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RESEARCH ARTICLE

Inhibition of human carbonic anhydrase isozymes I, II, IX and XII with a new series of sulfonamides incorporating aroylhydrazone-, [1,2,4]triazolo[3,4-b][1,3,4]thiadiazinyl- or 2-(cyanophenylmethylene)-1,3,4-thiadiazol-3(2H)-yl moieties

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Abstract

A series of benzenesulfonamides incorporating aroylhydrazone, piperidinyl, sulfone, [1,2,4]triazolo[3,4-b][1,3,4]thiadiazinyl- or 2-(cyanophenyl-methylene)-1,3,4-thiadiazol-3(2H)-yl moieties was investigated as inhibitors of four α-carbonic anhydrases (CAs, EC 4.2.1.1), the human (h) isoforms hCA I, II (cytosolic, offtarget enzymes) and hCA IX and XII (transmembrane, tumor-associated isoforms). Low nanomolar activity was observed against hCA II (K_Is of 0.56–17.1 nM) with these sulfonamides, whereas the slow cytosolic isoform hCA I was less inhibited by these compounds (K_Is of 86.4 nM–32.8 μM). Most of these sulfonamides significantly inhibited CA IX, with K_Is in the range of 4.5–47.0 nM, although some of the derivatives incorporating bulkier bicyclic moieties, as well as 2-thienyl fragments, showed a weaker activity against this isoform (K_Is in the range 50.1–553 nM). All the investigated compounds also inhibited CA XII with K_Is in the range 0.85–376 nM. The best inhibitors were those incorporating bulky [1,2,4]triazolo[3,4-b][1,3,4]thiadiazinyl moieties and 1,3,4-thiadiazol-3(2H)-yl groups.

Keywords

Cancer-associated isoform, carbonic anhydrase, enzyme inhibitor, sulfonamide

History

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Introduction

The primary sulfonamides, RSO_2NH_2, are the classical inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1)[1–4]. They have been widely used for almost 60 years as diuretic or systemically acting antiglaucoma drugs, since the introduction of acetazolamide (AAZ) in clinical use in 1954[5–9]. However, AAZ and the other clinically used sulfonamides/sulfamate CA inhibitors (CAIs), such as methazolamide and ethoxzolamide[1,4,6], target all mammalian CA isoforms (16 of them are known to date in vertebrates)[1,8–11], and as thus, they show a range of undesired side effects[1,4,6,11,12], motivating the continuous search of novel such agents with a selective inhibition profile against the desired isoform(s)[13–20].

These enzymes are also versatile catalysts of other hydrolytic reactions except the hydration of CO_2 to bicarbonate and protons, being esterases with a range of carboxylic acid esters, phosphate and sulfate esters[1,3,13,14].

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Recently, a large of number of sulfonamides and some of their derivatives started to be obtained which showed a good selectivity level for the inhibition of various CA isoforms[4,15–28], some of which are cytosolic (e.g. CA I, II, III, VII and XIII, as well as the acatalytic isoforms CA VIII, X and XI)[1,3,6,21], membrane associated/transmembrane (CA IV, IX, XII, XIV and XV)[1,2,8,25], mitochondrial (CA VA and VB)[1,4], 17 or secreted (CA VI)[1,4,15,20].

The tumor associated isoform CA IX is highly overexpressed in many cancer types by the hypoxia inducible factor-1α (HIF-1α) cascade[1–4]. Around 70% of the hypoxic tumors overexpress CA IX and show a bad response to classical chemo- and radiotherapies[2]. CA IX was shown to significantly contribute to the extracellular acidification of the tumor environment, by means of the protons resulted from the catalysis of carbon dioxide hydration[1–4]. This leads to the acquisition of metastasic phenotypes and chemoresistance towards many anticancer drugs. Ultimately, targeted structure-based drug design campaigns of CAIs against this novel target led to a range of interesting derivatives with a significant activity, selectivity and promising *in vivo* action against several types of tumors[29,30]. CA XII is also a tumor-associated CA and its inhibition was also shown to lead to anticancer effects[31].

