure–activity relationship (SAR) with this series of sulfonamides is straightforward, with the main features leading to good activity for each isoforms being established. The high sequence hCA alignment homology and molecular docking studies was performed in order to rationalize the activities reported and binding mode to different hCA as inhibitors.

1. Introduction

Carbonic anhydrases (CAs) also known as carbonate dehydratases are zinc (Zn^{+2}) metalloenzymes present in almost all living organism.[1,2] There are five genetically distinct CA families; α-CAs (vertebrates, bacteria, algae and cytoplasm of green plants); the β-CAs (bacteria, algae and chloroplasts of monocotyledons and dicotyledons); the γ-CAs (archaea and some bacteria); the δ-CAs (diatoms and other marine eukaryotes) and ζ-CAs (diatoms).[1,3] Human CAs (hCAs) all belong to the α-family and are present as fifteen isoforms, which differ by molecular features, oligomeric arrangement, cellular localization, distribution in organs and tissues, expression levels, and kinetic properties (Table 1).[2] There are five cytosolic forms (CA I, CA II, CA III, CA VII and CA XIII), five membrane associated (CA IV, CA IX, CA XII, CA XIV and CA XV), two mitochondrial (CA VA and CA VB), and a secreted CA isozyme (CA VI).[1–3] There are three additional 'acatalytic' CA isoforms (CA VIII, CA X, and CA XI) which are also cytosolic.[3]

CAs catalyze the interconversion between carbon dioxide and bicarbonate by using a metal hydroxide nucleophilic mechanism, and are involved in physiological processes connected with respiration and transport of CO_2 or bicarbonate ion, CO_2 homeostasis, electrolyte secretion in many tissues, biosynthetic reactions, calcification, etc. Inhibition and activation of these enzymes are well understood processes, with most classes of inhibitors binding to the metal centre, and activators binding at the entrance of the active site cavity.[1–3]

Abbreviations: CA, carbonic anhydrase; hCA, human carbonic anhydrase; CAI, carbonic anhydrase inhibitor; SAR, structure–activity relationship; Zn, zinc; AZM, acetazolamide; MZA, methazolamide; EZA, ethoxzolamide; DCP, dibromophenamide; DZA, dorzolamide; BRZ, brinzolamide; BZA, benzolamide; TPM, topiramate; ZNS, zonisamide; SLP, sulpiride; IND, indisulam; CLX, celecoxib; VLX, valdecoxib; CNS, central nervous system; VHL, von Hippel–Lindau protein; HIF, hypoxia inducible factor; PDB, protein data bank; XP, extra precision; RMSD, root mean square deviation; HIS, Histidine; ASN, asparagines; GLN, glutamine; THR, threonine; TRP, tryptophan; TLC, thin layer chromatography; UV, ultraviolet; FT-IR, Fourier transform infrared; DMSO, dimethyl sulfoxide; NMR, nuclear magnetic resonance; nM, nanomolar; mM, milimolar; E–I, enzyme–inhibitor; C:H:N:S:O, carbon:hydrogen:nitrogen:sulfur:oxygen.

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Table 1
Different hCA isoforms (hCA I, II, VII, IX and XII), organ/tissue distribution, drug targets/off targets in various diseases, kinetic parameters for CO_2 hydration reaction and affinity for sulfonamides[1]

Isoforms	Organ/tissue localization	Subcellular localization	Disease in which is involved	Off-targets other hCAs	K_{cat}/K_m (M^{-1} s^{-1})	Affinity for sulfonamides
hCA I	Erythrocytes, GI tract, Eye	Cytosol	Retinal/cerebral edema[8,9,18]	Unknown	5.0×10^7	Medium
hCA II	Erythrocytes, GI tract, eye, boneosteoclasts, kidney, lung, testis, brain	Cytosol	Glaucoma[9] Edema[8] Epilepsy[10] Altitude sickness[17] Cancer[16]	hCA I Unknown Unknown Unknown Unknown	1.5×10^8	Very high
hCA IX	Tumors, GI mucosa	Transmembrane	Cancer[16]	hCA I, hCA II	1.5×10^8	Very high
hCA XII	Renal, intestinal, reproductive epithelia, eye, tumors	Transmembrane	Cancer[16] Glaucoma[9] Retinopathies[19]	hCA I, hCA II Unknown Unknown	3.5×10^7	Low

K_{cat}/K_m results in the rate constant that measures catalytic efficiency.

Several studies demonstrated important roles of CAs in a variety of physiological processes, and showed that abnormal levels or activities of these enzymes have been often associated with different human diseases.[2] In the last few years, several CA isozymes have become interesting targets for the design of inhibitors or activators with biomedical applications.[4–7] Indeed originally CA inhibitors (CAIs) were clinically used (Fig. 1) as diuretic,[8] antiglaucoma,[9] and anticonvulsant[10] agent, whereas their employment as antiobesity drugs[11] or in the management of hypoxic tumors[12–16] were only recently validated.[4–19] Examples of clinically used CAIs: Acetazolamide AAZ, Methazolamide MZA, Ethoxzolamide EZA, Dichlorophenamide DCP, Dorzolamide DZA, Brinzolamide BRZ, Benzolamide BZA, Topiramate TPM, Zonisamide ZNS, Sulpiride SLP, Indisulam IND, Celecoxib CLX and Valdecoxib VLX. However, due to the large number of hCA isoforms, there is a constant need to improve the inhibition and selectivity profiles of the so far developed CAIs, to avoid side effects due to inhibition of isoforms not involved in a certain pathology.[1–3]

Derivatives D1 and D2 (Fig. 1) are investigational agents for targeting the tumor-associated isoform CA IX. The most useful CAIs for understanding the function of this protein in vivo were the fluorescent sulfonamide compound of type D1 that binds only to CA IX under hypoxic conditions in vivo.[2a] Compound D2 belongs to a class of positively charged trimethylpyridinium derivative, membrane-impermeable compounds. CA IX selective sulfonamide inhibitors (D1 and D2) reduced the medium acidity by inhibiting the catalytic activity of the enzyme, and thus the generation of H^+ ions, binding specifically only to hypoxic cells expressing CA IX.[2a] Derivatives D1 and D2 also participate in many other favourable interactions including stacking between the trimethylpyridinium ring of the

