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Carbonic anhydrase inhibitors: Synthesis and inhibition of the cytosolic mammalian carbonic anhydrase isoforms I, II and VII with benzene sulfonamides incorporating 4,5,6,7-tetrachlorophthalimide moiety

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ABSTRACT

A series of 4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl benzenesulfonamide derivatives (compounds **1**–**8**) was synthesized by reaction of benzene sulfonamides incorporating primary amino moieties with 4,5,6,7-tetrachlorophthalic anhydride. These sulfonamides were assayed as inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). Some of these compounds showed very good in vitro human carbonic anhydrase (hCA) isoforms I, II and VII inhibitory properties, with affinities in the low nanomolar range. Inhibition activities against hCA I were in the range of 159–444 nM; against hCA II in the range of 2.4–4515 nM, and against hCA VII in the range of 1.3–469 nM. The structure–activity relationship (SAR) with this series of sulfonamides is straightforward, with the main features leading to good activity for each isoform being established.

1. Introduction

Carbonic anhydrases (CAs) are 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, 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 isozymes (CA IV, CA IX, CA XII, CA XIV and CA XV), two mitochondrial forms (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) whose functions remain unclear.[3] CA which catalyze the interconversion between carbon dioxide and bicarbonate by using a metal hydroxide nucleophilic mechanism and other 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]

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] whereas their employment as antiobesity drugs[11] or in the management of hypoxic tumors were only recently validated.[12–16] 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, 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

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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)[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

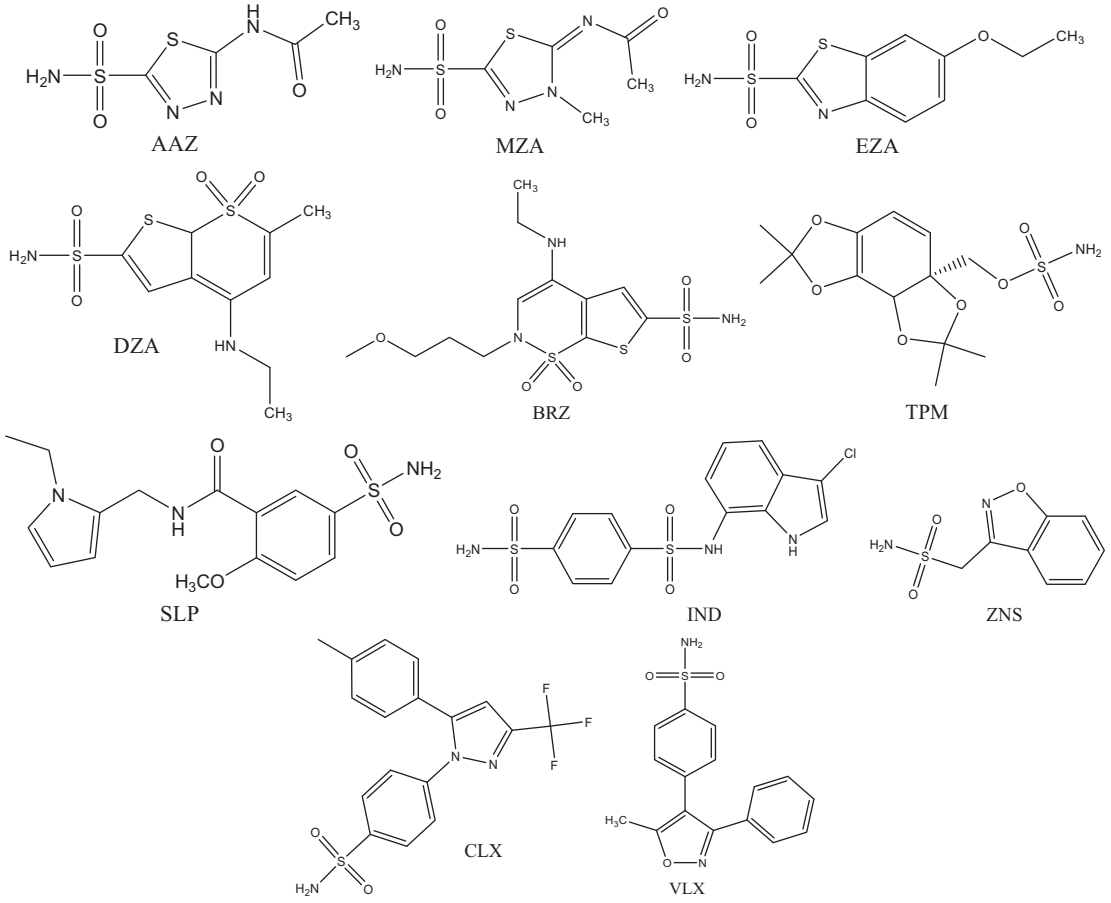


Figure 1. Clinically used sulfonamides and sulfamates.

