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ORIGINAL ARTICLE

Synthesis and inhibition potency of novel ureido benzenesulfonamides incorporating GABA as tumor-associated carbonic anhydrase IX and XII inhibitors

Mariangela Ceruso[1], Sabrina Antel[1], Andrea Scozzafava[1], and Claudiu T. Supuran[2]

[1]*Dipartimento di Chimica, Laboratorio di Chimica Bioinorganica, Università degli Studi di Firenze, Sesto Fiorentino (Firenze), Italy and* [2]*Neurofarba Department, Università degli Studi di Firenze, Sesto Fiorentino (Florence), Italy*

Abstract

New ureido benzenesulfonamides incorporating a GABA moiety as a linker between the ureido and the sulfonamide functionalities were synthesized and their inhibition potency determined against both the predominant cytosolic (hCA I and II) and the transmembrane tumor-associated (hCA IX and XII) isoforms of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). The majority of these compounds were medium potency inhibitors of the cytosolic isoform hCA I and effective hCA II inhibitors, whereas they showed strong inhibition of the two transmembrane tumor-associated isoforms hCA IX and XII, with K_Is in nanomolar range. Only one derivative had a good selectivity for inhibition of the tumor-associated hCA IX target isoform over the cytosolic and physiologically dominant off-target hCA I and II, being thus a potential tool to develop new anticancer agents.

Keywords

Anticancer agents, carbonic anhydrase inhibitors, GABA linker, transmembrane isoforms, ureido benzenesulfonamide

History

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Introduction

Sixteen human (h) α-carbonic anhydrase (CA, EC 4.2.1.1) isoforms have been discovered till now. They belong to a large family of metalloenzymes, which comprises six genetic families, i.e. α, β, γ, δ, ζ and η-classes. Carbonic anhydrases are mainly involved in the reversible hydration reaction of carbon dioxide to release bicarbonate and proton[1–3]. Among the various biological functions, CAs are involved in pH buffering of the intra- and extra-cellular spaces[4–7]. Mainly two carbonic anhydrase isoforms hCA IX[8–10] and CA XII[11,12], possessing extracellular active site, are over-expressed in most of hypoxic tumors, such as gliomas/ependymomas, mesotheliomas, carcinomas of the bladder, cervix, kidneys, lungs, breast, and other dysplasia resistant to classic chemo- and radiotherapy[7,13,14]. Indeed, in response to hypoxia event, such as the one occuring in the tumor area, the activated hypoxia inducible factor-1 (HIF-1) causes an over-expression of hCA IX[6], which increases the production of protons in the district involved. The pH in the tumor area decreases thus from the characteristic physiological value of 7.4 possessed by the normal tissue to about 6[6,7]. Since the main reason of this pH imbalance is the over-expression of hCA IX and XII isoforms, they are considered as interesting druggable target for imaging and treatment of hypoxic tumors[7,15–21]. Therefore, the selective inhibition of two transmembrane isoforms CA IX and XII over the ubiquitous and cytosolic ones, CA I/II, represents a promising approach for the development of an efficient and low-side effect antitumor therapy.

The most studied and clinically used pharmacophores as carbonic anhydrase inhibitor (CAI) are the sulfonamides[2,4,5,17], which is their mechanism of action within the enzymatic cavity is well reported with many representatives in clinical use for decades[15,16,22,23].

However, one of the main side effects of sulfonamide CAIs, especially those ones belonging to the first generation of such drugs, i.e. acetazolamide **AAZ**, is the fact that they are not able to discriminate different CA isoforms. The most efficient sulfonamide CAIs indeed inhibit the majority of the mammalian CA isoforms, thus leading to a wide range of side effects.

Only recently it has been developed several novel generations of sulfonamide CAIs, which posses a remarkable selectivity profile for inhibiting prevalently the two transmembrane hCA IX and XII isoforms[17–20,24]. Among such isoform-selective benzenesulfonamide CAIs, the ureido-containing inhibitors are very interesting, as some representatives of this class (among which compounds **A**–**D**) possessed nanomolar activity for the inhibition of the tumor-associated isoforms hCA IX and XII; whereas they were much weaker inhibitors of the widespread, cytosolic off-target isoforms hCA I and II[21]. The hCA IX inhibition with these sulfonamides *in vitro*, in cell cultures, and in animals with

Address for correspondence: Mariangela Ceruso, Dipartimento di Chimica, Laboratorio di Chimica Bioinorganica, Università degli Studi di Firenze, Via della Lastruccia 3, 50019 Sesto Fiorentino (Firenze), Italy. E-mail: mariangela.ceruso@unifi.it
Claudiu T. Supuran, Neurofarba Department, Università degli Studi di Firenze, Via U. Schiff 6, 50019 Sesto Fiorentino (Florence), Italy. E-mail: claudiu.supuran@unifit.it