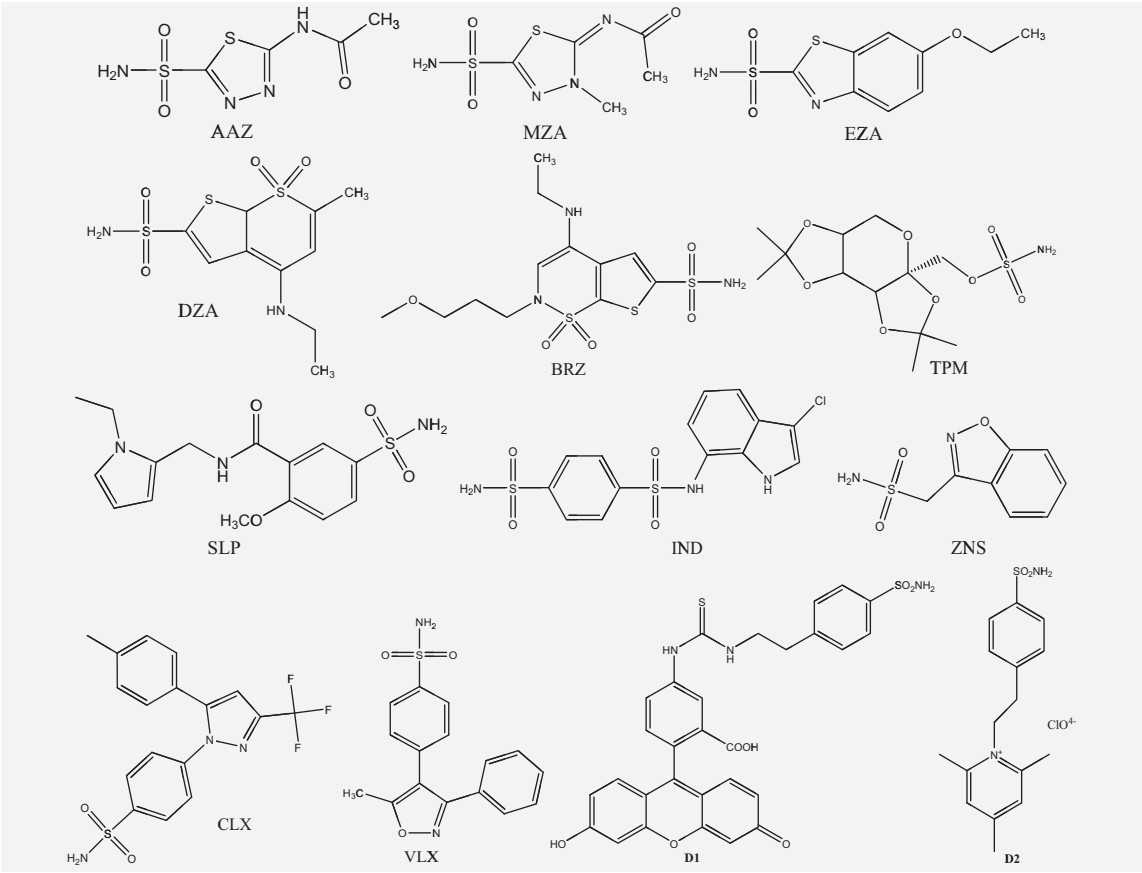


Figure 1. Carbonic anhydrase inhibitors of the sulfonamide and sulfamates type.

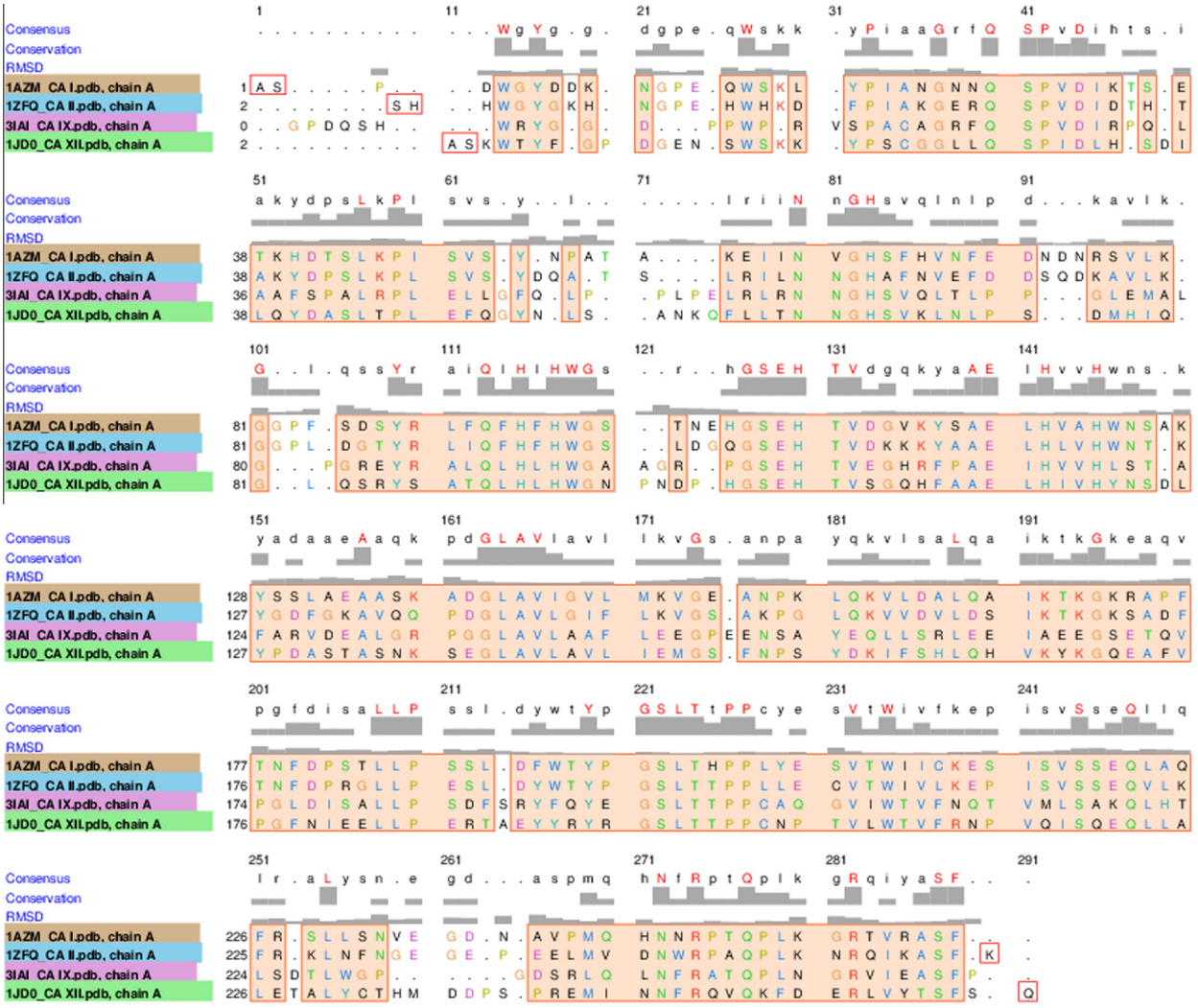


Figure 2. Sequence alignment for human α-CAs for which the crystal structures were reported with following PDB entries: 1AZM (hCA I); PDB: 1ZFQ (CA II); PDB: 1IAI (CA IX) and PDB: 1JD0 (CA XII).[20a–d]

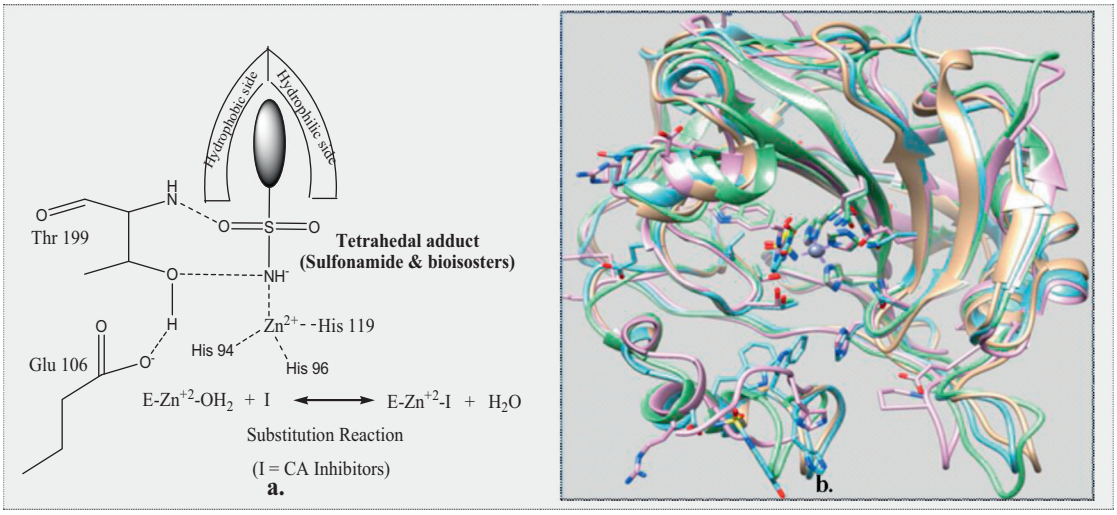


Figure 3. (a) Sulfonamide binding interactions to the Zn^{2+} ion and amino acid residues from the CA active site. Sulfonamides substitute the non-protein zinc ligand to generate a tetrahedral Zn(II) adduct;[1] (b) Homology modelling showing the helix and β-strand regions of five hCA (PDB files): 1AZM (hCA I); 1ZFQ (hCA II); 1IAI (hCA IX) and 1JD0 (hCA XII). The zinc ligands (His 94, 96 and 119) and AAZ or EZA bound within the active sites are also shown.[20a–d]