far developed CAIs, to avoid side effects due to inhibition of isoforms not involved in a certain pathology.[2]

A brief presentation of the hCA isoforms (hCA I, hCA II, hCA VII) as drug targets/off-targets is shown in Table 2. Specifically, CA 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. CA II is involved in several diseases, such as glaucoma, edema, epilepsy, and altitude sickness.[8–10,17,18] CA VII has been noted for its contributions to epileptic activity together with CA II and XIV.[10]

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 inhibitors directly bind to the metal ion within the enzyme active site, by substituting the zinc-bound hydroxide ion (Fig. 2). Other CAIs, such as some anions, add to the metal co-ordination sphere, leading to pentaco-ordinated Zn (II) complexes.[3,4]

These three isoforms are highly related to the central nervous system and responsible for many disease explained earlier and the similar compounds reported earlier were tested as CA I, II

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]	Unknown
CA II	Glaucoma[9]	hCA I
	Edema[8]	Unknown
	Epilepsy[10]	Unknown
	Altitude sickness[17,18]	Unknown
CA VII	Epilepsy[10]	Unknown

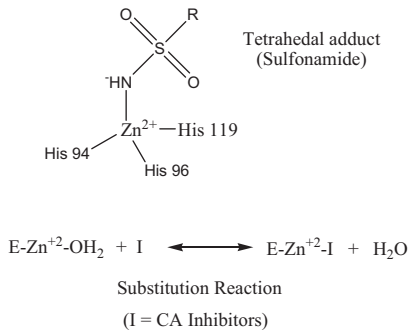


Figure 2. Sulfonamide binds to the Zn^{2+} ion of the enzyme by substituting the nonprotein zinc ligand to generate a tetrahedral adduct.[1]

and IV inhibitors, and showed excellent in vitro activity as well as antiglaucoma effects in rabbits which made an interest.[8–10,17–19,21] We report here the design of novel sulfonamide CAIs. The drug design has been based on the 'tail' strategy reported previously[20] 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 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 4-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (tetrachlorophthalimide) 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 4,5,6,7-tetrachlorophthalic anhydride.[21–25]

2. Chemistry

The lead molecules for the present drug design study were the sulfonamides incorporating phthalimido moieties reported earlier by one of these groups.[21] The 4,5,6,7-tetrachlorophthalic 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.[21]

A simple chemistry has been used to prepare the novel sulfonamides of types **1**–**8** incorporating tetrachlorophthalic anhydride moieties 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-tetrachlorophthalic anhydride.[21] It should be mentioned that the similar compounds reported earlier by reaction of aromatic/heterocyclic sulfonamides with phthalic anhydride, were tested as CA I, II and IV inhibitors, and showed excellent in vitro activity as well as antiglaucoma effects in rabbits.[21]

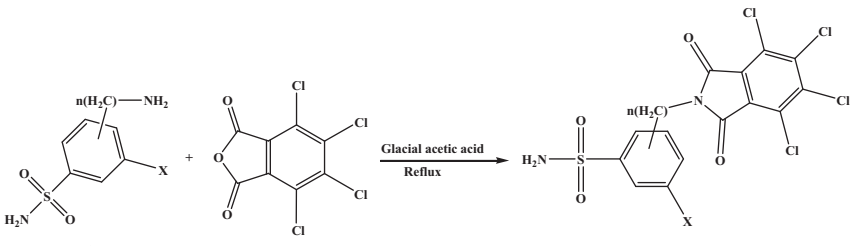
3. CA inhibition studies

The CA inhibition on hCA I, II, VII 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 moderately inhibited by the synthesized sulfonamides, with inhibition constants in the range of 150–>10,000 nM (Table 3). The best inhibitor in the series was the 3-chloro-4-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide **7** (K_i of 150 nM), whereas most other substitution patterns led to compounds with inhibition constants similar to that of acetazolamide (Table 3). The weak inhibition of this isoform may be considered a positive feature of this class of CAIs since hCA I, highly abundant in red blood cells, is undoubtedly an offtarget when considering other applications of the CAIs.[26]