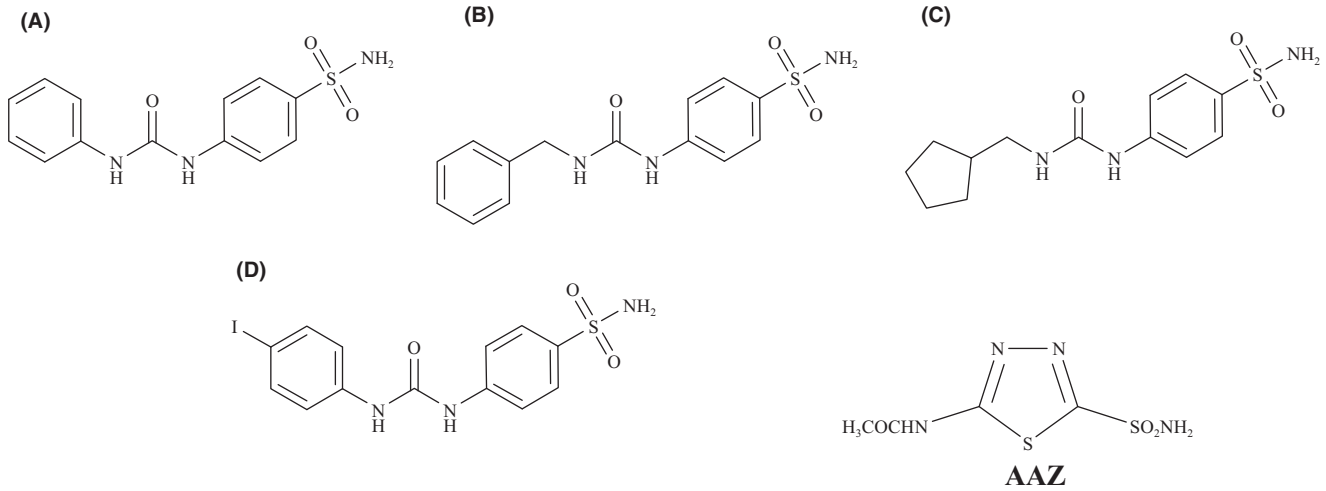


Chart 1. Structure of compounds **A–D** and the clinically used sulfonamide acetazolamide **AAZ**.

transplanted tumors, leads to a return to more normal extracellular pH values, with consequential delay of tumor growth[5,6]. One of these sulfonamide CA IX/XII inhibitors is currently in Phase I clinical trials for the treatment of primary tumors/metastases overexpressing these enzymes[6,21].

A large number of ureido-substituted benzenesulfonamide derivatives have been extensively studied from our group showing an interesting and excellent inhibition profile against the tumor-associated human carbonic anhydrase. Indeed, by only one-step synthesis occurring as well in high yield between sulfonamide and stoichiometric amount of aryl/alkyl isocyanates a large number of such derivatives were prepared. Generally, the ureido containing derivatives, among which compounds **A–D** are considered the most interesting representatives of the series (Chart 1), shown to possess an excellent low nanomolar inhibition potency demonstrated by a significant regression of the tumor volume *in vivo* experiments[1,2]. It has also been shown that depending on the substitution pattern on the urea side it was possible to obtain a good inhibition selectivity of the transmembrane CA isoforms over the cytosolic ones.

X-ray crystallographic studies of adduct formed between hCA II enzyme with ureido benzenesulfonamides, such as **A–D** derivatives, pointed out some important features of their inhibition mechanism. It is clear that the benzenesulfonamide moiety of various inhibitors belonging to this series was entirely superposable in the enzyme active site with the deprotonated sulfonamide portion (SO_2NH^- anion) coordinated to the Zn (II) ion, whereas the tails (R) of the ureido moiety adopted different orientations based on their specific chemical nature.

Therefore, for sulfanilamide derivatives incorporating the ureido linker, it has been shown that the tails attached to the urea portion are located in various hydrophobic pockets of CA II catalytic site and interacted with different amino acid residues according to the chemical properties of the R moiety[21].

Considering the inhibition profiles, the selectivity and the mechanism of inhibition of these compounds, it appeared of great interest to further explore these scaffolds for obtaining more potent CAIs. We extended consequently our earlier investigations on ureido benzenesulfonamide CAIs synthesizing and testing the inhibition potency of a small series of derivatives containing the same scaffold but incorporating instead also an elongation between the ureido and the sulfonamide moieties, such as a GABA portion[25].

Also, in this new series of GABA containing ureido benzenesulfonamide derivatives many low nanomolar hCA IX or CA XII inhibitors have been identified[25].

Since, in our previous studies some ureido benzenesulfonamide derivatives containing GABA were found highly efficient and selective CA IX/XII inhibitors and distinguished as excellent potential anticancer agents, we decided to continue our interest to the same pathway.