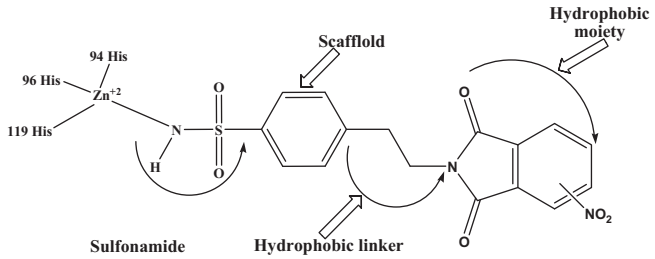


Figure 4. Representative virtual compound showing compliance to the general pharmacophoric features of sulfonamide compounds acting as carbonic anhydrase inhibitors.

inhibitor and the phenyl ring of Phe131 amino acid.[2a] Thus such structures can be used for the rational drug design of more selective and potent isozyme IX inhibitors.

A brief presentation of the hCA isoforms (CA I, CA II, IX and XII) as drug targets/off-targets is shown in Table 1. To date the three-dimensional structures of most hCA isoforms have been determined. The analysis of these structures shows that independently on their sub-cellular localization and, as expected on the basis of their high sequence alignment homology (Fig. 2), these enzymes present a rather similar structure, characterized by a central twisted β-sheet surrounded by helical connections and additional β-strands (Fig. 3). The active site is located in a large, conical cavity, approximately 12 Å wide and 13 Å deep, which spans from the protein surface to the centre of the molecule. The catalytic zinc ion is located at the bottom of this cavity, exhibiting a tetrahedral coordination with three conserved His residues and water molecule/hydroxide ion as the fourth ligand. In complex with sulfonamide inhibitors, a co-crystallized AAZ/EZA replacing the water molecule/hydroxide ion as ligand could also be seen (Fig. 3).

Specifically, hCA I found in many tissues its precise physiological function is unknown. However, a study from Feeener's group[19] demonstrated that this enzyme is involved in retinal and cerebral edema, and its inhibition may be a valuable tool for fighting these conditions. hCA II is ubiquitouos, being involved in several diseases, such as glaucoma, edema, epilepsy, and altitude sickness.[8–10,17,18] hCA IX is a therapeutic/imaging target for hypoxic cancers. hCA IX expression is increased upon hypoxia and is associated with poor prognosis, tumor progression and extracellular tumor acidification. Key pH regulators in tumor cells include: isoforms CA II, IX and XII, isoforms of anion exchangers, Na^+/HCO_3^- co-transporters, Na^+/H^+ exchangers, monocarboxylate transporters and the vacuolar ATP-ase.[16] In tumor cells, these processes are even more complex owing to the internal compartment being slightly more alkaline (pH 7.4 or more) and the external compartment being more acidic than in normal cells. A variation in the pHi/pHe ratio as low as 0.1 pH units may disrupt important biochemical and/or biological processes such as ATP synthesis, enzyme function and the proliferation, migration, invasion and metastasis of tumor cells; consequently, a tight regulation of these processes has evolved. Changes in the pHi as low as 0.1–0.2 pH units also trigger mechanisms of alternative splicing of extracellular matrix components that generate different isoforms of tenascin and fibronectin, which are typical of tumor cells and not normal cells. These alternatively spliced proteins are not involved in pH regulation but they may constitute a novel antitumor mechanism.[2,16] Possible off-targets among other hCAs for CA IX involved hCA I, and hCA II. CA IX expression is strongly increased in many types of tumors, such as gliomas/ependymomas, mesotheliomas, papillary/follicular carcinomas, carcinomas of the bladder, uterine cervix, nasopharyngeal carcinoma, head and neck, breast, oesophagus, lungs, brain, vulva, squamous/basal cell carcinomas, and kidney tumors, among others. In some cancer cells, the von Hippel-Lindau protein (VHL) gene is mutated leading to the strong upregulation of CA IX (up to 150-fold) as a consequence of constitutive hypoxia inducible factor (HIF) activation.[2,16]

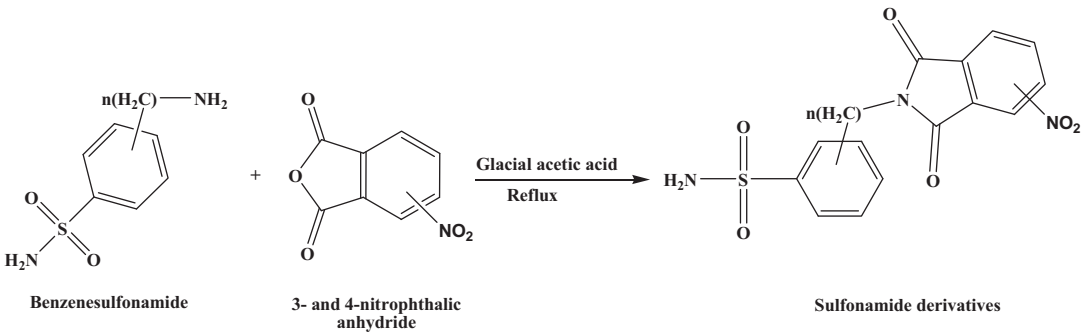
The potent CAIs incorporate various zinc-binding groups (ZBGs) and thus belong to various chemotypes, with the classical sulfonamide one, still constituting the main player in the field. The sulfonamides led to the development of several classes of pharmacological agents. Sulfonamide CAIs directly bind to the metal ion within the enzyme active site (1AZM; hCA I, 1FZQ; hCA II, 1IAI; hCA IX and 1JD0; hCA XII), by substituting the zinc-bound hydroxide ion (Fig. 3).[20a–d]

These isoforms are highly related and responsible for many diseases (Table 1) and the similar compounds reported earlier were tested as CA I and II inhibitors, and showed excellent in vitro activity as well as antiglaucoma effects in rabbits.[8–10,17–19,22]

We report here the design of novel sulfonamide CAIs. The drug design has been based on the 'tail' strategy reported previously,[21] which consists in attaching moieties that induce the desired physico-chemical properties to the molecules of aromatic sulfonamides possessing free amino groups (Fig. 4). These moieties should produce, inter alia, high affinity to the CA active site, acceptable water/lipid solubility, and good penetrability through the biological membranes, to the molecules of the newly obtained CAIs. The tails chosen to be incorporated in the compounds reported here are of the nitro-1,3-dioxoisoindolin-2-yl benzenesulfonamide (4- and 5-nitrophthalimide) type, since they may lead to interesting pharmacological properties for the new CAIs containing them. The compounds reported here were obtained by reaction of various benzene sulfonamides with 3- and 4-nitrophthalic anhydride.[22–27]