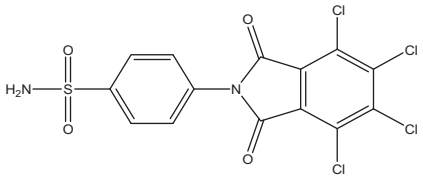
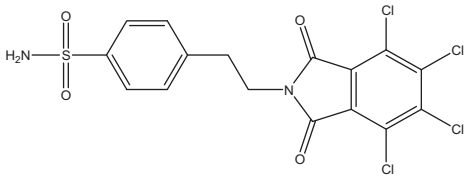
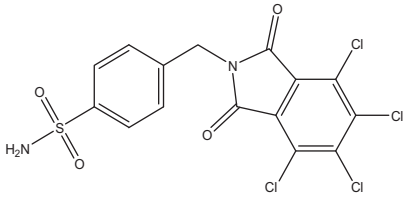
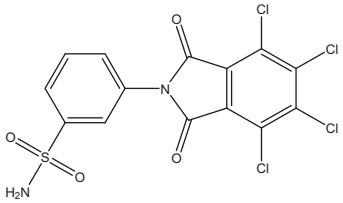
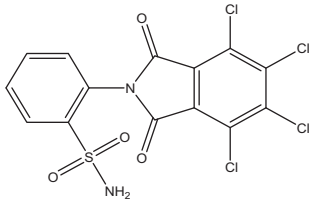
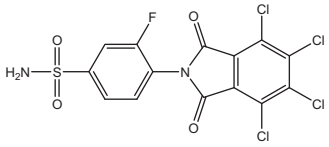
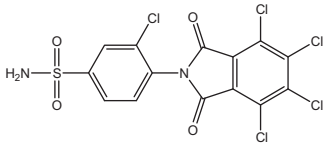
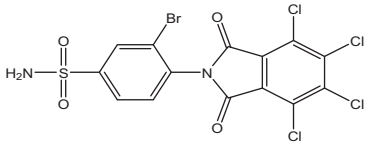
ii. The physiologically dominant cytosolic isoform hCA II was generally strongly inhibited by the synthesized sulfonamide, which showed K_{is} in the range of 2.4–4515 nM (Table 3). SAR is rather straightforward. For example, against hCA II, compound **8** was a potent inhibitor, with a K_i of 2.4 nM. The substitution patterns at the aromatic ring, such as halogens, led to compounds with similar or slightly improved inhibitory activity over the sulfanilamide derivative **1** (K_{is} in the range of 2.4–4.9 nM). Some steric hindrance effects led to a loss of the hCA II inhibition, such as for example those present in the *meta*- or *ortho*-substituted sulfonamides **4**, **5**. These compounds showed K_{is} in the range of 27.7–4515 nM. The linker between the two bulky groups (–CH_2– or –CH_2CH_2–) led to an increase of the activity of compounds **2** and **3** compared to the sulfanilamide derivative **1**. Overall, these sulfonamides showed a good inhibitory action towards hCA II, except for the orthanilamide derivative **5** which was a weak inhibitor.[26]



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**)
-(CH_2)n- = in *para* position for **1**, **2**, **3**, **6**, **7**, **8**; in *meta* position for **4**; in *ortho* position for **5**

Scheme 1. Scheme for the synthesis of sulfonamides **1**–**8** incorporating 4,5,6,7-tetrachloropthalic anhydride moiety to benzene sulfonamides.

Table 3
hCA I, II, VII Inhibition data with sulfonamides **1–8**, standard AZM and other clinically used CAIs, by a stopped-flow CO_2 hydrase assay method[27]

Compd. code	Structures/name	Ki (nM)*		
		hCA I	hCA II	hCA VII
1		332	7.1	2.1
2		326	2.9	3.5
3		427	5.2	3.1
4		444	27.7	3.5
5		>10,000	4515	46.9
6		368	3.4	1.3
7		159	4.9	4.7
8		281	2.4	2.2
AAZ	Acetazolamide {*N*-(5-sulfamoyl-1, 3, 4-thiadiazol-2-yl) acetamide}	250	12	2.5
MZA	Methazolamide	50	14	2.1
EZA	Ethoxzolamide	25	8	0.8
DZA	Dorzolamide	50,000	9	3.5
BRZ	Brinzolamide	45,000	3	2.8
TPM	Topiramate	250	10	0.9
SLP	Sulpiride	1200	40	3630
IND	Indisulam	31	15	122
ZNS	Zonisamide	56	35	117
CLX	Celecoxib	50,000	21	2,170
VLX	Valdecoxib	54,000	43	3900