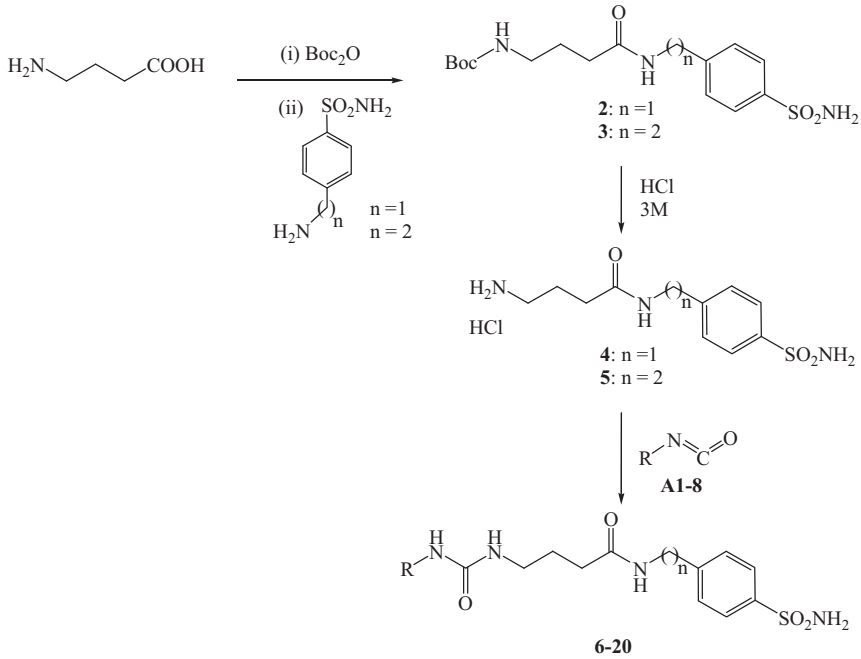
Therefore, in this paper we expanded the synthesis of the derivatives incorporating GABA as a linker between the ureido and the benzenesulfonamide moieties. The inhibition potency of these novel compounds against that over-expressed in both hypoxic tumors hCA IX and XII isoforms over the physiologically dominant, cytosolic hCA I and II ones have been determined and thus compared with the efficiency obtained from similar derivatives previously studied[25] and their smaller congeners containing sulfanilamide instead[21].

Materials and methods

Chemistry

Anhydrous solvents and all reagents were purchased from Sigma-Aldrich (Milan, Italy), Alfa Aesar (Milan, Italy) and Merck (Darmstadt, Germany). All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes techniques to transfer solutions. Nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectra were recorded using a Bruker Advance III 400 MHz spectrometer (Flawil, Switzerland) in DMSO-d_6. Chemical shifts are reported in parts per million (ppm) and the coupling constants (J) are expressed in Hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; sept, septet; t, triplet; q, quadruplet; m, multiplet; brs, broad singlet; brm, broad multiplet; dd, double of double; td, triplet of double; tt, triplet of triplet; appd, apparent double; appt, apparent triplet. The assignment of exchangeable protons (N*H*) was confirmed by the addition of D_2O. Electron ionization mass spectra (70 eV) were recorded on a Hewlett-Packard 5989 Mass Engine Spectrometer (Milan, Italy). Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel F-254 plates.

The synthesis and the full characterization of all compounds investigated here as hCA inhibitors are reported elsewhere, derivatives **6–11** in reference 24 and **12–20** in Supplemental information.



Scheme 1. Preparation of sulfonamides **6–20** by reaction of 4-amino-N-(4-sulfamoyl-benzyl)-butyramide hydrochloride ($n = 1$) (**4**) or 4-amino-N-[2-(4-sulfamoyl-phenyl)-ethyl]-butyramide hydrochloride ($n = 2$) (**5**) with arylisocyanates **A1–8** (R-NCO) in presence of diisopropylethylamine (DIPEA), in acetonitrile (dry).

CA inhibition studies

An Applied Photophysics stopped-flow instrument (Oxford, UK) has been used for assaying the CA catalyzed CO_2 hydration activity[26]. Phenol red (at a concentration of 0.2 mM) has been used as an indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.4), 10 mM Tris.HCl and 0.1 M $NaSO_4$ (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 mM 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 non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier[27–29], and represent the mean from at least three different determinations. All CAs were recombinant proteins obtained as reported earlier by these groups[28,29].

Results and discussion

Chemistry

After protecting the γ-amino group of GABA with tert-butyloxycabonyl (Boc) group via a well-known procedure[25,28,30,31] and consequently coupling of carboxylic group with 4-methyl or ethyl benzenesulfonamide in the presence of 1-hydroxybenzotriazole[30], the γ-Boc-GABA-substituted sulfonamides **2** and **3** were successfully synthesized (Scheme 1). The removal of Boc protecting group of derivatives **2** and **3** in acidic aqueous solution and subsequently treatment of the hydrolyzed products **4, 5** with various aryl isocyanates **A1–8** (R-N=C=O) (Table 1) gave the novel series of ureido benzenesulfonamides containing a GABA moiety reported here (Scheme 1).