2. Chemistry

The lead molecules for the present drug design study were the sulfonamides incorporating phthalimido moieties reported earlier



Scheme 1. Scheme for the synthesis of sulfonamides **1–8** incorporating 3- and 4-nitrophthalic anhydride moiety to different benzene sulfonamides.[22,29] Reagents and conditions: $n = 0$ (**1**, **4**, **5**, **7** and **8**); $n = 1$ (**2**, **6**); $n = 2$ (**3**). $-(CH_2)n-$ = in *para* position for **1**, **2**, **3** and **6**; in *meta* position for **4** and **7**; in *ortho* position for **5** and **8** in the benzene ring. 5-NO_2 (**1**, **2**, **3**, **4**, and **5**); 4-NO_2 (**6**, **7**, and **8**) in the benzene ring of anhydride.

by our groups.[22a–c] The 3- and 4-nitrophthalic anhydride moieties are interesting due to the presence of the nitro group, which led to compounds with interesting antitumor properties for a series of ureido-substituted sulfonamides.[22d]

A simple chemistry has been used to prepare the novel sulfonamides of types **1**–**8** incorporating 3 and 4-nitrophthalicanhydride moieties (Scheme 1). Aromatic sulfonamides having free amino groups were converted to the corresponding phthalimide by reaction with 3- and 4-nitrophthalicanhydride.[22a–c] It should be mentioned that the similar compounds reported earlier by reaction of aromatic/heterocyclic sulfonamides with phthalic anhydride derivatives, were tested as CA I, II,IV, IX and XII inhibitors, and showed excellent in vitro activity[22a–c] as well as antiglaucoma effects in rabbits.[22a]

3. CA inhibition studies

The CA inhibition on cytosolic hCA I and II membrane bound IX and XII (tumor associated human CA) data of compounds **1**–**8**, the standard AZM and other clinically used sulfonamides/sulfamate are shown in Table 2. Acetazolamide is clinically used for the adjunctive treatment of drug-induced edema, edema caused by congestive heart failure, petit mal and other types of epilepsies.[8–10] It has also been used to lower the intraocular pressure prior to surgery in acute conditions of angle-closure glaucoma, besides open-angle and secondary glaucoma and altitude sickness (Table 1).[17,18]

The following structure–activity relationship (SAR) was observed for this series of compounds:

i. The cytosolic isoform hCA I was inhibited by the synthesized sulfonamides, with inhibition constants in the range of 295 nM to >10,000 nM (Table 2). The best inhibitor in the series was the 4-(2-(5-nitro-1,3-dioxoisoindolin-2-yl)ethyl)benzenesulfonamide **3** (K_i of 295 nM), whereas some of the other substitution patterns led to compounds **1**, **2**, **6**, **4** and **7** with inhibition constants 318, 403, 490, 501 and 522 nM, respectively. The inhibition constants are somewhat close similar to that of clinically used acetazolamide/topiramate and better then sulpiride/dorzolamide/brinzolamide (Table 2). The weak inhibition of this isoform (compounds **5** and **8**) may be considered a positive feature of this class of CAIs since hCA I, highly abundant in red blood cells, is undoubtedly an off-target when considering other applications of the CAIs.[2,28] The increased carbon chain length between the two bulky groups (–CH_2– or –CH_2CH_2–) led to an increased activities of compounds **1** to **3** with 295 nM to 403 nM, respectively of the derivatives. The position of –SO_2NH_2 matters in the increase and decrease of activities. The decreased activity of compounds **1**, **4**, **5**, **7**, **8** according to position of –SO_2NH_2 in the benzene ring are 4th to 2nd (K_is 295 nM to >10,000 nM). It is very clear from the above inhibition data that, compounds having –SO_2NH_2 group in the *ortho* position of the benzene ring compatatively very less active then *meta* and then *para* position, respectively due to increased steric hindrance. Position of –NO_2 to the benzene ring slightly alter in the hCAI activities (5th > 4th) that is compound **2** (403 nM) >**6** (490 nM) and **4** (501 nM) >**7** (522 nM).

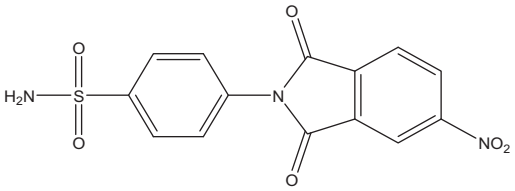
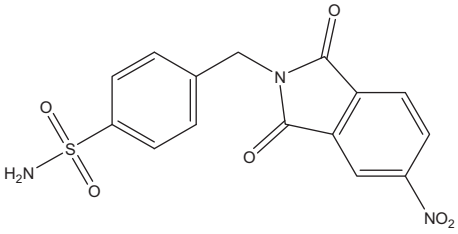
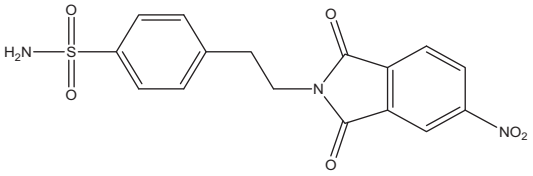
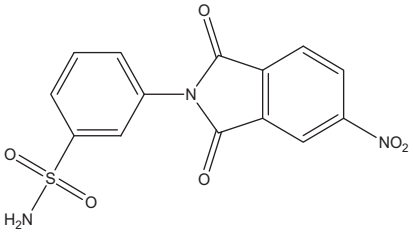
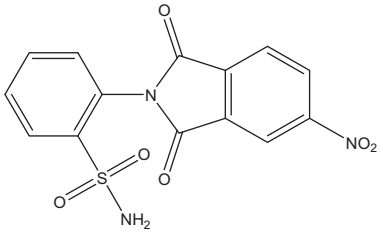
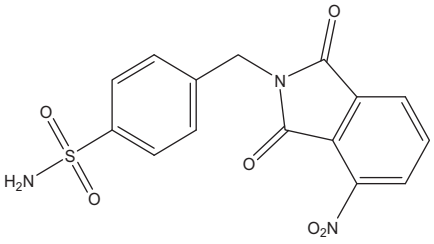
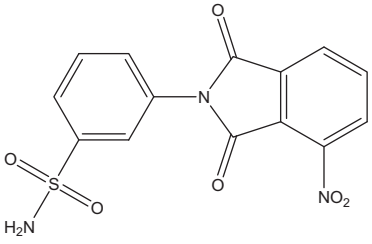
ii. The physiologically dominant cytosolic isoform hCA II was inhibited with low nanomolar concentration by the synthesized sulfonamide in comparison to standard clinically used AAZ and other clinically used sulfonamide, K_is in the range of 1.7–>10,000 nM (Table 2). Against hCA II, compound **8** was the most potent hCA inhibitor, with a K_i of 1.7 nM then the other compounds of the series. The compounds are excellent to moderate inhibitors for hCA II and SARs are straightforward. The increased carbon chain length between the two bulky groups (–CH_2– or –CH_2CH_2–) led to decreased activities of compounds **1** > **2** > **3** with K_is of 4.3, 238 and 589 nM, respectively. Compounds having –SO_2NH_2 in the *ortho*, *meta* and *para* substituted sulfonamides influence on the hCA II inhibition due to steric hindrance effects. That is compounds **5** < **4** < **1** (K_is of >10,000, 887 and 4.3 nM, respectively) and exactly reverse in case of compounds **6** < **7** < **8** (K_is of 34, 3.9 and 1.7 nM, respectively) may be due to presence of –NO_2 functional group in the 4th position. Position of –NO_2 to the benzene ring slightly change in the activity (5th < 4th) that is compound **2** (238 nM) <**6** (34 nM), **4** (887 nM) >**7** (3.9 nM) and **5** (>10,000 nM) >**8** (1.7 nM). Overall, most of these sulfonamides showed a potent inhibitory action towards hCA II, which is the main target isoform for designing antiglaucoma drugs, and the off-target one when other applications of the CAIs are envisaged.[28]

