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Carbonic anhydrase inhibitors: Synthesis and inhibition of the human carbonic anhydrase isoforms I, II, VII, IX and XII with benzene sulfonamides incorporating 4,5,6,7-tetrabromophthalimide moiety

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ABSTRACT

A series of 4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl benzenesulfonamide derivatives (compounds **1**–**8**) was synthesized by reaction of benzene sulfonamide derivatives with 4,5,6,7-tetrabromophthalic anhydride moiety. 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, II and VII and the transmembrane tumor-associated isoform hCA IX and XII. The new compounds were good hCA I inhibitors (Kis in the range of 143 to >10,000 nM), but were moderately effective, as hCA II inhibitors (Kis of 47–190 nM) and poor hCA VII inhibitors (Kis in the range of 54–175 nM) compared to acetazolamide. The tumor-associated hCA IX was effectively inhibited with Kis ranging between 8.5 and 234 nM and hCA XII with inhibition constants in the range of 6.1–197 nM with high selectivity ratio. 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 study of compounds was performed to rationalize the SAR reported over here.

1. Introduction

Carbonic anhydrases (CAs) 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 (marine diatoms) and ζ-CAs (bacteria, chemolithotrophs and marine cyanobacteria).[1,3] Human CAs (hCAs), all belong to the α-family and are present in fifteen isoforms (out of 16 mammalian isoforms), which are 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 (hCA I, hCA II, hCA III, hCA VII and hCA XIII), four membrane associated isozymes (hCA IV, hCA IX, hCA XII and hCA XIV), two mitochondrial forms (hCA VA and hCA VB), and a secreted hCA isozyme (hCA VI).[1–3] There are three additional 'acatalytic' hCA isoforms (hCA VIII, hCA X, and hCA XI) whose functions are remained unclear.[3] hCAs which catalyze the interconversion between carbon dioxide and bicarbonate by using a metal hydroxide nucleophilic mechanism and are involved in many physiological and pathological processes connected with respiration and transport of CO_2 or bicarbonate ion, CO_2 homeostasis, electrolyte secretion in many tissues, biosynthetic reactions, calcification, tumorigenicity, and

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; COX, cyclo-oxygenase enzyme; CLX, celecoxib; VLX, valdecoxib; CNS, central nervous system; VHL, von Hippel–Lindas protein; HIF, hypoxia inducible factor; PDB, protein data bank; XP, extra precision; RMSD, root mean square deviation; HIS, histidine; ASN, asparagine; 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.

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Table 1
Organ/tissue distribution, subcellular localization, CO_2 hydrase activity, and affinity for sulfonamides of the human CA isozymes (hCA I, II, VII, IX and XII)[1]

CA	Organ/Tissue distribution	Subcellular localization	Catalytic activity (CO_2 hydration)	Affinity for sulfonamides
CA I	Erythrocytes gastrointestinal tract, Eye	Cytosol	Low	Medium
CA II	Erythrocytes, Eye, Gastrointestinal tract, Bone osteoclasts, Kidney, Lung, Testis, Brain	Cytosol	High	Very high
CA VII	Central nervous system, liver	Cytosol	High	Very high
CA IX	Tumors, Gastrointestinal mucosa	Transmembrane	High	High
CA XII	Kidney, Intestine, Reproductive epithelia, Eye, Tumors	Transmembrane	Low	Very high

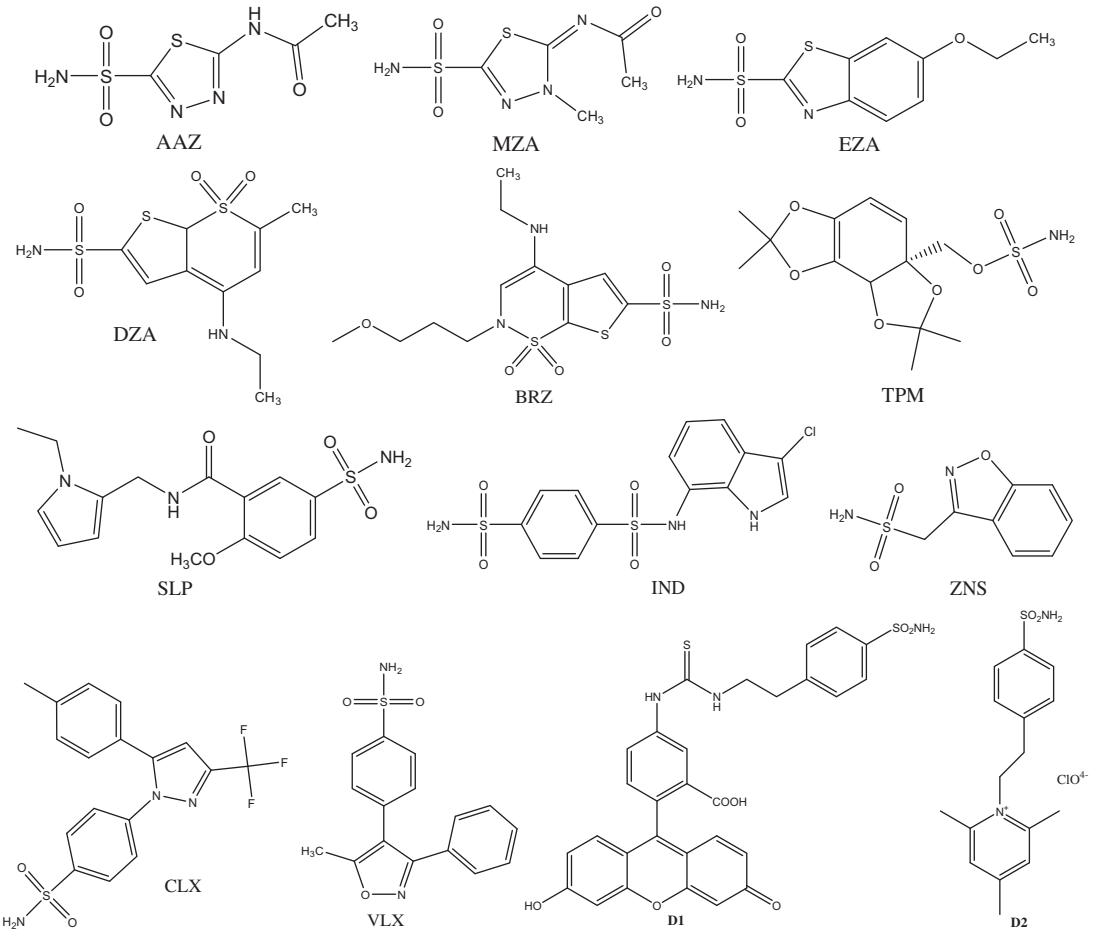


Figure 1. Carbonic anhydrase inhibitors sulfonamides and sulfamates.