In previous studies carried out by our group, we have determined the inhibition potency of some ureido sulfonamide derivatives, such as compounds **A–D**, which incorporate a sulfanilamide moiety in one side and an ureido tail in the other[21] (Chart 1). It has been demonstrated that this series of compounds act as highly effective hCA II inhibitors with potencies that correlate well with the orientation of the R moiety present in the ureido tail of the compound.

Similar to the leads **A–D** just reported, the new compounds obtained by the above-mentioned reactions showed as well remarkable properties as CAIs, against the cytosolic ubiquitous hCA I and CA II isoforms and the tumor-associated hCA IX and CA XII ones. Indeed, many such compounds were low nanomolar hCA IX/XII inhibitors whereas their affinity for the cytosolic off-target isozyme I was not as good, making them moderate tumor CA-selective inhibitors.

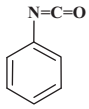
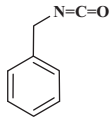
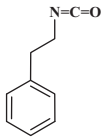
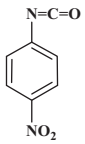
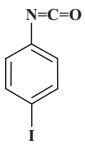
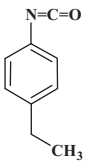
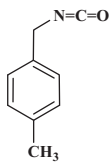
As mentioned above all hypoxic tumors overexpressed CA IX and CA XII isoforms after activation of the hypoxia inducible factor-1 (HIF-1)[6] with a pH imbalance between the tumor tissue and the normal tissue as main consequence[6,8,14]. The hCA IX/XII isoforms inhibition with sulfonamides was recently shown to reverse the effect of tumor acidification[6,8,14], leading to inhibition of the primary tumor and metastases growth, and CA IX/XII have been proposed as novel therapeutic antitumor targets[1,13].

Reported here, there are novel ureido-benzenesulfonamide CAIs obtained considering compounds **A–D** as leads, some of which incorporate the same R-ureido linker, where R was phenyl, benzyl, cyclopentyl and 4-iodophenyl[21] together with a GABA portion connecting the 4-aminomethyl or 4-aminoethylbenzene sulfonamide and the ureido moieties.

Therefore, the present findings extend our previous studies[21,25], and report here the synthesis of a series of ureido benzenzesulfonamide derivatives-containing the same ureido portion of such compounds or new ureido linker.

The reaction of γ-Boc-GABA with 4-(aminomethyl)-benzenesulfonamide and 4-(2-aminoethyl)-benzenesulfonamide and consequently hydrolysis of the coupling products afforded the key intermediates as hydrochlorides **4** and **5**, which were reacted with one equivalent of various arylisocyanates, such as 4-methylbenzyl, 4-iodiophenyl and 4-ethylphenyl isocyanates, as well as 4-

Table 1. Isocyanates used for the preparation of compounds **6–20**.

Isocyanates	R
A1	
A2	
A3	
A4	
A5	
A6	
A7	
A8	

nitrophenyl one (Table 1) in the presence of N-ethylisopropylamine (Scheme 1). These isocyanates were firstly chosen in such a way as to contain moieties which were shown earlier to lead to interesting CA inhibitory compounds in the ureido benzenesulfanilamide derivatives[21] for comparison reason.

Reaction of the key intermediate sulfonamide hydrochlorides **4** and **5**, possessing an amino group at the GABA moiety with arylisocyanates **A1–8** reported in Table 1 in the molar ratio of 1:1 in the presence of a weak base afforded the formation of a large series of ureido benzenesulfonamide derivatives (**6–20**) (Scheme 1). The equivalent amount of base used in this synthesis was required to form the nucleophilic free base of the amine group. Due to its low reactivity, a tertiary amine such as diisopropylethylamine (DIPEA) is always used as a base in this case (Scheme 1).

The characterization of new compounds by ^{1}H- and ^{13}C-NMR as well as by mass spectroscopy, confirmed their chemical structures (For further details, see Experimental Protocols reported in reference 24 and Supplemental information).

