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Carbonic Anhydrase Inhibitors: Design, Synthesis, and Biological Evaluation of Novel Sulfonyl Semicarbazide Derivatives

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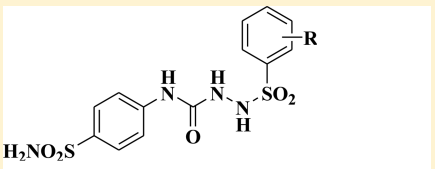
S *Supporting Information*

ABSTRACT: A series of novel sulfonyl semicarbazides **5**−**13** was designed, synthesized, and evaluated for human carbonic anhydrase (hCA) inhibition. The new sulfonyl semicarbazides were tested against a panel of hCA isoforms I, II, IX, and XII, using acetazolamide (AZA, **1**) as standard. All the sulfonyl semicarbazides showed subnanomolar affinity for hCA XII (pK_i range 0.59−0.79 nM) and high selectivity over hCA I (58−114-fold) and hCA IX (26−114-fold) compared to hCA II (5−20-fold except **11**, 121-fold). The importance of the nature of para-substitution on the sulfonyl substituted aromatic ring for potency and selectivity against one hCA isoform versus others is discussed. Overall, the research work led to the development of highly potent and selective hCA inhibitors.



12, R = 4-OCH_3
hCAI K_i = 47 nM; hCAII K_i = 3.8 nM
hCAIX K_i = 80.4 nM; hCAXII K_i = 0.66 nM

KEYWORDS: *Carbonic anhydrase, CA, sulfonyl semicarbazides, human isoform I, human isoform II, human isoform IX, human isoform XII*

Carbonic anhydrase (CA), the catalyst for the interconversion between carbon dioxide and bicarbonate, is an essential enzyme in bacteria, archaea, and eukaryotes. In humans, the CA-catalyzed reaction involves three simple chemical entities, CO_2, HCO_3^-, and H^+ essential in a host of physiological and pathological processes, such as respiration, pH and CO_2 homeostasis, electrolyte secretion, and lipogenesis.[1−5] A total of 16 CA isozymes have been described so far in mammals. Some of theses isoenzymes are cytosolic (CA I, CA II, CA III, CA VII, and CA XIII), others are membrane-bound (CA IV, CA IX, CA XII, and CA XIV), two are mitochondrial (CA VA and CA VB), and one is secreted in saliva and milk (CA VI). CA I and CA II are the two major CA isozymes present at high concentrations in the cytosol in erythrocytes, and CA II is the most active of all CAs.[1−5]

Sulfanilamide was the first organic inhibitor of these enzymes, discovered already in 1940.[6] It was subsequently showed that all primary aromatic or heterocyclic sulfonamides inhibit these enzymes, and pharmacologic agents started to be developed, initially as diuretics and antiglaucoma agents.[7,8] Acetazolamide (AAZ, compound **1**, Figure 1) is the best known such pharmacologic agent. Subsequently, other sulfonamide CA inhibitors (CAIs) showed applications as antiepileptics, antiobesity, and antitumor agents.[9−12] Different isoforms of the 16 known so far in mammals are targeted for these diverse pharmacologic applications.[5,9−12]

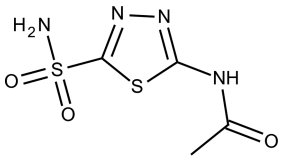


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Figure 1. Structure of acetazolamide (AAZ).