iii. The transmembrane isoform hCA IX was poorly inhibited by this new class of sulfonamides (**1**–**8**) CAIs, which showed K_is in the range of 873–>10,000 nM against hCA IX (Table 2). Compound **6** was the most potent hCA inhibitor, with a K_i of 873 nM then the other compounds of the series. The compounds are less effective inhibitors for hCA IX and SARs are straightforward. The increased carbon chain length between the two bulky groups (–CH_2– or –CH_2CH_2–) led to decreased activities of compounds **1** > **2** > **3** with K_is of 8654, 8837 and >10,000 nM, respectively of the derivatives. Some steric hindrance effects led to a loss of the hCA II inhibition, such as for example –SO_2NH_2 present in the *ortho*, *meta* and *para* substituted sulfonamides **5** < **4** < **1** (K_is in the range of >10,000, 8837 and 8654 nM) resulted due to steric hindrance and exactly reverse in **6** < **7** < **8** (K_is in the range of 873–8352 nM) may be due to 4th position of –NO_2 functional group. Position of –NO_2 to the benzene ring slightly improve the activity 5th < 4th that is compound **2** (8837 nM) <**6** (873 nM), **4** (10,000 nM) >**7** (8352 nM) and **5** (>10,000 nM) >**8** (7820 nM). Overall, most of these sulfonamides showed a very poor inhibitory action towards hCA IX.

iv. The transmembrane isoforms hCA XII on the other hand was moderately inhibited by most of the new class of sulfonamide CAIs, which showed K_is in the range of 90–>10,000 nM against hCA XII (Table 2). Compound **1** was the most potent hCA inhibitor, with a K_i of 90 nM then the other compounds of the series. The compounds are average inhibitors for hCA IX and SARs is straightforward. The increased carbon chain length between the two bulky groups (–CH_2– or –CH_2CH_2–) led to decreased activities of compounds **1** > **2** > **3** with K_is of 90, 119 and 620 nM, respectively of the derivatives. Some steric hindrance effects led to a loss of the hCA XII inhibition, such as for example –SO_2NH_2 present in the *ortho*, *meta* and *para* substituted sulfonamides **5** < **4** < **1** (K_is in the range of 3746, 96 and 90 nM) resulted due to steric hindrance and exactly in **6** > **7** > **8** (K_is in the range of 491, 838, >10,000 nM). Position of –NO_2 to the benzene ring slightly improve the activity 5th > 4th that is compound **2** (90 nM) >**6** (491 nM), **4** (96 nM) >**7** (838 nM) and **5** (3746 nM) >**8** (>10,000 nM). Overall, most of these sulfonamides showed a moderate inhibitory action towards hCA XII.

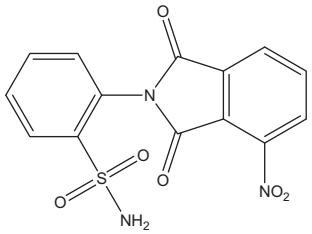
v. The selectivity ratio for inhibiting the tumor-associated isoforms hCA IX and XII over the cytosolic off-target hCA II,[28] was in the range of 0.00–0.08 and 0.0002–9.24 for the new sulfonamide reported here (Table 2). For hCA IX the selectivity ratio is too poor then hCA XII in which some the compounds shown a good result, since most sulfonamide CAIs (D1, D2, acetazolamide, ethoxzolamide, dichlorophenamide,

Table 2
hCA I, II, IX and XII inhibition data with sulfonamides **1–8**, standard AZM and other important CAIs, by a stopped-flow CO_2 hydrase assay method[30]

Comp. code	Structures/name	K_i (nM)*				Selectivity ratio#		Docking score (Glide XP)			
		hCA I	hCA II	hCA IX	hCAXII	A	B	1AZM	1ZFQ	3IAI	1JD0
1		318	4.3	8654	90	0.0005	0.58	−5.647	−5.132	−5.246	−4.244
2		403	238	8837	119	0.026	2	−5.931	−4.624	−5.515	−4.746
3		295	589	>10,000	620	0.06	0.95	−6.001	−5.509	−5.086	−5.046
4		501	887	10,000	96	0.088	9.24	−5.776	−4.781	−5.526	−4.751
5		>10,000	>10,000	>10,000	3746	0.00	2.669	−3.796	−3.856	−3.601	−3.618
6		490	34	873	491	0.039	0.069	−5.845	−5.112	−5.565	−5.335
7		522	3.9	8352	838	0.0005	0.005	−5.909	−5.103	−4.471	−4.801

(*continued on next page*)

Table 2 (*continued*)

Comp. code	Structures/name	K_i (nM)*				Selectivity ratio#		Docking score (Glide XP)			
		hCA I	hCA II	hCA IX	hCAXII	A	B	1AZM	1ZFQ	3IAI	1JD0
8		>10,000	1.7	7820	>10,000	0.0002	0.0002	−3.823	−4.794	−4.493	−4.377
AAZ	Acetazolamide	250	12	25	5.7	0.5	2.10	−4.8	−3.8	−4.4	−4.1
MZA	Methazolamide	50	14	27	3.4	0.5	4.11	−4.9	−3.8	−4.9	−3.5
EZA	Ethoxzolamide	25	8	34	22	0.2	0.36	−5.7	−4.1	−5.1	−4.2
DZA	Dorzolamide	50,000	9	52	3.5	0.2	2.57	−5.0	−4.5	−4.8	−4.4
BRZ	Brinzolamide	45,000	3	37	3	0.1	1	−5.5	−5.3	−4.9	−5.1
TPM	Topiramate	250	10	58	3.8	0.2	2.63	−6.1	−4.7	−5.0	−4.4
SLP	Sulpiride	1200	40	46	3.9	0.9	10.25	−6.5	−6.0	−5.7	−4.9
IND	Indisulam	31	15	24	3.4	0.6	4.41	−6.5	−5.0	−6.8	−5.2
ZNS	Zonisamide	56	35	5.1	11,000	6.9	0.003	−5.7	−3.7	−4.7	−4.5
CLX	Celecoxib	50,000	21	16	18	1.3	1.16	−6.5	−4.7	−4.7	−3.3
VLX	Valdecoxib	54,000	43	27	13	1.6	3.30	−6.1	−4.1	−4.5	−4.0
D1	—	1300	45	24	5	1.9	9	−3.9	−4.4	−5.0	−5.3
D2	—	4000	21	14	7	1.5	3	−4.1	−4.9	−5.1	−5.0

* Mean from three different assays, errors were in the range of 5–10% of the reported values (data not shown).
\# The K_i ratios are indicative of isozyme selectivity for transmembrane CAs IX, XII; A (hCA II/hCA IX) and B (hCA II/hCA XII).