CA inhibition

Inhibition data with the new group of ureido benzensulfonamides **6–20** reported here, against the human (h) CA isoforms hCA I, II (cytosolic) and hCA IX, XII (transmembrane and tumor associated), as well as the derivatives **A–D** reported earlier (for comparison reasons)[21] are shown in Table 2. Therefore, the following structure activity relationship (SAR) can be observed from data reported in Table 2:

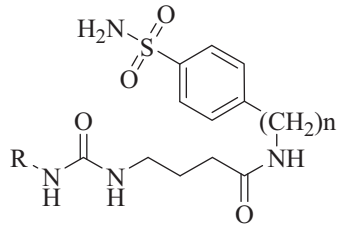
(i) The cytosolic and widespread isoform hCA I was moderately inhibited by almost all the compounds reported here, with inhibition constants in the range 192.7–373.4 nM. The best hCA I inhibitors in the series were the phenyl and the 4-nitrophenyl-ureido derivatives of 4-aminoethylbenzensulfonamide (**7** and **11**), with K_Is of 32.2 and 41.8 nM, respectively. The ethylbenzene-ureido derivative of 4-aminomethylbenzenesulfonamide (**10**) (K_I of 52.6 nM) was also a very effective hCA I inhibitor, and revealed an inhibition potency 5.4-fold higher compared to its longer congener **12** (K_I of 286.1 nM). On the other hand, the remaining substitution patterns R led to compounds with inhibition constants of the same order of magnitude as the clinically used drug acetazolamide, **AAZ**, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide (Table 2). The low inhibition of this isoform may be considered as a positive characteristic of this new series of compounds (shared also with the corresponding 4-substituted-ureido-benzenesulfonamide derivatives **A**, **C** and **D** reported earlier)[21] since the ubiquitous isoform hCA I is abundant in red blood cells, it can be certainly considered an important off-target when research of antitumor CAIs is involved[24].

(ii) Against the physiologically dominant isoform hCA II; derivatives **6–8**, **10**, **11**, **19** and **20** behaved as very strong inhibitors with K_Is in the range of 4.87–6.9 nM.

The slightly less effective compound was the benzyl ureido derivative of the ethylbenzensulfonamide **9** with a K_I value of 41 nM, whereas the remaining ones such as **9** and **12–18**, were all quite similar and medium potency hCA II inhibitors, with inhibition constants in a very narrow range 41.0–93.4 nM. Although these compounds possess a rather wide range of substitution patterns, SAR is almost impossible to define as all substituted moieties lead to effective inhibitors of this isoform (Table 2). Most probably, introduction of various substituents, such as halogens, methyl, ethyl and nitro group in the aromatic ring or cyclopenthyl ring, leads to a not significant increase in the inhibition potency. Interestingly, the new series of ureido benzenesulfonamides derivatives were very effective hCA II inhibitors compared to those reported earlier **A–D**, showing how an elongation of the linker chain between the ureido and the sulfonamide moieties can largely modify the inhibition potency of such compounds.

(iii) Several nanomolar hCA IX inhibitors are reported here, such as **6**, **7**, **13**, **14**, **16**, **17** and **20**, derivatives with K_Is in the range of 42.2–53.4 nM and thus about two times higher compared to that one of the clinically used **AAZ**. Although many of the highly effective hCA IX inhibitors reported here have not shown excellent selectivity ratios for inhibiting this transmembrane isoform over the cytosolic ones, the most interesting CA IX inhibitor listed here was derivative **12** which has shown selectivity ratios as high as 52.01 against hCA I and 16.98 against hCA II (Table 1). Indeed, this derivative resulted to be the best and the most isoform selective CA IX inhibitor. The least effective hCA IX inhibitors were derivatives **8**, **10**, **15**, **18** and **19**, which showed K_Is in the range of 289–370.4 nM, being thus low potency inhibitors of this isoform compared to the clinically

Table 2. Inhibition data of human CA isoforms hCA I, II, IX and XII with ureido-sulfonamides **6–20** reported here and the standard sulfonamide inhibitor acetazolamide (**AAZ**) by a stopped flow CO_2 hydrase assay[26].



6-20

			K_I (nM)*			
Compound	*n*	R	hCA I	hCA II	hCA IX	hCA XII
6†	1	C_6H_5	373.4	6.01	46	37.4
7†	2	C_6H_5	32.2	4.87	41.6	6.2
8†	1	$C_6H_5CH_2$	359	6.1	456.6	58.3
9†	2	$C_6H_5CH_2$	214.4	41	106.4	50.7
10†	1	$C_6H_5(CH_2)_2$	52.6	6.9	300.9	43.5
11†	2	4-$NO_2C_6H_4$	41.8	6.8	138.7	43
12	2	$C_6H_5(CH_2)_2$	286.1	93.4	5.5	10.4
13	1	C_5H_9	254.1	70.5	42.2	10
14	2	C_5H_9	240.4	60	45.3	28.5
15	1	4-C_2H_5Ph	247.9	71.4	289	5.4
16	2	4-C_2H_5Ph	232.4	57.5	53.4	6
17	1	4-CH_3PhCH_2	203.2	73.5	50.2	4.9
18	2	4-CH_3PhCH_2	192.7	64.3	328.2	6.2
19	1	4-IC_6H_4	217.8	6.8	370.4	6.1
20	2	4-IC_6H_4	213.4	6.3	46.7	5.1
AAZ	–	–	250	12	25	5.7
A‡	–	C_6H_5	760	3730	575	67.3
B‡	–	$C_6H_5CH_2$	92	2200	41.4	49.5
C‡	–	C_5H_9	470	226	7.3	7.0
D‡	–	4-IC_6H_4	5500	2634	24.5	4.3

†From reference 24; ‡From reference 23; *Mean from three different assay, by a stopped flow technique (errors were in the range of ±5–10% of the reported values).

used inhibitor **AAZ**. The remaining derivatives **9** and **11** have showed moderate inhibitory potency against this isoform (K_Is values of 106.4 and 138.7 nM, respectively).