so forth. 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]

Several studies demonstrated the important roles of hCAs 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 hCA isozymes have become interesting targets for the design of inhibitors or activators with biomedical applications.[4–7] Indeed originally hCA inhibitors (hCAIs) were clinically used (Fig. 1) as diuretic,[8] antiglaucoma,[9] and anticonvulsant,[10] whereas their employment as antiobesity drugs[11] or in the management of hypoxic tumors were only recently validated.[12–16] Examples of clinically used hCAIs: acetazolamide AZM, methazolamide MZA, ethoxzolamide EZA, dibromophenamide DCP, dorzolamide DZA, brinzolamide BRZ, benzolamide BZA, topiramate TPM, zonisamide ZNS, sulpiride SLP, indisulam IND, celecoxib CLX and valdecoxib VLX. However, because of the large number of hCA isoforms,[1–3] there is a constant need to improve the inhibition and selectivity profile of the so far developed CAIs, to avoid side effects due to inhibition of isoforms not involved in a certain pathology.[2]

Derivatives D1 and D2 (Fig. 1) are investigational agents for targeting the tumor-associated isoform hCA IX. The most useful CAIs for understanding the function of this protein in vivo were the fluorescent sulfonamide compounds of type D1.[2a] Compound D2 belongs to a class of positively charged trimethylpyridinium derivative.[2a] 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 hCA IX.[2a] It also participates in many other favorable interactions including stacking between the trimethylpyridinium ring of the inhibitor (D2) and the phenyl ring of Phe131.[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 (hCA I, hCA II, hCA VII, hCA IX and hCA XII) as drug targets/off-targets is shown in Table 2. To date the three-dimensional structures of all these 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 similar types of 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 a co-crystallized AZM/EZA replacing water molecule/hydroxide ion as ligand (Fig. 3).

Table 2
hCA isoforms as drug targets/offtargets in various diseases[1]

Isoform	Disease in which is involved	Possible offtargets among other hCAs
CA I	Retinal/Cerebral edema[8,9,19]	Unknown
CA II	Glaucoma[9]	hCA I
	Edema[8]	Unknown
	Epilepsy[10]	Unknown
	Altitude sickness[17,18]	Unknown
CA VII	Epilepsy[10]	Unknown
CA IX	Cancer[16]	hCA I, hCA II
CA XII	Cancer[16]	hCA I, hCA II
	Glaucoma[9]	Unknown

Specifically, hCA I is found in many tissues, but a study from Feener'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. Similarly hCA II is involved in several diseases, such as glaucoma, edema, epilepsy, and altitude sickness.[8–10,17,18] hCA VII mainly found in CNS has been noted for its contributions to epileptic activity together with hCA II and XIV.[10] hCA IX belongs to the highly active human α-CAs, its catalytic properties for the CO_2 hydration reaction being comparable with those of the highly evolved catalyst hCA II.[16] Carbonic anhydrase IX is considered as a potential target for hypoxia induced cancer therapy. hCA IX expression is increased upon hypoxia and has been proposed as a therapeutic target since it has been associated with poor prognosis, tumor progression and pH regulation. Key pH