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

iii. The cytosolic isoform hCA VII was also strongly inhibited by the synthesized sulfonamide, which showed K_{is} in the range of 1.3–46.9 nM (Table 3). Compound **6** of the series was the most potent inhibitor with a K_i of 1.3 nM, but most substitution patterns to the aromatic ring of sulfanilamide, such as the various halogens, led to compounds with similar inhibitory activity (K_{is} in the range of 1.3–4.7 nM). The steric hindrance of the *ortho*-substituted derivative compound **5** led to a weaker hCA VII inhibition (K_i = 46.9 nM). A linker between the two bulky moieties present in these compounds did not dramatically change the hCA VII inhibitory power of the compounds (compared to compounds **1**, **2** and **3**).

4. Conclusions

A small series of 4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl benzenesulfonamide was prepared and investigated for the inhibition of three physiologically relevant CA isoforms CA I, CA II and CA VII. These compounds were generally relevantly less potent inhibitors of CA I, but many of them were effective, low nanomolar CA II and CA VII. Such compounds may constitute interesting candidates for the development of novel antiglaucoma, antiepileptic, edema, or altitude sickness drugs.

5. Experimental section

5.1. 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. *p*-Amino benzene sulphanilamide (Sigma–Aldrich), 4,5,6,7-tetrachlorophthalic 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 Micro-Mass ZQ.

5.2. Chemistry

A mixture of selected sulfonamide and tetrachlorophthalic anhydride in glacial acetic acid was refluxed with stirring under nitrogen environment for desirable time to complete the reaction lead to compounds **1**–**8**.[21–25]

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

0.002 mol (0.344 g) of 4-aminobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.572 g) of 4,5,6,7-tetrachlorophthalic anhydride to produce 0.002 mol of 4-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**1**) (0.880 g) in the presence of glacial acetic acid as solvent for 2 h at 130 °C. The reaction was 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 to purify the synthesized compounds.[21–25]

White crystalline and solid; % yield = 98%; mp = 315–320 °C; solubility; insoluble: water, acetic acid glacial; partially soluble: ethanol; fully soluble: DMSO and methanol, chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1778.43, 1716.70 (C=O imide); 1321.28, 1163.11 (S=O) and 3414.12 (NH_2). MS (ESI+); m/z: 440.8 [M], 436.8 [M−H]$^-$, 438.8 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.533 (s, 2H, SO_2NH_2), 7.699–7.678 (d, 2H, Ar−H from benzenesulfonamide), 8.050–8.029 (d, 2H, Ar−H from benzenesulfonamide). ^{13}C NMR (100 MHz, DMSO-d_6): 167.29, 144.38, 138.98, 134.49, 128.9, 128.8, 128.23, 127.04.

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

0.002 mol (0.400 g) of 4-(2-aminoethyl)benzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.572 g) of 4,5,6,7-tetrachlorophthalic anhydride to produce 0.002 mol of 4-(2-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)ethyl)benzenesulfonamide (**2**) (0.936 g) in the presence of glacial acetic acid as solvent for 1.5 h at 130 °C. The reaction was 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 to purify the synthesized compounds.[21–25]

White crystalline and solid; % yield = 98%; mp = 286 °C; solubility; insoluble: water, acetic acid glacial; partially soluble: ethanol, methanol; fully soluble: DMSO and chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1776.50, 1712.92 (C=O imide); 1303.92, 1159.29 (S=O) and 3387.11 (NH_2), 2955.04 (aliphatic CH_2). MS (ESI+); m/z: 465.2 [M], 283.2 [M−184.24]$^-$, 450.8 [M−16.02]$^-$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.347 (s, 2H, SO_2NH_2), 7.473–7.494 (d, 2H, Ar−H from benzenesulfonamide), 7.763–7.785 (d, 2H, Ar−H from benzenesulfonamide), 3.011, 3.029, 3.048 (t, 2H, CH_2), 3.849, 3.868, 3.885 (t, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6): 164.19, 143.41, 138.143.21, 139.17, 130.16, 129.23, 129.03, 126.81, 34.13.