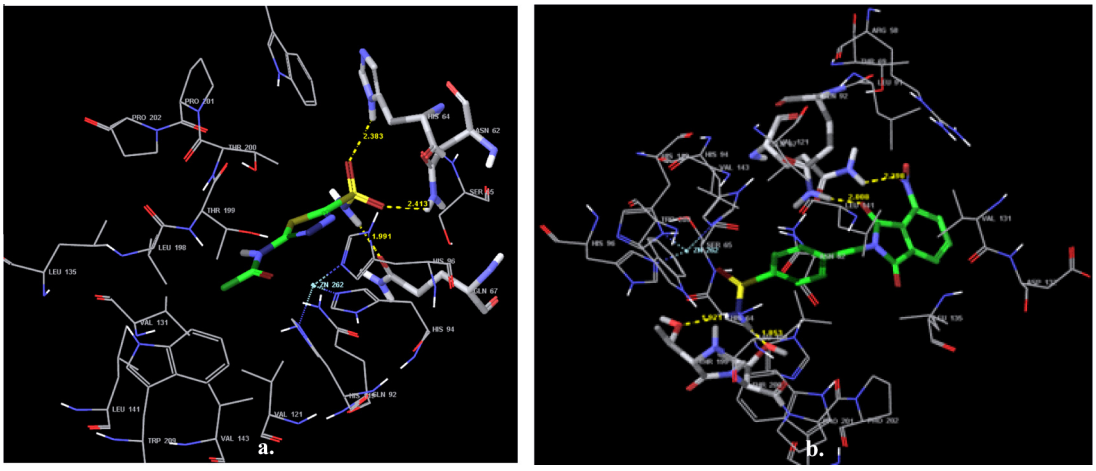


Figure 5. Docked conformations in the CA IX (PDB: 3IAI) catalytic site. (a) AZM in the binding pocket formed H-bond to HIS 64, ASN 62, GLN 67; (b) Compound **6** (highest docking score −5.565) formed hydrogen bond to THR 119, THR 200 and GLN 67, GLN 92.

etc) show better hCA II than hCA IX inhibitory action.[2] In the new series reported here, most of the compounds had a poor selectivity ratio, being thus relatively better CA I and II selective rather then hCA IX but being thus somewhat highly CA XII selectivity (compared to the inhibition of CA I and II).

4. Docking studies

Molecular docking of eight compounds and other clinically used sulfonamides/sulfamate were performed to rationalize the SARs reported and to study the Zn^{+2} binding mode to different hCA as inhibitors. Docking study was performed in the crystal structure of hCA preferably in 1AZM (hCA I), 1FZQ (hCA II), 3IAI (hCA IX) and 1JD0 (hCA XII).[20a–d] All the 'A' chain of the hCA crystal structures catalytic domains was considered for docking study. The Glide (XP) score of the co-crystallized ligand AZM is −3.8 to −4.8 and the RMSD values range from 1.8 to 2.3 which is considered as good for docking of the ligands (Table 2). Most of the docking scores of the synthesized compounds are so good enough then standard AZM and other clinically used sulfonamides/sulfamate, D1 and D2 also (Table 2). Figure 5 showed the docked conformations of AZM and compound **6** (highest docking score) in catalytic site in the binding pocket of CA IX (PDB: 3IAI).

The main objective of our molecular docking study was to rationalize the SARs reported over here and Zn^{+2} binding mode interactions to different hCA as inhibitors. In each and every case we have observed that the SAR tally to the results obtained by means of docking study. For examples: compounds **5** and **8** showed the lowest docking scores found in each and every case. The increased carbon chain length between the two bulky groups (–CH_2– or –CH_2CH_2–) led to improve docking score of the synthesized derivatives. Some steric hindrance effects result loss of the hCAI docking score, for example –SO_2NH_2 present in the *ortho*-, *meta*- and *para*-substituted sulfonamides **5** < **4** < **1**. Addition of –NO_2 to the 4th and 5th position to the compounds provide the clear indication of variable scores of compounds (Table 2) and interaction to different amino acid (Fig. 5).

5. Conclusions

A small series of 4- and 5-nitro-1,3-dioxoisoindolin-2-yl benzenesulfonamide (compounds **1**–**8**)was prepared and investigated for the inhibition of four physiologically relevant hCA isoforms hCA I, hCA II, hCA IX and hCA XII. These compounds were generally potent inhibitors of CA I, but many of them were relevantly good to moderate effective with nanomolar hCA II. Such compounds may constitute interesting candidates for the development of novel antiglaucoma, antiepileptic, edema, or altitude sickness drugs. The compounds inhibit the transmembrane isoforms hCA IX with high nanomolar concentration and hCA XII with moderately nanomolar concentration. In the new series reported here, most of the compounds had a poor selectivity ratio, being thus relatively better hCA I and II selective rather then hCA IX but being thus somewhat high CA XII selectivity (compared to the inhibition of hCA I and II). Such compounds may promote interesting candidates for the development of more selective and potent novel hypoxia induced cancer drug therapy and retinopathies. The compounds showed better hCA I and II may be further interesting candidates for the development of retinal/cerebral edema, glaucoma, altitude sickness.

6. Experimental section

6.1. Reagents and instruments

All reagents and solvents were of commercial quality and used without further purification, unless otherwise specified. All reactions were carried out under an inert nitrogen atmosphere. These are the following chemicals obtained from different sources that is *p*-amino benzene sulphanilamide (Sigma–Aldrich), 3- and 4-nitrophthalic anhydride (Sigma–Aldrich), acetic acid glacial, chloroform, methanol, DMSO (Central Drug House), silica gel 60 F254 plates (Merck Art. 1.05554). Spots were visualized under 254 nm (short) and 365 nm (long) UV illumination and/or by ninhydrin solution spraying. FT-IR spectra were recorded on 8400S, Shimadzu. ^{1}H and ^{13}C NMR spectra were recorded on Bruker DRX-400 spectrometer using DMSO-d_6 as solvent and tetramethylsilane as internal standard. For ^{1}H NMR spectra, chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane, and coupling constants (J) are expressed in Hertz. Electron ionization mass spectra were recorded in positive or negative mode on a Water MicroMass ZQ. CHNSO elemental analysis recorded by Elementar, Vario EL III.

6.2. Chemistry

A mixture of selected sulfonamide and 3- or 4-nitrophthalic anhydride in glacial acetic acid was refluxed with stirring under nitrogen environment for desirable time to complete the reaction leading to compounds **1**–**8**.[22,29]

6.2.1. Synthesis of 4-(5-nitro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (1)

0.002 mol (0.344 g) of 4-aminobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.386 g) of 5-nitroisobenzofuran-1,3-dione to produce 0.002 mol of 4-(5-nitro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**1**) (0.695 g) in the presence of glacial acetic acid as solvent for 12 h at 130 °C. The reaction monitored in each 30 min with the help of TLC (Chloroform/Methanol; 3:1). After completion of reaction, the reaction mixture was cooled and 30 ml of cold water was added. The product was filtered and washed with cold water repeatedly for 4/5 times. The product was recrystallized from ethanol leading to compound **1**.[22,29]

White crystalline and solid; % yield = 88%; mp = 250.2 °C; Solubility; Insoluble: water, acetic acid glacial; Partially Soluble: ethanol; Fully Soluble: DMSO and methanol, chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1780.36, 1716.70 (C=O imide); 1338.64, 1161.19 (S=O) and 3363.97 (NH_2), 1539.25, 1390.72 (NO_2). MS (ESI+); *m*/*z*: 346.08 [M−H]$^-$, 378.08 [M−H]$^-$+MeOH. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.527 (s, 2H, SO_2NH_2), 7.715–7.749, 8.023–8.056 (d, 2H, Ar–H from benzenesulfonamide), 8.278–8.299, 8.73–8.735 (d, 2H, Ar–H from nitrobenzene), 8.654–8.659 (s, H, Ar–H from nitrobenzene). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 119.21, 125.95, 127.40, 128.43, 130.84, 133.92, 135.32, 137.16, 144.57, 152.56, 166.05. Elem. Anal. calcd for $C_{14}H_9N_3O_6S$: C, 48.42; H, 2.61; N, 12.10; O, 27.64; S, 9.23; found C, 50.50; H, 2.647; N, 12.80; O, 23.883; S, 10.17.