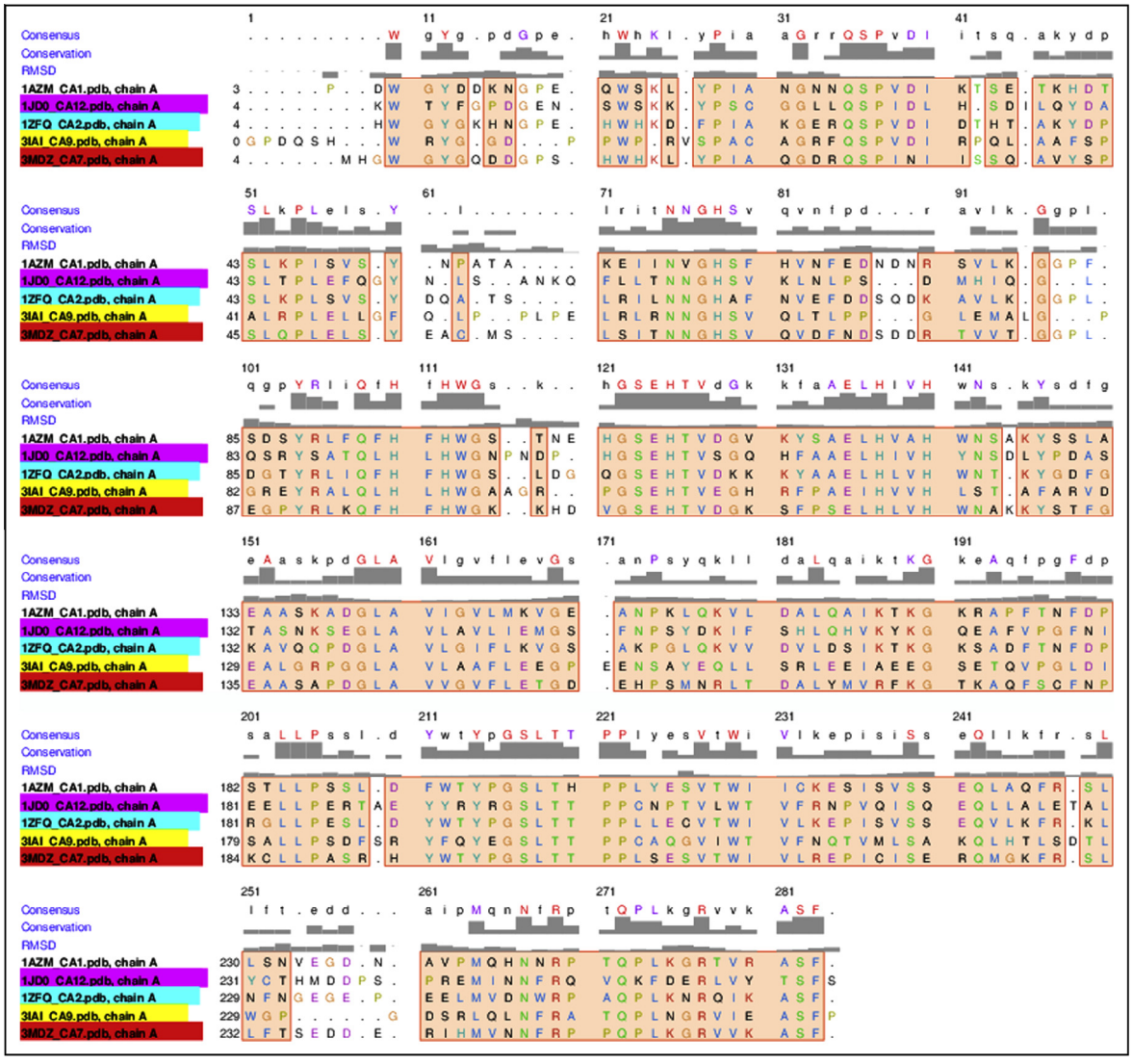


Figure 2. Structure-based sequence alignment of α-CAs with known three-dimensional structure. The following PDB entries were used in the alignment: 1AZM (hCA I); PDB: 1ZFQ (CA II); PDB: 3MDZ (hCA VII); PDB: 1IAI (CA IX) and PDB: 1JD0 (CA XII).[20a–e]

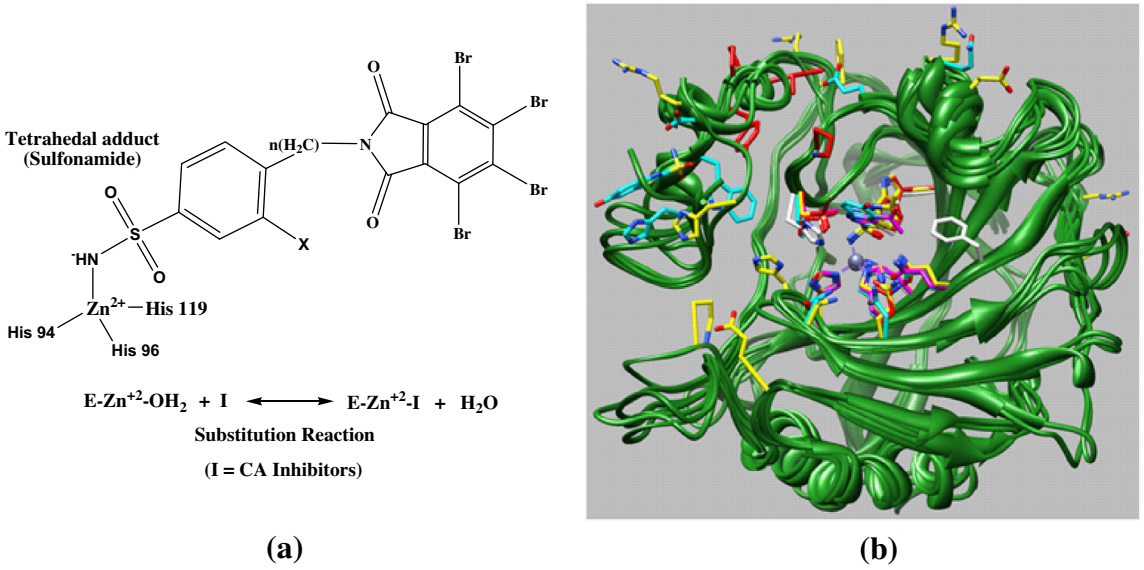
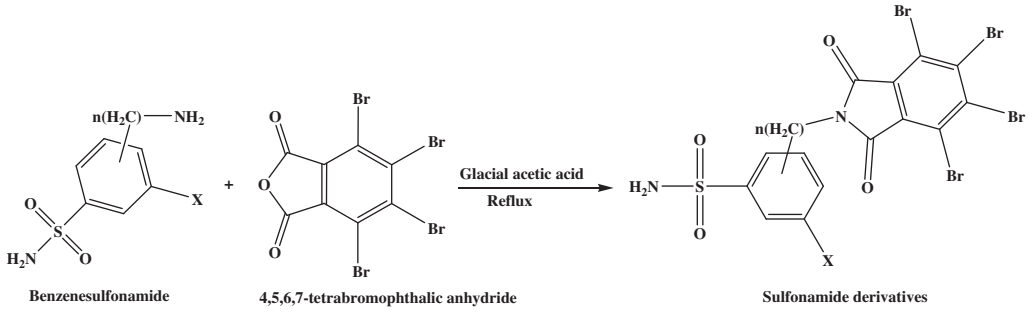


Figure 3. (a) Sulfonamide binds to the Zn^{2+} ion of the enzyme by substituting the non-protein zinc ligand to generate a tetrahedral adduct.[1] (b) Crystal structures alignment showing helix and β-strand regions of five hCA of PDB: 1AZM (hCA I); PDB: 1ZFQ (CA II); PDB: 3MDZ (hCA VII); PDB: 1IAI (CA IX) and PDB: 1JD0 (CA XII) binds to standard sulfonamide co-crystalized ligand AAZ and EZA to the Zn and His 94, His 96, and His 119 in the binding pocket.[20a–e]

regulators in tumor cells include: isoforms II, IX and XII of carbonic anhydrase, isoforms of anion exchangers, Na^+/HCO_3^- co-transporters, Na^+/H^+ exchangers, monocarboxylate transporters and the vacuolar ATPase.[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] hCA IX isoform is a dynamic drug target and a key feature of many hypoxia induced tumors.[2,16] Possible offtargets among other hCAs for hCA IX involved hCA I, hCA II (Table 2). hCA 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, , lungs, brain, vulva, squamous/basal cell carcinomas, and kidney tumors, among others. In some cancer cells, the von Hippel–Lindas protein (VHL) gene is mutated leading to the strong upregulation of hCA IX (up to 150-fold) as a consequence of constitutive hypoxia inducible factor (HIF) activation.[2,16]