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

0.002 mol (0.445 g) of 4-(aminomethyl)benzenesulfonamide hydrochloride stirred under nitrogen environment with 0.002 mol (0.572 g) of 4,5,6,7-tetrachlorophthalic anhydride to produce 0.002 mol of 4-(2-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)ethyl)benzenesulfonamide (**3**) (0.955 g) in the presence of glacial acetic acid as solvent for 1.5 h at 130 °C. The reaction was 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 to purify the synthesized compounds.[21–25]

White crystalline and solid; % yield = 90%; mp = 305 °C; solubility; insoluble: water, acetic acid glacial; partially soluble: ethanol and methanol; fully soluble: DMSO, chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1774.57, 1714.77 (C=O imide); 1338.64, 1161.19 (S=O) and 3350.46 (NH_2). MS (ESI+); m/z: 454.7 [M], 450.8 [M−H]$^-$, 452.8 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.38 (s, 2H, SO_2NH_2), 7.565, 7.586 (d, 2H, Ar−H from benzenesulfonamide), 7.81–7.831 (d, 2H, Ar−H from benzenesulfonamide), 4.891 (s, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6): 164.28, 144.26, 140.63, 139.1, 129.49, 129.1, 128.89, 126.81, 42.1.

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

0.002 mol (0.344 g) of 3-aminobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.572 g) of 4,5,6,

7-tetrachlorophthalic anhydride to produce 0.002 mol of 3-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**4**) (0.880 g) in the presence of glacial acetic acid as solvent for 2 h at 130 °C. The reaction was 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 to purify the synthesized compounds.[21–25]

White crystalline and solid; % yield = 92%; mp = 308 °C; solubility; insoluble: water, acetic acid glacial; partially soluble: ethanol and methanol; fully soluble: DMSO, chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1782.29, 1718.68 (C=O imide); 1301.99, 1132.25 (S=O) and 3356.25 (NH_2). MS (ESI+); *m*/*z*: 440.7 [M], 436.8 [M−H]$^-$, 438.7 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.61 (s, 2H, SO_2NH_2), 7.7, 7.72 (d, 2H, Ar−H from benzenesulfonamide), 7.799–7.84 (d, 2H, Ar−H from benzenesulfonamide), 7.969–7.84 (d, 2H, Ar−H from benzenesulfonamide), 7.98–7.988 (d, 2H, Ar−H from benzenesulfonamide). ^{13}C NMR (100 MHz, DMSO-d_6): 163.42, 145.99, 139.4, 132.56, 131.68, 130.92, 129.39, 126.86, 125.6.

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

0.002 mol (0.344 g) of 2-aminobenzenesulfonamide stirred under nitrogen environment with 0.002 mol (0.572 g) of 4,5,6,7-tetrachlorophthalic anhydride to produce 0.002 mol of 2-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**5**) (0.880 g) in the presence of glacial acetic acid as solvent for 2 h at 130 °C. The reaction was 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 to purify the synthesized compounds.[21–25]

White crystalline and solid; % yield = 90%; mp = 313 °C; solubility; insoluble: water, acetic acid glacial; partially soluble: ethanol and methanol; fully soluble: DMSO and chloroform. IRν_{max} (cm^{-1}; KBr pellets); 1782.29, 1712.85 (C=O imide); 1305.85, 1139.97 (S=O) and 3387.11 (NH_2). MS (ESI+); *m*/*z*: 440.7 [M], 436.7 [M−H]$^-$, 438.7 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.661 (s, 2H, SO_2NH_2), 7.802–7.844 (d, 2H, Ar−H from benzenesulfonamide), 8.102–8.126 (d, 2H, Ar−H from benzenesulfonamide). ^{13}C NMR (100 MHz, DMSO-d_6): 165.71, 163.11, 143.27, 139.53, 134.05, 132.74, 131.63, 129. 62, 129.48, 129.26.