6.2.2. Synthesis of 4-((5-nitro-1,3-dioxoisoindolin-2-yl)methyl)benzenesulfonamide (2)

0.002 mol (0.444 g) of 4-(aminomethyl)benzenesulfonamide hydrochloride stirred under nitrogen environment with 0.002 mol (0.386 g) of 5-nitroisobenzofuran-1,3-dione to produce 0.002 mol of 4-((5-nitro-1,3-dioxoisoindolin-2-yl)methyl)benzenesulfonamide (**2**) (0.722 g) in the presence of glacial acetic acid as solvent for 12 h at 130 °C. The reaction monitored in each 30 min with the help of TLC (Chloroform/Methanol; 1:1). After completion of reaction, the reaction mixture was cooled and 30 ml of cold water was added. The product was filtered and washed with cold water repeatedly for 4/5 times. The product was recrystallized from ethanol leading to compound **2**.[22,29]

White crystalline and solid; % yield = 75%; mp = 214 °C; Solubility; Insoluble: acetic acid glacial; Partially Soluble: ethanol, methanol; Fully Soluble: water, DMSO and chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1768.78, 1707.06 (C=O imide); 1310.10, 1157.33 (S=O) and 3365.90 (NH_2), 1537.32, 1350.30 (NO_2), 2990.10 (aliphatic CH_2). MS (ESI+); *m*/*z*: 360.17 [M−H]$^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.375 (s, 2H, SO_2NH_2), 7.569–7.59, 7.806–7.827 (d, 2H, Ar–H from benzenesulfonamide), 8.189–8.21, 8.675–8.7 (d, 2H, Ar–H from nitrobenzene), 8.566–8.57 (s, H, Ar–H from nitrobenzene), 4.933 (s, 2H, CH_2 aliphatic). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 41.95, 118.96, 125.68, 126.84, 128.81, 130.61, 134.05, 137.31, 140.91, 144.21, 152.29, 166.8 Elem. Anal. calcd for $C_{15}H_{11}N_3O_6S$: C, 49.86; H, 3.07; N, 11.63; O, 26.57; S, 8.87; found C, 51.92; H, 3.104; N, 12.21; O, 23.052; S, 9.714.

6.2.3. Synthesis of 4-(2-(5-nitro-1,3-dioxoisoindolin-2-yl)ethyl)benzenesulfonamide (3)

0.002 mol (0.401 g) of 4-(2-aminoethyl)benzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.386 g) of 5-nitroisobenzofuran-1,3-dione to produce 0.002 mol of 4-(2-(5-nitro-1,3-dioxoisoindolin-2-yl)ethyl)benzenesulfonamide (**3**) (0.750 g) in the presence of glacial acetic acid as solvent for 12 h at 130 °C. The reaction monitored in each 30 min with the help of TLC (Chloroform/Methanol; 1:1). After completion of reaction, the reaction mixture was cooled and 30 ml of cold water was added. The product was filtered and washed with cold water repeatedly for 4/5 times. The product was recrystallized from ethanol leading to compound **3**.[22,29]

White crystalline and solid; % yield = 88%; mp = 207 °C; Solubility; Insoluble: water, acetic acid glacial; Partially Soluble: ethanol and Methanol; Fully Soluble: DMSO, chloroform. DMSO and methanol, chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1770.71, 1705.13 (C=O imide); 1332.86, 1151.54 (S=O) and 3369.75 (NH_2), 1539.25, 1350.30 (NO_2), 2949.26 (aliphatic CH_2). MS (ESI+); *m*/*z*: 376.08 [M−H]$^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.338 (s, 2H, SO_2NH_2), 7.455–7.475, 7.741–7.762 (d, 2H, Ar–H from benzenesulfonamide), 8.14–8.16, 8.641–8.666 (d, 2H, Ar–H from nitrobenzene), 8.516–8.521 (s, H, Ar–H from nitrobenzene), 3.05–3.085, 3.914–3.95 (d, 2H, CH_2 aliphatic). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 34.18, 39.91, 118.78, 125.47, 126.7, 130.1, 130.56, 133.8, 137.04,

143.22, 152.35, 166.7, 166.97. Elem. Anal. calcd for $C_{16}H_{13}N_3O_6S$: C, 51.20; H, 3.49; N, 11.19; O, 25.57; S, 8.54; found C, 53.41; H, 3.661; N, 11.80; O, 21.691; S, 9.438.

6.2.4. Synthesis of 3-(5-nitro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (4)

0.002 mol (0.344 g) of 3-aminobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.386 g) of 5-nitroisobenzofuran-1,3-dione to produce 0.002 mol of 3-(5-nitro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**4**) (0.694 g) in the presence of glacial acetic acid as solvent for 12 h at 130 °C. The reaction monitored in each 30 min with the help of TLC (Chloroform/Methanol; 3:1). After completion of reaction, the reaction mixture was cooled and 30 ml of cold water was added. The product was filtered and washed with cold water repeatedly for 4/5 times. The product was recrystallized from ethanol leading to compound **4**.[22,29]

Reddish white crystalline and solid; % yield = 73%; mp = 265 °C; Solubility; Insoluble: Water, Acetic acid glacial; Partially Soluble: ethanol and Methanol; Fully Soluble: DMSO, chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1791.93, 1716.70 (C=O imide); 1346.36, 1116.82 (S=O) and 3369.75 (NH_2), 1533.46, 1386.86 (NO_2). MS (ESI+); *m*/*z*: 346.08 $[M-H]^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.604 (s, 2H, SO_2NH_2), 7.741–7.769, 7.96–7.987 (d, 2H, Ar–H from benzenesulfonamide), 7.801–7.84 (t, H, Ar–H from benzenesulfonamide), 8.02–8.029 (s, H, Ar–H from benzenesulfonamide), 8.271–8.279, 8.72–8.755 (d, 2H, Ar–H from nitrobenzene), 8.645–8.646 (s, H, Ar–H from nitrobenzene). ^{13}C NMR (100 MHz, DMSO-d_6) δ:119.13, 125.32, 125.88, 126.57, 130.76, 131.46, 132. 86, 133.99, 137.25, 145.88, 152.51, 165.94, 166.18. Elem. Anal. calcd for $C_{14}H_9N_3O_6S$: C, 48.42; H, 2.61; N, 12.10; O, 27.64; S, 9.23; found C, 50.45; H, 2.829; N, 12.83; O, 23.791; S, 10.10.

6.2.5. Synthesis of 2-(5-nitro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (5)

0.002 mol (0.344 g) of 2-aminobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.386 g) of 5-nitroisobenzofuran-1,3-dione to produce 0.002 mol of 2-(5-nitro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**5**) (0.694 g) in the presence of glacial acetic acid as solvent for 12 h at 130 °C. The reaction monitored in each 30 min with the help of TLC (Chloroform/Methanol; 3:1). After completion of reaction, the reaction mixture was cooled and 30 ml of cold water was added. The product was filtered and washed with cold water repeatedly for 4/5 times. The product was recrystallized from ethanol leading to compound **5**.[22,29]

White crystalline and solid; % yield = 97%; mp = 260 °C; Solubility; Insoluble: water, acetic acid glacial; Partially Soluble: ethanol and methanol; Fully Soluble: DMSO and chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1791.93, 1728.28 (C=O imide); 1342.50, 1170.83 (S=O) and 3410.26 (NH_2), 1541.18, 1388.79 (NO_2). MS (ESI+); *m*/*z*: 346.8 $[M-H]^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.632 (s, 2H, SO_2NH_2), 7.787–7.862 (t, 2H, Ar–H from benzenesulfonamide), 7.69–7.719, 8.105–8.129 (d, 2H, Ar–H from benzenesulfonamide), 8.269–8.290, 8.7–8.75 (d, 2H, Ar–H from nitrobenzene), 8.64 (s, 2H, Ar–H from nitrobenzene). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 119.28, 126.02, 129.46, 130.68, 131.4, 132.75, 133.89, 134.32, 137.5, 143.14, 152.51, 165.80, 166.05. Elem. Anal. calcd for $C_{14}H_9N_3O_6S$: C, 48.42; H, 2.61; N, 12.10; O, 27.64; S, 9.23; found C, 51.38; H, 2.355; N, 12.37; O, 23.998; S, 9.897.