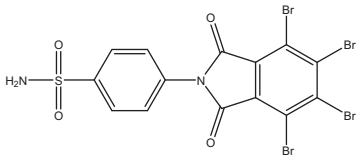
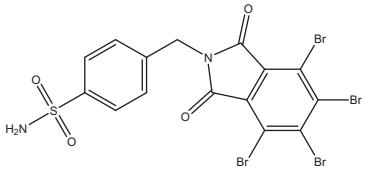
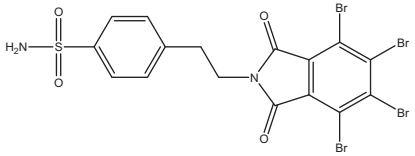
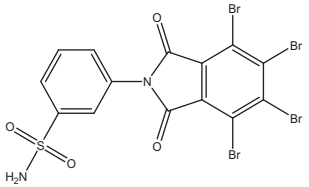
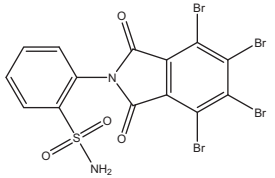
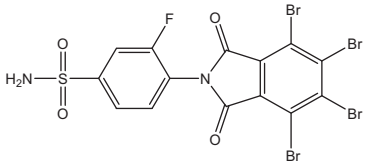
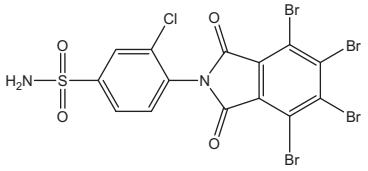
The potent hCAIs incorporate various zinc-binding groups (ZBGs) and thus belong to various chemotypes with the classical sulfonamide one, still constituting the main player in the field. The sulfonamides led to the development of several classes of pharmacological agents. Sulfonamide inhibitors directly bind to the metal ion within the enzyme active site (i.e. 1AZM; hCA I, 1FZQ; hCA II, 3MDZ; hCA VII, 3IAI; hCA IX and 1JD0; hCA XII), by substituting the zinc-bound hydroxide ion (Fig. 3).[20a–e] Other hCAIs, such as some anions, add to the metal co-ordination sphere, leading to pentaco-ordinated Zn (II) complexes.[3,4]

Some of these hCA isoforms are highly related and responsible for many diseases explained earlier and the similar compounds reported earlier were tested as hCA I, II and IV inhibitors, and showed excellent in vitro activity as well as antiglaucoma effects in rabbits which made an interest.[8–10,17–19,22] We reported here the structure based virtual evidence of the design of novel sulfonamide hCAIs. 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. These moieties should



Scheme 1. Scheme for the synthesis of sulfonamides **1–8** incorporating 4,5,6,7-tetrabromopthalic anhydride moiety to benzene sulfonamides. *n* = 0 for (**1**, **4**, **5**, **6**, **7**, **8**); *n* = 1 for **3**; *n* = 2 for **2**. X = H (**1**, **2**, **3**, **4**, **5**); F (**6**); Cl (**7**); Br (**8**). –(CH2)n– = in *para* position for **1**, **2**, **3**, **6**, **7**, **8**; in *meta* position for **4**; in *ortho* position for **5**.

Table 3
hCA I, II, VII, IX and XII inhibition data with sulfonamides **1**–**8**, standard AZM and other important CAIs, by a stopped-flow CO_2 hydrase assay method[28]

Compounds code	Structures/name	K_i[a] (nM)					Selectivity ratio	Docking score (Glide XP)				
		hCA I	hCA II	hCA VII	hCA IX	hCA XII	hCA II/hCA IX	1AZM	1ZFQ	3MDZ	3IAI	1JD0
1		432	68	54	9.1	6.1	7.5	−5.6	−4.7	−4.6	−4.7	−3.2
2		251	71	59	12.3	12.3	5.8	−6.0	−4.7	−5.7	−5.4	−4.6
3		185	80	77	15.9	10.2	5.0	−6.1	−4.8	−5.3	−5.2	−5.0
4		340	79	80	24	19	3.3	−5.8	−5.2	−5.7	−5.0	−4.3
5		>10,000	190	175	234	197	0.8	−3.9	−4.8	−3.2	−4.6	−4.1
6		770	96	84	9.4	7.5	10.2	−5.9	−4.7	−4.5	−4.7	−3.2
7		208	55	72	8.5	5.9	6.5	−6.0	−4.7	−4.9	−5.2	−3.6

(*continued on next page*)

Table 3 (*continued*)

Compounds code	Structures/name	K_i[a] (nM)					Selectivity ratio		Docking score (Glide XP)			
		hCA I	hCA II	hCA VII	hCA IX	hCA XII	hCA II/hCA IX	1AZM	1ZFQ	3MDZ	3IAI	1JD0
8		143	47	68	8.8	6.5	5.3	−6.1	−4.7	−4.7	−4.9	−4.0
AZM	Acetazolamide	250	12	2.5	25	5.7	0.5	−4.8	−3.8	−4.0	−4−4	−4.1
MZA	Methazolamide	50	14	2.1	27	3.4	0.5	−4.9	−3.8	−4.1	−4.9	−3.5
EZA	Ethoxzolamide	25	8	0.8	34	22	0.2	−5.7	−4.1	−4.9	−5.1	−4.2
DZA	Dorzolamide	50,000	9	3.5	52	3.5	0.2	−5.0	−4.5	−4.6	−4.8	−4.4
BRZ	Brinzolamide	45,000	3	2.8	37	3	0.1	−5.5	−5.3	−5.8	−4.9	−5.1
TPM	Topiramate	250	10	0.9	58	3.8	0.2	−6.1	−4.7	−5.2	−5.0	−4.4
SLP	Sulpiride	1200	40	3630	46	3.9	0.9	−6.5	−6.0	−6.9	−5.7	−4.9
IND	Indisulam	31	15	122	24	3.4	0.6	−6.5	−5.0	−6.2	−6.8	−5.2
ZNS	Zonisamide	56	35	117	5.1	11,000	6.9	−5.7	−3.7	−4.7	−4.7	−4.5
CLX	Celecoxib	50,000	21	2170	16	18	1.3	−6.5	−4.7	−5.5	−4.7	−3.3
VLX	Valdecoxib	54,000	43	3900	27	13	1.6	−6.1	−4.1	−4.8	−4.5	−4.0
D1	—	1300	45	18	24	5	1.9	−3.9	−4.4	−5.7	−5.0	−5.3
D2	—	4000	21	15	14	7	1.5	−4.1	−4.9	−5.7	−5.1	−5.0