5.2.6. Synthesis of 3-fluoro-4-(4,5,6,7-tetrachloro-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 (0.572 g) of 4,5,6,7-tetrachlorophthalic anhydride to produce 0.002 mol of 3-fluoro-4-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**6**) (0.916 g) in the presence of glacial acetic acid as solvent for 12 h at 130 °C. The reaction was 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 to purify the synthesized compounds.[21–25]

White crystalline and solid; % yield = 95%; mp = 278 °C; solubility; insoluble: water, acetic acid glacial; partially soluble: ethanol; fully soluble: DMSO, chloroform and methanol. IRν_{max} (cm^{-1}; KBr pellets); 1790.00, 1720.56 (C=O imide); 1301.99, 1168.90 (S=O) and 3385.18 (NH_2). MS (ESI+); *m*/*z*: 458.7 [M], 454.7 [M−H]$^-$, 456.7 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.704 (s, 2H, SO_2NH_2), 7.796–7.818 (d, 2H, Ar−H from benzenesulfonamide), 7.835 (s, 2H, Ar−H from benzenesulfonamide), 7.893–7.941 (d, 2H, Ar−H from benzenesulfonamide). ^{13}C NMR (100 MHz, DMSO-d_6): 162.74, 159.07, 156.53, 147.76, 139.89, 132.35, 129.78, 123.52, 122.74, 115.39.

5.2.7. Synthesis of 3-chloro-4-(4,5,6,7-tetrachloro-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 (0.572 g) of 4,5,6,7-tetrachlorophthalic anhydride to produce 0.002 mol of 3-chloro-4-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**7**) (0.949 g) in the presence of glacial acetic acid as solvent for 12 h at 130 °C. The reaction was 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 to purify the synthesized compounds.[21–25]

White crystalline and solid; % yield = 94%; mp = 303 °C; solubility; insoluble: water, acetic acid glacial; partially soluble: ethanol; fully soluble: DMSO, chloroform and methanol. IRν_{max} (cm^{-1}; KBr pellets); 1788.07, 1722.49 (C=O imide); 1305.87, 1172.76 (S=O) and 3387.11 (NH_2). MS (ESI+); *m*/*z*: 470.6 [M−H]$^-$, 472.6 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.724 (s, 2H, SO_2NH_2), 7.861, 7.882 (d, 2H, Ar−H from benzenesulfonamide), 8.017–8.042 (d, 2H, Ar−H from benzenesulfonamide), 8.155 (s, 2H, Ar−H from benzenesulfonamide). ^{13}C NMR (100 MHz, DMSO-d_6): 162.64, 147.69, 140.16, 133.91, 133.12, 132.82, 129.91, 129.11, 129.31, 126.65.

5.2.8. Synthesis of 3-bromo-4-(4,5,6,7-tetrachloro-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 (0.572 g) of 4,5,6,7-tetrachlorophthalic anhydride to produce 0.002 mol of 3-bromo-4-(4,5,6,7-tetrachloro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide (**8**) (1.038 g) in the presence of glacial acetic acid as solvent for 12 h at 130 °C. The reaction was 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 to purify the synthesized compounds.[21–25]

White crystalline and solid; % yield = 90%; mp = 265 °C; solubility; insoluble: water, acetic acid glacial; partially soluble: ethanol; fully soluble: DMSO, chloroform and methanol. IRν_{max} (cm^{-1}; KBr pellets); 1772.64, 1720.56 (C=O imide); 1300.00, 1170.83 (S=O) and 3400.0 (NH_2). MS (ESI+); *m*/*z*: 518.5 [M], 514.5 [M−H]$^-$, 516.5 [M+1]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.715 (s, 2H, SO_2NH_2), 7.842, 7.863 (d, 2H, Ar−H from benzenesulfonamide), 8.051–8.077 (d, 2H, Ar−H from benzenesulfonamide), 8.292 (s, 2H, Ar−H from benzenesulfonamide). ^{13}C NMR (100 MHz, DMSO-d_6): 162.64, 147.69, 140.16, 133.91, 133.12, 132.82, 129.91, 129.11, 129.31, 126.65.

5.3. 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 1.7–17 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 to-

tal 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.[27–29]

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, dichlorophenamide; DZA, Dorzolamide; BRZ, brinzolamide; BZA, benzolamide; TPM, topiramate; ZNS, zonisamide; SLP, Sulpiride; IND, indisulam; COX2, cyclo-oxygenase enzyme 2; CLX, celecoxib; VLX; valdecoxib; TLC, thin layer chromatography; UV, ultraviolet; FT-IR, Fourier transform infrared; DMSO, dimethyl sulfoxide; NMR, Nuclear Magnetic Resonance.

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