6.2.6. Synthesis of 4-((4-nitro-1,3-dioxoisoindolin-2-yl)methyl)benzenesulfonamide (6)

0.002 mol (0.444 g) of 4-(aminomethyl)benzenesulfonamide hydrochloride stirred under nitrogen environment with 0.002 mol (0.386 g) of 4-nitroisobenzofuran-1,3-dione to produce 0.002 mol of 4-((5-nitro-1,3-dioxoisoindolin-2-yl)methyl)benzenesulfonamide (**6**) (0.722 g) in the presence of glacial acetic acid as solvent for 12 h at 130 °C. The reaction monitored in each 30 min with the help of TLC (Chloroform/Methanol; 3:1). After completion of reaction, the reaction mixture was cooled and 30 ml of cold water was added. The product was filtered and washed with cold water repeatedly for 4/5 times. The product was recrystallized from ethanol leading to compound **6**.[22,29]

White crystalline and solid; % yield = 76%; mp = 221 °C; Solubility; Insoluble: Water, acetic acid glacial; Partially Soluble: ethanol; Fully Soluble: DMSO, chloroform and methanol. IRν_{max} (cm^{-1}; KBr pellets); 1770.71, 1708.99 (C=O imide); 1336.71, 1157.33 (S=O) and 3358.18 (NH_2), 1535.39, 1346.36 (NO_2). MS (ESI+); *m*/*z*: 360.17 $[M-1]^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.375 (s, 2H, SO_2NH_2), 7.567–7.588, 7.803–7.824 (d, 2H, Ar–H from benzenesulfonamide), 8.094–8.133, 8.23–8.25 (t, 2H, Ar–H from nitrobenzene), 8.335–8.337 (d, H, Ar–H from nitrobenzene), 4.89 (s, 2H, CH_2 aliphatic). ^{13}C NMR (100 MHz, DMSO-d_6) δ:41.92, 124.13, 126.8, 127.94, 128.83, 129.32, 134.54, 137.17, 140.86, 144.18, 145.31, 164.11, 166.75. Elem. Anal. calcd for $C_{15}H_{11}N_3O_6S$: C, 49.86; H, 3.07; N, 11.63; O, 26.57; S, 8.87; found C, 50.59; H, 2.646; N, 11.60; O, 26.053; S, 9.111.

6.2.7. Synthesis of 3-(4-nitro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (7)

0.002 mol (0.344 g) of 3-aminobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.386 g) of 4-nitroisobenzofuran-1,3-dione to produce 0.002 mol of 3-(4-nitro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**7**) (0.694 g) in the presence of glacial acetic acid as solvent for 12 h at 130 °C. The reaction monitored in each 30 min with the help of TLC (Chloroform/Methanol; 3:1). After completion of reaction, the reaction mixture was cooled and 30 ml of cold water was added. The product was filtered and washed with cold water repeatedly for 4/5 times. The product was recrystallized from ethanol leading to compound **7**.[22,29]

White crystalline and solid; % yield = 65%; mp = 214.2 °C; Solubility; Insoluble: Water, acetic acid glacial; Partially Soluble: ethanol; Fully Soluble: DMSO, chloroform and methanol. IRν_{max} (cm^{-1}; KBr pellets); 1780.36, 1716.70 (C=O imide); 1338.64, 1159.26 (S=O) and 3362.04 (NH_2), 1541.18, 1388.79 (NO_2). MS (ESI+); *m*/*z*: 346.17 $[M-1]^-$, 364 $[M-1]^-+H_2O$ (adduct). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.597 (s, 2H, SO_2NH_2), 7.711–7.739, 7.951–7.978 (d, 2H, Ar–H from benzenesulfonamide), 7.785–7.824 (t, H, Ar–H from benzenesulfonamide), 7.994–7.998 (s, 2H, Ar–H from benzenesulfonamide), 8.29–8.33, 8.38–8.41 (d, 2H, Ar–H from nitrobenzene), 8.25–8.29 (t, H, Ar–H from nitrobenzene). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 123.86, 125.59, 126.61, 128.01, 129.35, 130.72, 131.7, 132.76, 134.49, 137.37, 145.44, 145.84, 163.34, 165.89. Elem. Anal. calcd for $C_{14}H_9N_3O_6S$: C, 48.42; H, 2.61; N, 12.10; O, 27.64; S, 9.23; found C, 49.15; H, 2.829; N, 12.82; O, 26.091; S, 9.11.

6.2.8. Synthesis of 2-(4-nitro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (8)

0.002 mol (0.344 g) of 2-aminobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.386 g) of 4-nitroisobenzofuran-1,3-dione to produce 0.002 mol of 2-(4-nitro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**8**) (0.694 g) in the presence of glacial acetic acid as solvent for 12 h at 130 °C. The reaction monitored in each 30 min with the help of TLC (Chloroform/Methanol; 3:1). After completion of reaction, the reaction mixture was cooled and 30 ml of cold water was added. The product was filtered and washed with cold water repeatedly for 4/5 times. The product was recrystallized from ethanol leading to compound **8**.[22,29]

White crystalline and solid; % yield = 65%; mp = 252.4 °C; Solubility; Insoluble: Water, acetic acid glacial; Partially Soluble: ethanol; Fully Soluble: DMSO, chloroform and methanol. IRν_{max} (cm^{-1}; KBr pellets); 1793.86, 1732.13 (C=O imide); 1301.99, 1114.89 (S=O) and 3385.18 (NH_2), 1533.46, 1363.72 (NO_2). MS (ESI+); *m*/

z: 346.17 $[M-H]^-$. ^1H NMR (400 MHz, DMSO-d_6) δ: 7.633 (s, 2H, SO_2NH_2), 7.773–7.849 (t, 2H, Ar–H from benzenesulfonamide), 7.603–7.706, 8.094–8.118 (d, H, Ar–H from benzenesulfonamide), 8.163–8.202 (t, 2H, Ar–H from nitrobenzene), 8.308–8.33, 8.39–8.43 (d, H, Ar–H from nitrobenzene). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 124.28, 128, 21, 129.41, 129.51, 131.4, 132.84, 133.88, 134.79, 137.33, 143.22, 145.53, 163.04, 165.73. Elem. Anal. calcd for $C_{14}H_9N_3O_6S$: C, 48.42; H, 2.61; N, 12.10; O, 27.64; S, 9.23; found C, 48.47; H, 2.81; N, 12.83; O, 25.68; S, 10.21.

6.3. CA inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. 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 a buffer and 20 mM Na_2SO_4 (for maintaining a 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. 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 inhibitor solutions (0.1 mM) were prepared in distilled–deionized water, and dilutions of up to 0.01 nM were made thereafter with distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min to 72 h at room temperature (15 min) or 4 °C (all other incubation times) prior to the assay, to allow the formation of the E–I complex or the eventual active site-mediated hydrolysis of the inhibitor. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3.[30]

Acknowledgments

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References and notes

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