[a] Mean from three different assays, errors were in the range of 5–10% of the reported values (data not shown).

produce, inter alia, high affinity to the hCA active site, acceptable water/lipid solubility, and good penetrability through the biological membranes, to the molecules of the newly obtained hCAIs. The tails chosen to be incorporated in the compounds reported here are of the 4-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (tetrabromophthalimide) type, since they may lead to interesting pharmacological properties for the new hCAIs containing them. The compounds reported here were obtained by reaction of various benzene sulfonamides with 4,5,6,7-tetrabromophthalic anhydride.[22–26]

2. Chemistry

The lead molecules for the present drug design study were the sulfonamides incorporating phthalimide moiety reported earlier by one of these groups.[22] The 4,5,6,7-tetrabromophthalic anhydride moiety is more hydrophobic compared to the phthalimide one contained in the derivatives reported previously, which showed interesting antiglaucoma properties in an animal model of the disease.[22a]

A simple chemistry has been used to prepare the novel sulfonamides of types **1**–**8** incorporating tetrabromophthalimide moiety to simple aromatic sulfonamides (Scheme 1). Simple aromatic sulfonamides having free amino groups were converted to the corresponding phthalimide by reaction with 4,5,6,7-tetrabromophthalic anhydride.[22a,b] It should be mentioned that the similar compounds reported earlier by reaction of aromatic/heterocyclic sulfonamides with phthalic anhydride derivatives, were tested as hCA I, II IV and VII inhibitors, and showed excellent in vitro activity as well as antiglaucoma effects in rabbits.[22]

3. hCA inhibition studies

The hCA inhibition on cytosolic hCA I, II, VII and membrane bound IX and XII (tumor associated hCA) data of compounds **1**–**8**, the standard AZM and other clinically used sulfonamides/ sulfamate are shown in Table 3. 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 3).[17,18]

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

i. The cytosolic, widespread isoforms hCA I was inhibited by the synthesized sulfonamides, with inhibition constants Kis in the range of 143 to >10,000 nM (Table 3). The best inhibitor in the series was the 3-bromo-4-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide 8 (K_i of 143 nM), whereas most other substitution patterns led to compounds **3**, **7** and **2** with inhibition constants 185, 208 and 251 nM, respectively are good/similar to that of acetazolamide (Table 3). The relatively weak inhibition of this isoform (compounds **4**, **1**, **6**, **5**) may be considered a positive feature of this class of CAIs since hCA I, highly abundant in red blood cells and is undoubtedly an offtarget when considering other applications of the CAIs.[2,27] The increased carbon chain length between the two bulky groups (–CH_2– or –CH_2CH_2–) led to an increased activities of compounds **1** < **2** < **3** with 432, 251 and 185 nM respectively of the derivatives. The position of –SO_2NH_2 in the benzene ring matters in the increase and decrease of activities. The increased activity of compounds according to position of –SO_2NH_2 in the benzene ring are 4th to 2nd (Kis 432 nM to >10,000 nM). Addition of halogen (F,

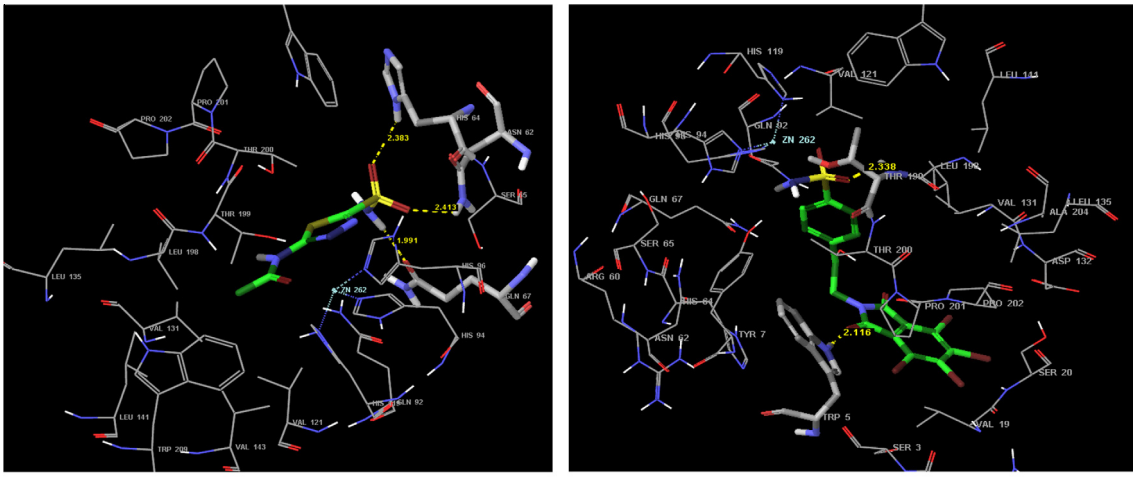


Figure 4. Docked conformations in the CA IX (PDB: 3IAI) catalytic site. (a) Acetazolamide in the binding pocket formed H-bond to HIS 64, ASN 62, GLN 67; (b) compound **3** (highest docking score) formed hydrogen bond to THR 190 and TRP 5.