Although some of the compounds reported here contain same substitution pattern at the R moiety as part of ureido sulfonamides previously studied possessed, it is interesting to notice that their shorter congeners containing sulfanilamide **B–D** are generally about 10-fold more effective as hCA IX inhibitors compared to the new derivatives incorporating a GABA moiety in their structures (**8** and **9** towards **B**; **13** and **14** corresponding to **C**; **19** and **20** with **D**). However, only derivatives **6** and **7** showed higher inhibition potency against hCA IX isoform with K_I 10 times lower compared to their shorter congener **A** (with K_Is of 41.6–46 nM for compounds **6** and **7** versus 575 nM of derivatives **A**), revealing to be more effective hCA IX inhibitors.

However, it is also interesting to observe that among the ureido-derivatives incorporating a GABA moiety the ones containing 4-aminoethylbenzenesulfonamide are generally stronger hCA IX inhibitors than the shorter 4-aminomethylbenzenesulfonamide congeners of the same series (Table 2).

(iv) Most of the compounds reported here are shown to be very efficient hCA XII inhibitors possessing inhibition activity at low nanomolar range in the same order of magnitude as **AAZ** (Table 2). Indeed, it is interesting to notice that this second transmembrane tumor-associated isoform, hCA XII, was much better inhibited by the new ureido benzenesulfonamides investigated here, compared to the other transmembrane tumor-associated isoform, hCA IX. The best inhibitors were derivatives **7**, **12**, **13**, **15–17**, **19** and **20** with K_I values in a narrow range between 4.9 and 10.4 nM. The remaining derivatives were slightly less effective hCA XII inhibitors, with inhibition constants in the range of 28.5–58.3 nM, which is about 10-fold higher compared to that of the standard drug **AAZ**. However, the elongation of the main skeleton between the ureido linker and the benzenesulfonamide portion with a GABA moiety did not significantly improve the inhibition potency of these compounds compared to their smaller congeners (such as in the case of derivatives **8** and **9** with **B**, **13** and **14** with **C** and **19**, **20** with **D**). Indeed, benzyl ureido derivatives **8** and **9** have shown extremely similar inhibition potency against hCA XII isoform compared to their sulfanilamide congener **B**, revealing K_I values of 58.3 and 50.7 nM, respectively, like that one of the compound **B** equal to 49.5 nM. The same occurred both for the cyclopentyl derivatives **13** and **14**, which have shown K_I values of 10 and 28.5 nM, respectively, revealing to be slightly less effective hCA XII inhibitors than their congener **C** with K_I of 7 nM and for the 4-iodophenyl derivatives **19** and **20**, with inhibition constants of 6.1 and 5.1 nM of the same order of magnitude as compound **D** possessing a K_I value equal to 4.3 nM.

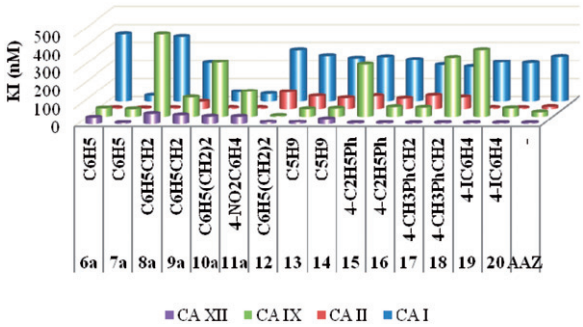


Figure 1. Graphical comparison of inhibition constants of the new ureido-sulfonamides **6–20** reported here and the standard drug **AAZ** against hCA I, II, IX and XII.

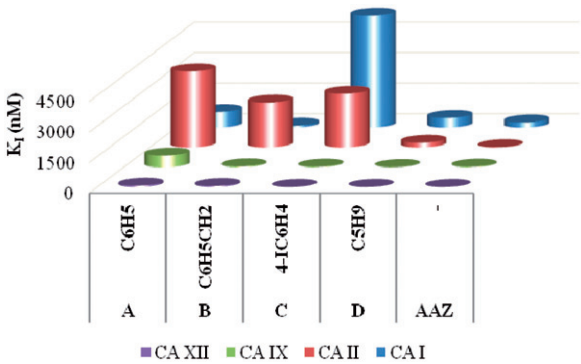


Figure 2. Graphical comparison of inhibition constants of the ureido-sulfonamides **A–D** previously reported and the standard drug **AAZ** against hCA I, II, IX and XII.