Continuing our interest in the design of CA IX and XII inhibitors of the sulfonamide type, here we report that a series of benzenesulfonamides incorporating aroylhydrazone, [1,2,4]triazolo[3,4-b][1,3,4]thiadiazinyl- or 2-(cyanophenyl-methylene)-1,3,4-thiadiazol-3(2H)-yl moieties, reported earlier by us for the inhibition of bacterial enzymes from extremophiles[26], shows interesting inhibition profile against the two tumor-associated isoforms CA IX and XII.

Materials and methods

Chemistry

Compounds **3**–**10** investigated here were reported in a previous study from our group[26].

Enzymology

hCA I, II, IX and XII were recombinant enzymes obtained in-house as described earlier[1–7].

CA catalytic and inhibition assay

An applied photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity[27]. 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 8.4) 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 room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier[12–17,28], and represent the mean from at least three different determinations.

Results and discussion

Chemistry

Sulfonamides investigated here were obtained from oxo-*N*′-(4-sulfamoylphenyl)propanehydrazonoyl chloride (**1**)[26] which was reacted with aroylhydrazines **2** to form the key intermediates of type **3** (Scheme 1). The 2-(2-arylhydrazono)-*N*′-(4-sulfamoylphenyl)propanehydrazonoyl chlorides **3a–d** were then reacted with piperidine in ethanol led to the formation of piperidine derivatives **4a–d** or with sodium benzenesulfinate to afford the corresponding sulfones **5a–d**, respectively (Scheme 1)[26].

Alternatively, treatment of **1** with 4-amino-5-(methyl/phenyl)-4*H*-1,2,4-triazole-3-thiol **6a**, **b** gave 1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazines **7a**, **b** (Scheme 2). In another approach, **1** was reacted with thioanilide derivatives **8a–d** in the presence of triethylamine, which afforded the 1,3,4-thiadiazoles **10a–d**, as depicted in Scheme 2.

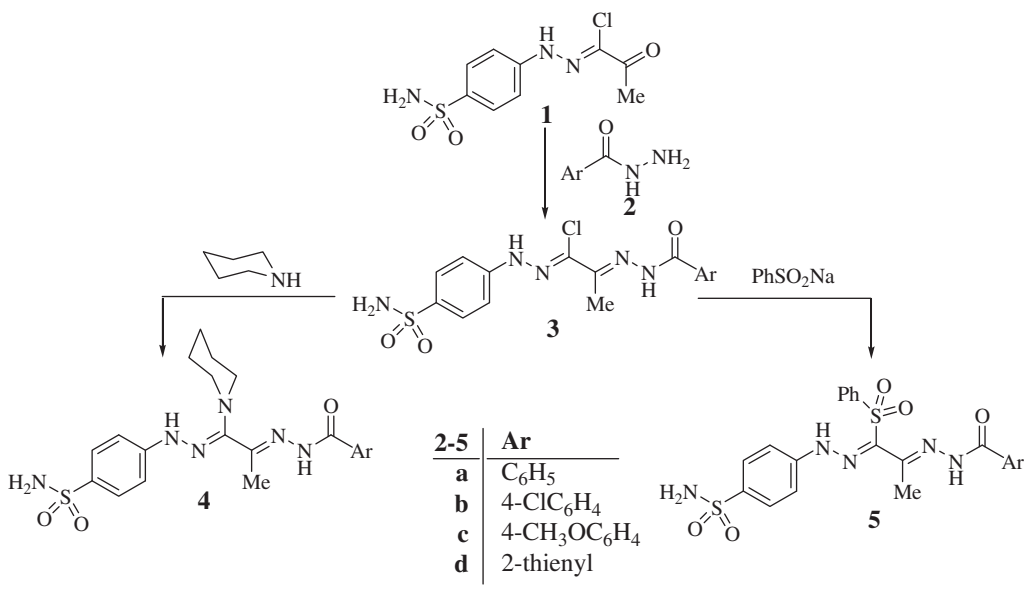
All these compounds have been characterized by physico-chemical and spectral techniques confirming the proposed structures (see ref.[26] for details).