Cl and Br) to the 3rd position of the benzene ring scaffold improved the activities (Kis 770 < 208 < 145 nM) with respect to increase the electro negativity of the halogen groups that is the compounds **6**, **7** and **8**.

ii. The physiologically dominant cytosolic isoform hCA II was generally moderately inhibited by the synthesized sulfonamides compared to standard AZM, which showed Kis in the range of 47–190 nM (Table 3). Against hCA II, compound 8 was found to be good inhibitor, with a K_i of 47 nM then the other compounds of the series. Although the compounds are moderate inhibitors for hCA II but SARs is rather straightforward. Addition of halogen (F, Cl and Br) to the 3rd position to the benzene ring that is compounds **6**, **7** and **8** improved the activities (Kis 96 < 55 < 47 nM) with respect to increase in the electro negativity of the halogen groups. The increased carbon chain length between the two bulky groups (–CH_2– or –CH_2CH_2–) led to an decreased activities of compounds 1 > 2 > 3 with Kis of 68, 71 and 80 nM, respectively of the derivatives. The 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** (Kis in the range of 190, 79 and 68 nM). Overall, these sulfonamides show a moderate inhibitory action towards hCA II, which is the main offtarget isoform among the various hCAs.[27]

iii. The cytosolic isoform hCA VII was also poorly inhibited by the synthesized sulfonamide, Kis in the range of 54–175 nM (Table 3) then the standard AZM. Compound **1** of the series was the most potent inhibitor with a K_i of 54 nM, but most substitution patterns to the aromatic ring of sulfonamide, such as the various halogens, led to compounds with improved inhibitory activity (Kis in the range of 84 < 72 < 68 nM). The 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 (Kis in the range of 175, 80 and 54 nM). 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 54, 59 and 77 nM, respectively of the derivatives. Overall, these sulfonamides show a poor inhibitory action towards hCA VII, which is one of a major isoform among the various hCAs in CNS.[1]

iv. The transmembrane isoforms hCA IX (tumor associated) was effectively inhibited by this new class of sulfonamide, which showed Kis in the range of 8.5–234 nM (Table 3). SAR is rather straightforward. For example, against hCA IX, in comparisons to AZM the compounds **7** and **8** were potent inhibitors, with a K_i of 8.5 and 8.8 nM, respectively. Addition of halogen (F, Cl and Br) to the 3rd position to the compounds **6**, **7** and **8** scaffold 'benzene' carrying sulfonamide improved the activities (Kis in the range of 9.4–8.8 nM) with respect to increase in the electro negativity of the halogen groups. The increased carbon chain length between the two bulky groups (–CH_2– or –CH_2CH_2–) led to an decreased activities of compounds **1** > **2** > **3** with Kis of 9.1, 12.3 and 15.9 nM, respectively of the derivatives. Some steric hindrance effects led to a loss of the hCA IX inhibition activities, for example –SO_2NH_2 present in the *ortho*-, *meta*- and *para*-substituted sulfonamides **5** < **4** < **1** (showed Kis of 234, 24 and 9.1 nM).

v. The transmembrane isoforms hCA XII (tumor associated) on the other hand also effectively inhibited by this new class of sulfonamide hCAIs, which showed Kis in the range of 5.9–197 nM in comparisons to AZM (Table 3). Here also the SARs are straightforward. For example, against hCA XII, compounds **1**, **7** and **8** was potent inhibitor, with a Kis of 6.1, 5.9 and 8.8 nM, respectively. 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_i of 6.1, 12.3 and 10.2 nM respectively of the derivatives. Some steric hindrance effects led to a loss of the hCA XII inhibition activity, for example –SO_2NH_2 present in the *ortho*-, *meta*- and *para*-substituted sulfonamides **5** < **4** < **1** (showed Kis of 197, 19 and 6.1 nM). Addition of halogen (F, Cl and Br) to the 3rd position to the compounds **6**, **7** and **8** scaffold 'benzene' carrying sulfonamide improved the activity of compounds. The improved activities (Kis in the range of 7.5–6.5 nM) with respect to increase in the electro negativity of the halogen groups.

vi. The selectivity ratio for inhibiting the tumor-associated isoforms hCA IX over the cytosolic offtarget one hCA II,[27] was in the range of 0.8–10.2 for the new sulfonamide reported here (Table 3). This is a good result, since most sulfonamide hCAIs (D1, D2, acetazolamide, ethoxzolamide, dichlorophenamide, etc.) show better hCA II than hCA IX inhibitory action.[2] In the new series reported here, only compound **5** had a low selec-

tivity ratio in the of 0.8, being thus relatively little hCA IX-selective. The remaining ones had selectivities ratios in the range of 3.3–10.2, being thus highly selective hCA IX/XII inhbitors (compared to the inhibition of hCA II and I).

4. Docking studies

Molecular docking of eight synthesized compounds and other clinically available sulfonamides/sulfamate was performed to rationalize the SARs reported. Docking study was performed preferably by interest in crystal structures of hCA preferably in 1AZM (hCA I), 1FZQ (hCA II), 3MDZ (hCA VII), 3IAI (hCA IX) and 1JD0 (hCA XII).[20a–e] All the 'A' chain of the crystal structures catalytic domains of the hCA was considered for docking. The Glide (XP) score of the co-crystallized ligand AZM is −3.8 to −4.8 and the RMSD values ranges from 1.8 to 2.3 which is considered as good for docking of the ligands (Table 3). 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 3). The docked conformations of AZM (Fig. 4) and compound **2** (highest docking score) in catalytic site in the binding pocket of hCA IX (PDB: 3IAI).

The main objective of our molecular docking study of compounds was to rationalize the in vitro SARs reported over here. In each and every case we have observed that the concluded SAR of the compounds tally to the results obtained by means of docking study. For examples: in case of compound **5**, the docking scores found to be lowest 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 scores in case of hCA I and decrease the docking scores in hCA II, hCA VII, hCA IX and hCA XII of the synthesized derivatives. Some steric hindrance effects led to a loss of docking scores, for example –SO_2NH_2 present in the *ortho*-, *meta*- and *para*-substituted sulfonamides decrease in scores of compounds **5** < **4** < **1**. Addition of halogen (F, Cl and Br) to the 3rd position to the compounds **6**, **7** and **8** scaffold 'benzene' carrying sulfonamide improved the scores of compounds with respect to increase in the electro negativity of the halogen groups.