On the other hand, the only phenyl ureido benzenesulfonamide derivatives **6** and **7** containing GABA were more effective hCA XII inhibitors than their small congener **A**, showing K_I values approximately 2 and 10 times lower than that shown by **A**, respectively.

(v) Regarding the selectivity of the new derivatives reported here against the two transmembrane tumor-associated hCA IX and XII isoforms over the off-target cytosolic hCA I and II ones, some interesting results emerged. Firstly, the selectivity ratio for inhibiting the target isoforms over the off-target isoforms for most of this series of derivatives is rather low compared to that shown by their smaller congeners **A–D** (Figures 1 and 2). However, only one compound is shown to be very selective inhibitor against both the tumor-associated CA isoforms. Indeed, the phenethyl derivative **12** possessed a selectivity ratio of 52.01 for inhibiting hCA IX over hCA I and of 16.98 for inhibiting hCA IX over hCA II, thus being the best selective inhibitor of the series against the tumor-associated isoforms over the cytosolic ones, as shown in Figure 1. It should also be noted that acetazolamide has quite low selectivity ratios for inhibiting the tumor-associated isoforms against hCA I (and as well hCA II), which are 10 and 0.48, respectively.

Although the selectivity ratios of derivative **12** against the transmembrane isoforms over the cytosolic ones is lower compared to those of the corresponding sulfanilamide derivatives **A–D**, those value are still significantly higher than the selectivity ratios shown by **AAZ**. Therefore, this result revealed that compound **12** has better selectivity ratios for inhibiting the tumor-associated isoforms over the cytosolic isoforms compared to **AAZ**.

We can conclude that in this new series of compounds an elongation of the linker between the ureido and the sulfonamide moieties with a GABA scaffold, mostly brought an increase of the inhibition potency against the tumor-associated target isoforms, decreasing on the other hand their selectivity ratio against the off-target cytosolic isoforms.

Conclusions

A novel series of ureido benzenesulfonamides incorporating GABA scaffold and some of them containing same ureido linker as their sulfanilamide smaller congeners, were explored here as a new generation of selective tumor-associated hCAIX and XII inhibitors. The compounds investigated contain a GABA portion as linker between the ureido and the benzenesulfonamide moieties. Most of the new derivatives were medium potency inhibitors of the ubiquitous cytosolic hCA I isoform and efficient inhibitors against hCA II, but they showed significant inhibition potency against both the transmembrane isoforms hCA IX and XII, with inhibition constants in nanomolar range. The inhibition profile of these new GABA containing ureidosulfonamides is very different from the corresponding analogs not incorporating GABA, which were previously investigated as inhibitors of these enzymes.

Considering the selectivity ratios of the derivatives reported here for inhibiting the tumor-associated hCA IX over the physiologically dominant hCA I and II isoforms, the phenethyl derivative is the most selective inhibitor, thus representing an interesting tool for the development of new anticancer agents.

Declaration of interest

Most of the authors declare no financial interest. CTS reports conflict of interest as author of many patents on CA inhibitors.

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References

1. Supuran CT. Carbonic anhydrases. Bioorg Med Chem 2013;21:1377–8.
2. Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nat Rev Drug Discov 2008;7:168–81.
3. Esbaugh AJ, Tufts BL. The structure and function of carbonic anhydrase isozymes in the respiratory system of vertebrates. Respir Physiol Neurobiol 2006;154:185–98.
4. Supuran CT. Structure-based drug discovery of carbonic anhydrase inhibitors. J Enzyme Inhib Med Chem 2012;27:759–72.
5. Supuran CT. Carbonic anhydrase inhibitors. Bioorg Med Chem Lett 2010;20:3467–74.
6. Neri D, Supuran CT. Interfering with pH regulation in tumors as a therapeutic strategy. Nat Rev Drug Discov 2011;10:767–77.
7. Švastová E, Hulíková A, Rafajová M, et al. Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH. FEBS Lett 2004;577:439–45.
8. McIntyre A, Patiar S, Wigfield S, et al. Carbonic anhydrase IX promotes tumor growth and necrosis *in vivo* and inhibition enhances anti-VEGF therapy. Clin Cancer Res 2012;18:3100–11.
9. Hussain SA, Ganesan R, Reynolds G, et al. Hypoxia-regulated carbonic anhydrase IX expression is associated with poor survival in patients with invasive breast cancer. Br J Cancer 2007;96:104–9.
10. Proescholdt MA, Merrill MJ, Stoerr EM, et al. Function of carbonic anhydrase IX in glioblastoma multiforme. Neurooncology 2012;14:1357–66.
11. Barnett DH, Sheng S, Charn TH, et al. Estrogen receptor regulation of carbonic anhydrase XII through a distal enhancer in breast cancer. Cancer Res 2008;68:3505–15.
12. Haapasalo J, Hilvo M, Nordfors K, et al. Identification of an alternatively spliced isoform of carbonic anhydrase XII in diffusely infiltrating astrocytic gliomas. Neuro-Oncology 2008;10:131–8.
13. Said HM, Hagemann C, Carta F, et al. Hypoxia induced CA9 inhibitory targeting by two different sulfonamide derivatives