CA inhibition

The inhibition studies against four mammalian CA isoforms of this series of benzenesulfonamides containing the interesting tails of the [1,2,4]triazolo[3,4-b][1,3,4]thiadiazinyl- or 2-(cyanophenyl-methylene)-1,3,4-thiadiazol-3(2H)-yl type (**3**–**10**), i.e. hCA I, II, IX and XII are shown in Table 1.

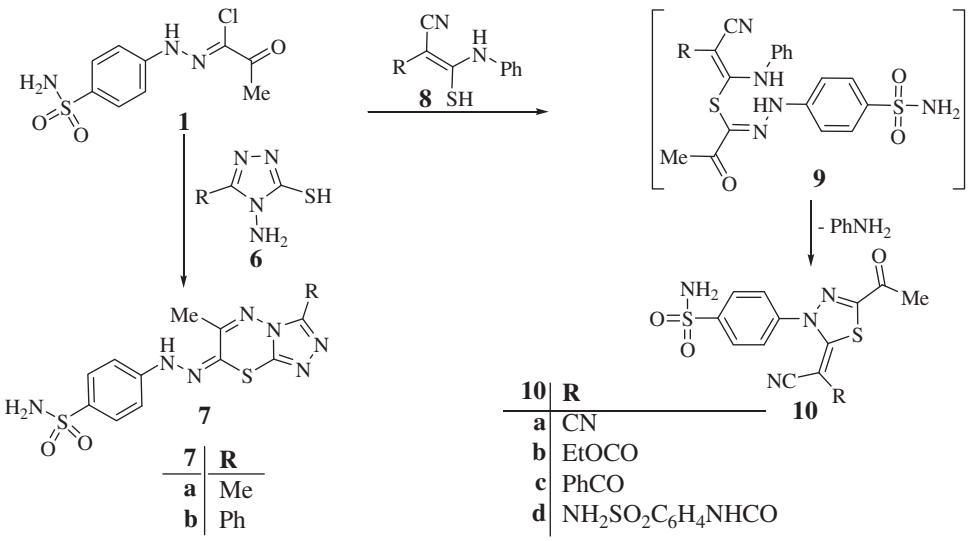
As seen from data of Table 1, sulfonamides **3a–10d** (and acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, AAZ, as standard inhibitor) showed interesting inhibitory properties against all four investigated CAs. The following structure-activity relationship (SAR) has been delineated:

(i) The slow cytosolic isoform hCA I was moderately inhibited by sulfonamides **3a–3d**, **4a–4d**, **5a–5d** and **7a**, **7b**, with inhibition constants in the range of 86.4–753 nM, and weakly inhibited by derivatives **10a–10d** (K_Is in the range of 6.54–32.8 μM). It is interesting to note that the Schiff's bases **3a–3d** showed a rather similar behavior as hCA I inhibitors, with a relatively small variation of the inhibition constants irrespective of the nature of moiety Ar. The strongest inhibitor in the subseries was the phenyl derivative **3a**, but its substitution with 4-Cl, 4-MeO groups, or its



2-5	Ar
a	C_6H_5
b	4-ClC_6H_4
c	4-$CH_3OC_6H_4$
d	2-thienyl

Scheme 1. Synthesis of compounds **3a–d**, **4a–d** and **5a–d**.



Scheme 2. Synthesis of compounds **7a, b** and **10a–d**.

Table 1. Inhibition data against the human isoforms hCA I, II, IX and XII with sulfonamides **3–10** by a stopped-flow, CO_2 hydrase assay[27].