Aromatic/heterocyclic sulfonamides (mainly sulfanilamide **2**) also incorporating ureido or thioureido moieties in their molecules have been extensively investigated as CAIs.[7,13] Some of these derivatives were the first ones reported to have some selectivity for the inhibition of some human (h) CA isoforms, such as hCA I, II, or IV.[7,13,14] Furthermore, some derivatives of this type were also shown to possess significant antitumor/antimetastatic properties *in vivo*, in animal models of the disease.[14]

Considering the interesting biologic activity of benzenesulfonamides incorporating ureido moieties, we report here a small series of such compounds that incorporate a longer linker between the benzenesulfonamide and the tail of the semicarbazide type. These derivatives were obtained by an original

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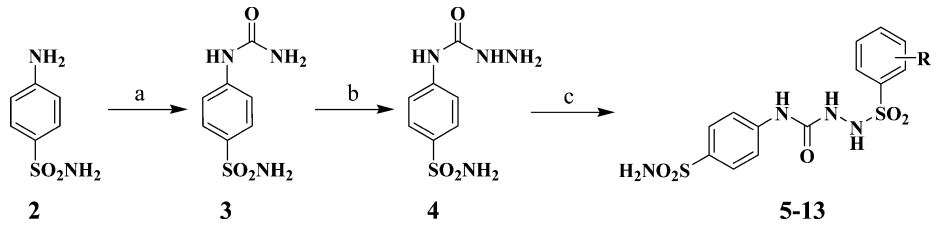
Scheme 1. Synthesis of Title Compounds 5−13



[f]Reagents and conditions: (a) NaCNO, glacial AcOH, heat; (b) $NH_2NH_2 \cdot H_2O$, EtOH, reflux, 48 h.; (c) $ArSO_2Cl$, pyridine, RT.

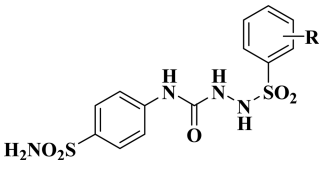
procedure and investigated for the inhibition of four physiologically relevant human isoforms, hCA I and II (cytosolic enzymes, targets for antiglaucoma agents)[5] and hCA IX and XII (transmembrane enzymes, targets for antitumor therapies).[12,14]

Results and Discussion. *Chemistry.* Syntheses of the title compounds **5−13** are outlined in Scheme 1. Sulfanilamide (**2**) was reacted with sodium cyanate in glacial acetic acid and water to yield the corresponding urea derivative **3**. Further, reaction of **3** with hydrazine hydrate gave semicarbazide **4** in good yields. The title compounds were prepared by reacting substituted aromatic sulfonyl chlorides with semicarbazide **4**. The used arylsulfonyl halides incorporated 4-halogeno, 4-methyl, 4-nitro-, 4-methoxy, 4-acetamido-, and 3-nitro-4-chlorophenylmoieties, as these groups present in other series of reported CAIs[7,14] usually showed good activity and interesting contacts with the enzyme active site.[1,2] Column purification was not required since all the compounds were obtained in high purity (>95%) after recrystallization. Our attempt to react **4** with 2-nitrobenzenesulfonyl chloride failed. Hence, the corresponding title compound from the proposed sulfonyl semicarbazides list could not be included for biological evaluation. All the sulfonyl semicarbazides were characterized by HPLC, IR, ^{1}H NMR, ^{13}C NMR, and LC−MS.

CA Inhibition. Inhibition data of compounds **5−13** (and AAZ **1** as standard) against four physiologically relevant isoforms, i.e., hCA I, II (cytosolic enzymes), IX, and XII (transmembrane, tumor-associated isoforms) are shown in Table 1. The following structure−activity relationship (SAR) can be observed:

(i) The predominant (in blood) cytosolic isoform hCA I was effectively inhibited by compounds **5−13**, which showed inhibition constants in the range of 40.2−82.3 nM: The unsubstituted compound (R = H) **5** was already one of the best hCA I inhibitors (K_i of 41.2 nM) and the substitution by halogens at the para-position on the aromatic ring was either detrimental (the fluorine derivative **6** was much less efficient as a CAI) or only slightly changed the affinity for this isoform (compounds incorporating Cl and Br, **7** and **8**). The same behavior was observed for the 4-Me derivative **9** or the nitro one **10**. Other substituents in this position, such as methoxy or acetamido, as well as R moieties in other positions (3,4-disubstituted compounds) did not show great variations of the inhibitory power against hCA I (Table 1). This is probably due to the fact that the R-arylsulfonyl fragment of the molecule does not make crucial contacts with the enzyme active site, as obsereved earlier for other congeneric series of arylureido-substituted sulfonamides.[7,14] All these compounds were found to be more efficient hCA I inhibitors compared to the clinically used drug **1**.