5. Conclusions

A small series of 4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl benzenesulfonamide was prepared and investigated for the inhibition of the five physiologically relevant hCA isoforms hCA I, hCA II, hCA VII, hCA IX and hCA XII. These compounds were generally relevantly good to moderate potent inhibitors of hCA I, but were moderate to poorly effective with low nanomolar in hCA II and poorly inhibit hCA VII. Such compounds may constitute interesting candidates for the development of novel anti-glaucoma, antiepileptic, edema, or altitude sickness drugs. The compounds are excellent inhibitors of transmembrane isoforms hCA IX and hCA XII effectively in low nanomolar and with high selectivity ratio by this new class of sulfonamide hCAIs. Such potent compounds may promote as novel and interesting candidates for the development of more selective and potent hypoxia induced cancer drug therapy.

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 atmosphere of nitrogen. The list of chemicals used are *p*-amino benzene sulfanilamide (Sigma–Aldrich), 4,5,6,7-tetrabromophthalic anhydride (Sigma–Aldrich), glacial acetic acid, Bromoform, 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.

6.2. Chemistry

A series of 4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl benzenesulfonamide derivatives (compounds **1**–**8**) was synthesized by refluxing mixture of selected sulfonamide and tetrabromophthalic anhydride in glacial acetic acid as solvent with stirring under nitrogen environment for desired reaction time.[22–26]

6.2.1. Synthesis of 4-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (1)

0.002 mol (0.344 g) of 4-aminobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.927 g) of 4,5,6,7-tetrabromo isobenzofuran-1,3-dione to produce 0.002 mol of 4-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**1**) (1.236 g) in the presence of glacial acetic acid as solvent for 2 h at 130 °C. The reaction monitored in each 30 min with the help of TLC (chloroform/methanol; 3:1). After that the 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 in ethanol for further purification.[22–26]

White crystalline and solid; yield = 75%; mp = 284 °C; solubility; insoluble: water, glacial acetic acid; partially poluble: ethanol; fully soluble: DMSO and methanol, chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1774.56, 1718.63 (C═O imide); 1313.57, 1157.33 (S═O) and 3486.18 (NH_2). MS (ESI+); m/z: 612.7 [M−H]$^-$, 614.7 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.532 (s, 2H, SO_2NH_2), 7.666–7.687 (d, 2H, Ar–H from benzenesulfonamide), 8.018–8.040 (d, 2H, Ar−H from benzenesulfonamide). ^{13}C NMR (100 MHz, DMSO-d_6):121.88, 127.46, 128.81, 131.99, 135.30, 137.72, 144.79, 163.63.

6.2.2. Synthesis of 4-((4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)methyl)benzenesulfonamide hydrochloride (2)

0.002 mol (0.445 g) of 4-(aminomethyl)benzenesulfonamide hydrochloride stirred under nitrogen environment with 0.002 mol (0.927 g) of 4,5,6,7-tetrabromo isobenzofuran-1,3-dione to produce 0.002 mol of 4-(2-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)ethyl) benzenesulfonamide (**2**) (1.264 g) in the presence of glacial acetic acid as solvent for 1.5 h at 130 °C. The reaction monitored in each 30 min with the help of TLC (chloroform/methanol; 1:1). After that the 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 in ethanol for further purification.[22–26]

White crystalline and solid; yield = 94%; mp = 293°C; solubility; insoluble: water, glacial acetic acid; partially soluble: ethanol and methanol; fully soluble: DMSO, chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1766.85, 1712.86 (C═O imide); 1338.64, 1159.26 (S═O) and 3356.25 (NH_2). MS (ESI+); m/z: 626.7 [M−H]$^-$, 628.7 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.375 (s, 2H, SO_2NH_2), 7.56, 7.581 (d, 2H, Ar–H from benzenesulfonamide), 7.804–7.825 (d, 2H, Ar–H from benzenesulfonamide), 4.880 (s, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6): 41.79, 121.07, 126.33, 128.38, 131.71, 136.86, 140.24, 143.75, 164.12.

6.2.3. Synthesis of 4-(2-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)ethyl)benzenesulfonamide (3)

0.002 mol (0.400 g) of 4-(2-aminoethyl)benzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.927 g) of 4,5,6,7-tetrabromo isobenzofuran-1,3-dione to produce 0.002 mol of 4-(2-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)ethyl)benzenesulfonamide (**3**) (1.292 g) in the presence of glacial acetic acid as solvent for 1.5 h at 130 °C. The reaction monitored in each 30 min with the help of TLC (chloroform/methanol; 1:1). After that the 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 in ethanol for further purification.[22–26]

White crystalline and solid; yield = 99%; mp = 278 °C; solubility; insoluble: water, glacial acetic acid; partially soluble: ethanol, methanol; fully soluble: DMSO and chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1772.64, 1716.70 (C=O imide); 1334.78, 1156 (S=O) and 3358.18 (NH_2), 2953.12 (aliphatic CH_2). MS (ESI+); *m*/*z*: 658.5 [M−H+H_2O]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.351 (s, 2H, SO_2NH_2), 7.476–7.497 (d, 2H, Ar–H from benzenesulfonamide), 7.772–7.793 (d, 2H, Ar–H from benzenesulfonamide), 3.004, 3.022, 3.041 (t, 2H, CH_2), 3.837, 3.857, 3.874 (t, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6): 33.63, 120.93126.32, 129.65, 131.48, 136.87, 142.80, 142.91, 164.01.