	K_I (nM)*			
Compound	hCA I	hCA II	hCA IX	hCA XII
3a	134	17.1	9.3	11.7
3b	185	6.4	16.8	17.1
3c	180	3.1	7.6	38.5
3d	193	0.94	354	207
4a	151	5.8	63.0	15.1
4b	132	0.57	35.1	4.6
4c	247	1.1	47.0	29.3
4d	91.3	0.89	553	376
5a	130	8.6	64.8	36.3
5b	367	0.97	85.1	32.8
5c	162	0.55	42.7	41.2
5d	753	4.5	57.3	8.5
7a	86.4	1.0	52.7	4.3
7b	390	0.93	72.5	0.85
10a	16 300	4.2	8.9	7.1
10b	27 500	5.4	4.5	3.4
10c	32 800	0.91	8.8	5.2
10d	6540	0.56	50.1	7.5
AAZ	250	12.1	25.0	5.8

The standard, clinically used sulfonamide acetazolamide (AAZ, 5-acetamido-1,3,4-thidiazole-2-sulfonamide) is also included for comparison reasons.

*Errors were in the range of ±10% of the reported values from three different assays (data not shown).

replacement by a thienyl scaffold, led to a slight diminution of the inhibitor potency in compounds **3b–3d**. For piperidines **4a–4d**, the range of inhibition was already higher compared to derivatives **3**, with the thienyl derivative **4d** being the most active in the subseries (K_I of 91.3 nM) and the 4-methoxyphenyl one **4c** the least active (K_I of 247 nM, Table 1). Sulfones **5** also showed a significant variation of the inhibitory power with the nature of the Ar group, with the best inhibitor being the phenyl derivative **5a** (K_I of 130 nM) and the least effective one the thienyl derivative **5d** (K_I of 753 nM). For compounds **7**, the methyl derivative **7a** showed effective hCA I inhibitory properties (this was the best inhibitor of this isoform among the reported derivatives, being almost 3-fold more effective than the clinically used sulfonamide (AAZ) whereas the bulkier Ph derivative **7b** was much less effective as inhibitor of this isoform. Even a stronger loss of activity was then observed for sulfonamides **10**, which probably due to their more rigid scaffold (the thiadiazole ring is directly connected to the benzene one in these compounds) can be less well-accommodated within the restricted space of the hCA I active site[28].

(ii) All new compounds reported here were excellent inhibitors of the physiologically dominant (in humans) isoform hCA II. Indeed, only **3a** showed an inhibition constant of 17.1 nM (comparable to that of acetazolamide, 12.1 nM) whereas all the remaining derivatives were sub-nanomolar or low nanomolar hCA II inhibitors, with K_Is in the range of 0.55–8.6 nM. The SAR is thus almost impossible to delineate as all these substitution patterns seem to be highly favorable for obtaining tight-binding hCA II inhibitors. Among the best such compounds were the Schiff's base **3d** (incorporating the thienyl moiety), the piperidine **4b** (incorporating a 4-chlorophenyl moiety), the sulfones **5b** and **5c**, incorporating 4-Cl- and 4-MeO-phenyl moieties, the bulky phenyl-substituted **7b** as well as the 1,3,4-thiadiazoles **10c** and **10d**. All these compounds showed inhibition constants <1 nM, being among the most effective hCA II inhibitors reported up until now.

(iii) Against hCA IX the derivatives 3–10 showed with K_Is in the range of 4.5–553 nM. The most ineffective inhibitors of this isoform were **3d** and **4d** (with K_Is of 354–553 nM), which both incorporate 2-thienyl fragment at the end of the bulky tail derivatizing the sulfanilamide scaffold. It is interesting to note that other chloro derivatives **3** or piperidines **4**, incorporating a different substitution patterns (e.g. in **3a–3c** or **4a–4c**) were quite effective (**3a** and **3c**) or medium potency (**3b, 4a–4c**) hCA IX inhibitors (the last group of compounds showed with K_Is in the range of 16.8–63.0 nM, Table 1). Interestingly, irrespective of the substitution pattern, all the sulfones **5** and the [1,2,4]triazolo[3,4-b][1,3,4]thiadiazinyl-substituted derivatives **7** were medium potency hCA IX inhibitors, with K_Is in the range of 42.7–85.1 nM. The same is true for **10d**, incorporating two sulfamoyl functionalities but on a quite bulky scaffold (K_I of 50.1 nM), whereas the remaining 2-(cyanophenyl-methylene)-1,3,4-thiadiazol-3(2H)-yl derivatives **10a–10c**, which incorporate more compact functionalities compared to **10d**, were quite efficient hCA IX inhibitors, with K_Is in the range of 4.5–8.9 nM. These compounds are more effective compared to acetazolamide AAZ as hCA IX inhibitors (together with **3a** and **3c**). Thus, SAR for hCA IX inhibition with compounds **3–10** is much more complicated compared