Table 1. Structures and *in Vitro* Carbonic Anhydrase Inhibition Data of Sulfonyl Semicarbazides against Isoforms hCA I, II, IX, and XII[19]



		K_i (nM)[a]			
compd	R	hCAI	hCAII	hCAIX	hCAXII
1 (AZA)		250	12	25	5.8
5	H	41.2	4.3	73.9	0.68
6	4-F	82.3	14.4	25.1	0.72
7	4-Cl	40.2	3.5	23.1	0.63
8	4-Br	48.9	3.9	30.4	0.79
9	4-Me	47.7	6.1	24.0	0.65
10	4-NO_2	49.3	6.0	20.5	0.79
11	4-Cl-3-NO_2	52.5	71.8	38.7	0.59
12	4-OCH_3	47.0	3.8	80.4	0.66
13	4-$NHCOCH_3$	41.3	3.6	81.3	0.71

[a]Mean from 3 different assays. Errors were in the range of ±10% of the reported value (data not shown).

(ii) The physiologically dominant hCA II was also highly inhibited by most of these derivatives. Indeed, except for the 4-chloro-3-nitrophenyl-substituted compound **11**, which with a K_i of 71.8 nM was a medium potency inhibitor, all the remaining derivatives reported here were highly efficient CAIs, with inhibition constants in the range of 3.5−14.4 nM (Table 1). Thus, all other substitution patterns present in these derivatives (i.e., the nature of the R moiety from the tail of the inhibitors) lead to compounds that are equipotent or better than **1** as hCA II inhibitors.

(iii) The tumor-associated hCA IX was also inhibited efficiently by the new sulfonyl semicarbazides **5−13** reported here (Table 1). The unsubstituted (R = H) derivative **5** as well as **12** and **13** were medium potency hCA IX inhibitors, with inhibition constants of 73.9−81.3 nM, whereas the remaining derivatives showed a better potency, comparable to that of acetazolamide (**1**) (K_i of 25 nM), with inhibition constants in the range of 20.5−38.7 nM. The best inhibitor was the 4-nitro-substituted derivative **10**, and the weakest one **13** (incorporating the 4-acetamido-phenyl moiety), as they differ in K_is almost by a factor of 4. Thus, rather small structural changes lead to drastic effects on the inhibitory power in this small series of CAIs.

(iv) All sulfonyl semicarbazides **5−13** reported here were subnanomolar hCA XII inhibitors, with potencies one order of magnitude better than that of acetazolamide **1**. Indeed,

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compounds **5**−**13** had K_is in the range of 0.59−0.82 nM, with basically no SAR being obvious at all, except that all the substitution patterns present in these derivative lead to excellent inhibition of this transmembrane, tumor-associated CA (Table 1). Overall, the title compounds exhibited interesting potency and selectivity profiles [selective for hCA XII over hCA I (58−114-fold), hCA IX (26−114-fold,) and hCA II (5−20-fold except **11**, 121-fold).

Conclusions. Our attempts in developing potent hCA inhibitors useful as diuretics, antiglaucoma, anticancer agents, etc., led to the identification of a series of sulfonyl semicarbazides. Preliminary investigations involving *in vitro* inhibition studies against a panel of hCA isoforms I, II, IX, and XII validated our design strategy. The title compounds were highly potent and selective for at least two hCA isoforms II (cytosolic) and XII (membrane-bound). Such interesting selectivity profiles of these NCEs may be useful for deciding their therapeutic utility as hCA inhibitors.