6.2.4. Synthesis of 3-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (4)

0.002 mol (0.344 g) of 3-aminobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.927 g) of 4,5,6,7-tetrabromo isobenzofuran-1,3-dione to produce 0.002 mol of 3-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**4**) (1.236 g) in the presence of glacial acetic acid as solvent for 2 h at 130 °C. The reaction monitored in each 30 min with the help of TLC (chloroform/methanol; 3:1). After that the 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 in ethanol for further purification.[22–26]

Reddish white crystalline and solid; yield = 92%; mp = 306 °C; solubility; insoluble: water, glacial acetic acid; partially soluble: ethanol and methanol; fully soluble: DMSO, chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1770.64, 1716.70 (C=O imide); 1338.64, 1161.19 (S=O) and 3367.82 (NH_2). MS (ESI+); *m*/*z*: 612.7 [M−H]$^-$, 614.6 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.6 (s, 2H, SO_2NH_2), 7.689–7.694 (d, 2H, Ar–H from benzenesulfonamide), 7.706–7.714 (d, 2H, Ar–H from benzenesulfonamide), 7.787–7.828 (t, 2H, Ar–H from benzenesulfonamide), 7.958–7.977 (d, 2H, Ar–H from benzenesulfonamide). ^{13}C NMR (100 MHz, DMSO-d_6): 121.81, 125.78, 126.87, 130.94, 131.88, 132.2, 132.98, 137.71, 146.0, 163.89.

6.2.5. Synthesis of 2-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (5)

0.002 mol (0.344 g) of 2-aminobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.927 g) of 4,5,6,7-tetrabromo isobenzofuran-1,3-dione to produce 0.002 mol of 2-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**5**) (1.236 g) in the presence of glacial acetic acid as solvent for 2 h at 130 °C. The reaction monitored in each 30 min with the help of TLC (chloroform/methanol; 3:1). After that the 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 in ethanol for further purification.[22–26]

White crystalline and solid; yield = 80%; mp = 272 °C; solubility; insoluble: water, glacial acetic acid; partially soluble: ethanol and methanol; fully soluble: DMSO and chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1770.71, 1708.99 (C=O imide); 1340.57, 1172.76 (S=O) and 3390.97 (NH_2). MS (ESI+); *m*/*z*: 612.6 [M−H]$^-$, 614.6 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.646 (s, 2H, SO_2NH_2), 7.663–7.668 (d, 2H, Ar–H from benzenesulfonamide), 7.772–7.854 (t, 2H, Ar–H from benzenesulfonamide), 8.097–8.12 (d, 2H, Ar−H from benzenesulfonamide). ^{13}C NMR (100 MHz, DMSO-d_6): 121.93, 129.6, 131.6, 131.53, 132.38, 132.77, 134.0, 137.69, 139.03, 143.23, 163.49.

6.2.6. Synthesis of 3-fluoro-4-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (6)

0.002 mol (0.380 g) of 4-amino-2-fluorobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.927 g) of 4,5,6,7-tetrabromo isobenzofuran-1,3-dione to produce 0.002 mol of 3-fluoro-4-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**6**) (1.272 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 that the 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 in ethanol for further purification.[22–26]

White crystalline and solid; yield = 90%; mp = 281 °C; solubility; insoluble: water, glacial acetic acid; partially soluble: ethanol; fully soluble: DMSO, chloroform and methanol. IRν_{max} (cm^{-1}; KBr pellets); 1780.36, 1726.35 (C=O imide); 1339.61, 1158.29 (S=O) and 3372.65 (NH_2). MS (ESI+); *m*/*z*: 632.66 [M], 632.66 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.785 (s, 2H, SO_2NH_2), 7.785–7.824 (d, 2H, Ar–H from benzenesulfonamide), 7.884–7.907 (d, 2H, Ar–H from benzenesulfonamide), 7.931 (s, 2H, Ar–H from benzenesulfonamide). ^{13}C NMR (100 MHz, DMSO-d_6): 114.95, 122.10, 123.27, 131.95, 138.00, 147.48, 156.40, 158.94, 162.98.

6.2.7. Synthesis of 3-chloro-4-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (7)

0.002 mol (0.412 g) of 4-amino-2-chlorobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.927 g) of 4,5,6,7-tetrabromo isobenzofuran-1,3-dione to produce 0.002 mol of 3-chloro-4-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**7**) (1.305 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 that the 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 in ethanol for further purification.[22–26]

White crystalline and solid; yield = 92%; mp = 265 °C; solubility; insoluble: water, glacial acetic acid; partially soluble: ethanol; fully soluble: DMSO, chloroform and methanol. IRν_{max} (cm^{-1}; KBr pellets); 1780.36, 1722.49 (C=O imide); 1339.61, 1166.97 (S=O) and 3366.86 (NH_2). MS (ESI+); *m*/*z*: 646.83 [M−1], 648.83 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): i 7.717 (s, 2H, SO_2NH_2), 7.851–7.872 (d, 2H, Ar–H from benzenesulfonamide), 8.008–8.034 (d, 2H, Ar–H from benzenesulfonamide), 8.152 (s, 2H, Ar–H from benzenesulfonamide). ^{13}C NMR (100 MHz, DMSO-d_6): 122.24, 126.42, 128.09, 131.69, 133.77, 136.96, 138.27, 147.48, 162.84, 166.77.

6.2.8. Synthesis of 3-bromo-4-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (8)

0.002 mol (0.502 g) of 4-amino-2-bromobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.927 g) of 4,5,6,7-tetrabromo isobenzofuran-1,3-dione to produce 0.002 mol of 3-bromo-4-(4,5,6,7-tetrabromo-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**8**) (1.394 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 that the 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 in ethanol for further purification.[22–26]

White crystalline and solid; yield = 90%; mp = 255 °C; solubility; insoluble: water, glacial acetic acid; partially soluble: ethanol; fully soluble: DMSO, chloroform and methanol. IRν_{max} (cm^{-1}; KBr pellets); 1767.82, 1723.45 (C=O imide); 1340.57, 1120.86 (S=O) and 3379.40 (NH_2). MS (ESI+); *m/z*: 690.75 $[M-H]^-$, 692.75 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.708 (s, 2H, SO_2NH_2), 7.832–7.853 (d, 2H, Ar–H from benzenesulfonamide), 8.043–8.069 (d, 2H, Ar–H from benzenesulfonamide), 8.288 (s, 2H, Ar–H from benzenesulfonamide). ^{13}C NMR (100 MHz, DMSO-d_6): 122.25, 124.26, 126.98, 131.90, 132.95, 136.99, 138.37, 147.57, 162.77, 166.74.

6.3. hCA inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the hCA-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 hCA-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.[28–30]

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