including acetazolamide in human glioblastoma. Bioorg Med Chem 2013;21:3949–57.
14. Dubois L, Lieuwes NG, Maresca A, et al. Imaging of CA IX with fluorescent labelled sulphonamides distinguishes hypoxic and (re)-oxygenated cells in a xenograft tumor model. Radiother Oncol 2009; 92:423–8.
15. Alterio V, Hilvo M, Di Fiore A, et al. Crystal structure of the catalytic domain of the tumor-associated human carbonic anhydrase IX. Proc Natl Acad Sci USA 2009;106:16233–8.
16. De Simone G, Supuran CT. Carbonic anhydrase IX: biochemical and crystallographic characterization of a novel antitumor target. Biochim Biophys Acta 2010;1804:404–9.
17. Alterio V, Di Fiore A, D'Ambrosio K, et al. Multiple binding modes of inhibitors to carbonic anhydrases: how to design specific drugs targeting 15 different isoforms? Chem Rev 2012;112: 4421–68.
18. Supuran CT. Carbonic anhydrase inhibitors and activators for novel therapeutic applications. Future Med Chem 2011;3:1165–80.
19. De Simone G, Alterio V, Supuran CT. Exploiting the hydrophobic and hydrophilic binding sites for designing carbonic anhydrase inhibitors. Expert Opin Drug Discov 2013;8:793–810.
20. Supuran CT, McKenna R. Carbonic anhydrase inhibitors drug design. In: McKenna R, Frost S, eds. Carbonic anhydrase: mechanism, regulation, links to disease, and industrial applications. Heidelberg: Springer Verlag; 2014:291–323.
21. Pacchiano F, Carta F, McDonald PC, et al. Ureido-substituted benzenesulfonamides potently inhibit carbonic anhydrase IX and show antimetastatic activity in a model of breast cancer metastasis. J Med Chem 2011;54:1896–902.
22. Carta F, Scozzafava A, Supuran CT. Sulfonamides (RSO_2NH_2): a patent review 2008–2012. Expert Opin Ther Pat 2012;22:747–58.
23. Cecchi A, Hulikova A, Pastorek J, et al. Carbonic anhydrase inhibitors. Sulfonamides inhibit isozyme IX mediated acidification of hypoxic tumors. Fluorescent sulphonamides design as probes of membrane-bound carbonic anhydrase isozymes involvement in tumorigenesis. J Med Chem 2005;48:4834–41.
24. Supuran CT. Carbonic anhydrases: off-targets, add-on activities, or emerging novel targets? In: Peters JU, ed. Polypharmacology in drug discovery. Hoboken: Wiley; 2012:457–89.
25. Ceruso M, Antel S, Vullo D, et al. Inhibition studies of new ureido-substituted sulfonamides incorporating a GABA moiety against human carbonic anhydrase isoforms I-XIV. J Med Chem 2014;22: 6768–75.
26. Khalifah RG. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. J Biol Chem 1971;246:2561–73.
27. Supuran CT, Mincione F, Scozzafava A, et al. Carbonic anhydrase inhibitors. Part 52. Metal complexes of heterocyclic sulfonamides: a new class of strong topical intraocular pressure-lowering agents with potential use as antiglaucoma drugs. Eur J Med Chem 1998;33: 247–54.
28. Wilkinson BL, Bornaghi LF, Houston TA, et al. Carbonic anhydrase inhibitors: inhibition of isozymes I, II and IX with triazole-linked O-glycosides of benzene sulphonamides. J Med Chem 2007;50: 1651–7.
29. Supuran CT, Mincione F, Scozzafava A, et al. Carbonic anhydrase inhibitors. Part 52. Metal complexes of heterocyclic sulfonamides: a new class of strong topical intraocular pressure-lowering agents with potential use as antiglaucoma drugs. Eur J Med Chem 1998;33: 247–54.
30. Ceruso M, Del Prete S, Alothman Z, et al. Sulfonamides with potent inhibitory action and selectivity against the α-carbonic anhydrase from *Vibrio cholera*. ACS Med Chem Lett 2014;5:826–30.
31. Ceruso M, Bragagni M, AlOthman Z, et al. New series of sulfonamides containing amino acid moiety act as effective and selective inhibitors of tumor-associated carbonic anhydrase XII. J Enzyme Inhib Med Chem 2014. [Epub ahead of print]. doi: 10.3109/14756366.2014.942659.

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