EXPERIMENTAL PROCEDURES

Chemistry. Reagents and solvents were obtained from commercial suppliers and were used as received unless otherwise indicated. Solvents were dried, wherever necessary, according to standard procedures. All reactions were performed under an inert atmosphere (N_2) unless otherwise indicated. Analytical silica gel 60 F_{254}-coated TLC plates were purchased from EMD Chemicals and were visualized with UV light. ^{1}H NMR spectra were routinely obtained with a Varian Mercury Plus 300 MHz NMR (Agilent Technologies, Santa Clara, USA), and Me_4Si was used as an internal standard. LC−MS spectra were recorded on 6110 AA Series Quadrupole LC/MS system (Agilent Technologies, Santa Clara, USA). IR spectra were recorded on Shimadzu-FTIR 8400S instrument (Shimadzu Corp. Tokyo, Japan) using KBr pellet technique. Analytical HPLC analyses ware carried out with a Kromasil 100-5C18 column (150 mm × 4.6 mm) on PerkinElmer Series 200 HPLC system with autosampler and PDA detector (PerkinElmer, Inc., Waltham, USA). Melting points were recorded using a Veego (VMP)-D capillary melting point apparatus (Veego Instruments Copr. Mumbai, India) and are uncorrected.

*General Procedure for Synthesis of N^3-(4-Sulfamoylphenyl) Urea (**3**).*[15,16] Sulfanilamide (**2**) (17.2 g, 0.1 mol) was dissolved in a mixture of glacial HOAc (20 mL) and hot water (80 mL) in a 250 mL beaker. The solution was heated until **2** dissolved completely. To this, a solution of sodium cyanate (6.5 g, 0.1 mol) in warm water (80 mL) was added with stirring. The reaction mixture was allowed to stand for 30 min at RT, then cooled in ice-bath and further kept for 30 min. The resulting precipitate was filtered, washed several times with ice-cold water, and recrystallized from EtOH.

*General Procedure for Synthesis of N-(4-Sulfamoylphenyl) Hydrazine Carboxamide (**4**).*[16,17] Equimolar quantities of N^3-(4-sulfamoylphenyl) urea (**3**) (10.75 g, 0.05 mol) and hydrazine hydrate (2.5 mL, 0.05 mol) in abs. EtOH (25 mL) were refluxed for 48 h with continuous stirring. Two-thirds volume of ethanol was removed by vacuum distillation, and the contents of the flask were poured onto crushed ice. The crude product was filtered, washed with water, dried, and recrystallized from 90% EtOH.

*General Procedure for Synthesis of 1-(Substituted benzenesulfonylamido)-3-(4-sulfamoylphenyl) Urea (**5**−**13**).*[13,18] In a 250 mL RBF, an alcoholic solution of **4** (2.3 g, 0.01 mol) was taken. To this solution, substituted benzenesulfonyl chlorides (0.01 mol) and pyridine (1 mL) were added dropwise with stirring. The mixture was stirred for 48 h at RT. After completion of the reaction (monitored by TLC), the solution was poured onto crushed ice. Enough dil. HCl was added to remove pyridine. The resulting solid was filtered, washed with ice-cold water, and recrystallized using EtOH/water (50:50) mixture.

Synthetic and spectral characterization details for compounds **5**−**13** can be found in Supporting Information.

CA Inhibition Studies. An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity.[19] 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 7.4) and 20 mM $NaBF_4$ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10−100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5−10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled−deionized water, and dilutions up to 0.01 nM were done thereafter with distilled−deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at RT prior to assay, in order to allow for the formation of the E−I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver−Burk plots, as reported earlier,[11,13,14] represent the mean from at least three different determinations. All CAs were recombinant proteins obtained as reported earlier by these groups.[20−23]

ASSOCIATED CONTENT

Supporting Information

Synthetic, spectral details along with ^{1}H NMRs, ^{13}C NMRs, and mass spectrum of the title compounds **5**−**13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): CTS reports conflict of interest as author of many patents on CA IX/XII inhibitors..

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ABBREVIATIONS USED

CA, carbonic anhydrase; AAZ, acetazolamide; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; TLC, thin layer chromatography

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