to what we observed for the inhibition of the cytosolic isoforms hCA I and II discussed earlier.

(iv) The second transmembrane isoforms, hCA XII; was also inhibited by sulfonamides **3**–**10** investigated here, with K_Is in the range of 0.85–376 nM (Table 1). As for hCA IX discussed earlier, the least effective hCA XII inhibitors were the same two compounds (**3d** and **4d**) incorporating the 2-thienyl tail (K_Is of 207–376 nM). Medium potency inhibition, with K_Is in the range of 11.7–41.2 nM was observed for aroylhydrazones **3a**–**3c**, piperidines **4a** and **4c** as well as sulfones **5a**–**5c**. The remaining derivatives, i.e. **4b, 5d, 7a**, **b** and **10a**–**10d**, were highly effective hCA XII inhibitors with K_Is in the range of 0.85–8.5 nM (Table 1). The best and only subnanomolar hCA XII inhibitor was **7b** (K_I of 0.85 nM). Many of the investigated compounds were more effective or similar to acetazolamide for the inhibition of this isoform.

(v) The inhibition profile of the four subclasses of derivatives investigated here, which all carry the sulfanilamide head group, but highly diverse tail moieties, was very different and specific for each of them. For example derivatives **7** were highly effective as hCA II and XII inhibitors, medium potency hCA IX inhibitors and rather weak hCA I inhibitors. Although no highly hCA IX/XII – selective compounds were detected in this study, the inhibition profiles of these derivatives are of great interest considering the many applications that CAIs possess in various pharmacological fields, for obtaining diuretics[32], antiepileptics[33], antiobesity[34] and antiglaucoma[35,36] agents.

Conclusions

We investigated a series of recently reported benzenesulfonamides, incorporating aroylhydrazone, [1,2,4]triazolo[3,4-b][1,3,4]thiadiazinyl- or 2-(cyanophenyl-methylene)-1,3,4-thiadiazol-3(2H)-yl moieties as inhibitors of four α-CAs, the isoforms hCA I, II (cytosolic, offtarget enzymes) and hCA IX and XII (transmembrane, tumor-associated isoforms). Low nanomolar activity was observed against hCA II (K_Is of 0.56–17.1 nM) with these sulfonamides, whereas the slow cytosolic isoform hCA I was less inhibited by these compounds (K_Is of 86.4 nM–32.8 μM). Most of the sulfonamides investigated here also significantly inhibited CA IX, with K_Is in the range of 4.5–47.0 nM, although some of the derivatives incorporating bulkier bicyclic moieties as well as thienyl fragments, showed a weaker activity against this isoform (K_Is in the range 50.1–553 nM). All the investigated compounds also inhibited CA XII with K_Is in the range 0.85–376 nM. The best inhibitors were those incorporating bulky [1,2,4]triazolo[3,4-b][1,3,4]thiadiazinyl moieties and 1,3,4-thiadiazol-3(2H)-yl groups. Although no hCA IX/XII – selective compounds were detected in this study, the inhibition profiles of these derivatives are of great interest considering the many applications of CAIs for obtaining diuretics, antiepileptics, antiobesity and antiglaucoma agents.

Declaration of interest

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