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Carbonic anhydrase inhibitors: Inhibition of the human transmembrane isozyme XIV with a library of aromatic/heterocyclic sulfonamides

Özen Özensoy,[a,†] Isao Nishimori,[b] Daniela Vullo,[a] Luca Puccetti,[c] Andrea Scozzafava[a] and Claudiu T. Supuran[a,*]

[a]*Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino (Florence), Italy*
[b]*Department of Gastroenterology and Hepatology, Kochi Medical School, Nankoku, Kochi 783-8505, Japan*
[c]*Ospedale San Lazzaro, Divisione di Urologia, Via Pierino Belli 26, 12051 Alba, Cuneo, Italy*

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Abstract—The first inhibition study of the transmembrane carbonic anhydrase (CA, EC 4.2.1.1) isozymes hCA XIV with a library of aromatic and heteroaromatic sulfonamides synthesized earlier is reported. Most of the inhibitors were sulfanilamide, homosulfanilamide and 4-aminoethyl-benzenesulfonamide derivatives, to which tails that would induce diverse physicochemical properties have been attached at the amino moiety. Several of these compounds were metanilamide, benzene-1,3-disulfonamide or the 1,3,4-thiadiazole/thiadiazoline-2-sulfonamide derivatives. The tails incorporated in these molecules were of the alkyl/aryl-carboxamido/sulfonamido-, ureido- or thioureido-types. The sulfanilamides acylated at the 4-amino group with short aliphatic/aromatic moieties incorporating 2-6 carbon atoms showed modest hCA XIV inhibitory activity (K_I-s in the range of 1.25–4.2 μM) which were anyhow better than that of sulfanilamide (K_I of 5.4 μM). Better activity showed the homosulfanilamide and 4-aminoethyl-benzenesulfonamide derivatives bearing arylsulfonamido/ureido and thioureido moieties, with K_I's in the range of 203–935 nM. The best activity was observed for the heteroaromatic compounds incorporating 1,3,4-thiadiazole/thiadiazoline-2-sulfonamide and 5-arylcarboxamido/sulfonamido moieties, with K_I's in the range of 10–85 nM. All these compounds were generally also much better inhibitors of the other two transmembrane CA isozyme, hCA IX and XII. Thus, highly potent hCA XIV inhibitors were detected, but isozyme-specific inhibitors were not discovered for the moment.

1. Introduction

Isozyme XIV of carbonic anhydrase (CA, EC 4.2.1.1) was the last one to be discovered[1] (together with isozymes CA VB,[2] and XIII[3]) among the 15 CA isoforms of this widespread metalloprotein known up to now in humans.[4] By catalysing the reversible hydration of carbon dioxide to bicarbonate and a proton at neutral pH, these enzymes play a host of physiological/pathological roles in many processes in which these three chemical species are involved.[4–7] As carbon dioxide is the main waste product in all metabolic processes and H^+/HCO_3^- ions are critical for the pH homeostasis, electrolyte secretion in many tissues/organs, biosynthetic reactions, transport of anions across membranes, tumorigenesis, etc., to cite only some of the most important ones, it starts to be even more obvious why evolution selected a rather large family of such proteins (i.e., 15 isoforms in humans) to deal specifically with these tasks. Similar to the isozymes CA IX and XII, CA XIV is also a transmembrane protein with an extracellular active site,[1,8] but unlike the first two proteins, isozyme XIV is not associated to cancerous tissues.[9–15] Indeed, work followed in several laboratories showed CA XIV to be a highly abundant protein in the brain, kidney, colon, small intestine, urinary bladder, liver and spinal cord.[9–13] It has then been shown by Parkkila's group that in kidneys, the luminal CA XIV is involved in bicarbonate reabsorption (a function previously considered to be played by CA IV)[11] whereas the same group also showed that in hepatocytes plasma

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* Corresponding author. Tel.: +39 055 457 3005; fax: +39 055 4573 385; e-mail: claudiu.supuran@unifi.it
† On leave from Balikesir University, Turkey.

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membranes CA XIV is involved in the control of pH and ion transport processes between the hepatocytes, sinusoids, and bile canaliculi.[12] Finally, this extracelullar CA isozyme is highly abundant in neurons and axons in the murine and human brain, where it seems to play an important role in modulating excitatory synaptic transmission.[13]

The structure of the murine isoform XIV has been reported by Christianson and co-workers,[8] whereas very recently our group[16] reported the complete kinetic characterization of the human isozyme (full length hCA XIV). Similar to the isozymes CA XII (the most closely related philogenetically)[1] and CA I (a highly abundant cytosolic blood isoform), hCA XIV shows a medium–low catalytic activity for the CO_2 hydration reaction, with $k_{cat} = 3.12 \times 10^5\ s^{-1}$, and $k_{cat}/K_M = 3.9 \times 10^7\ M^{-1}\ s^{-1}$.[16] In this work,[16] for the first time the inhibition of hCA XIV with a series of simple aromatic and heteroaromatic sulfonamides (such as among others sulfanilamide **SA**, homosulfanilamide, and 4-aminoethylbenzenesulfonamide **AEB**), as well as with the clinically used derivatives from this class of pharmacological agents, among them acetazolamide **AZA** and ethoxzolamide **EZA**, were investigated. It was thus observed that isozyme XIV shows a completely different inhibition profile as compared to the other transmembrane isoforms CA IX and XII, which we showed earlier to be important druggable targets, mainly in the design of novel antitumor or antiglaucoma therapies.[17–25]

Considering the wide distribution of this isozyme throughout the human body and its involvement in critical physiological processes, it is important to explore the possibility of designing potent and possibly also isozyme-specific hCA XIV inhibitors. Here, we report an inhibition study of this new isozyme with a class of aromatic/hetroaromatic sulfonamides.

2. Chemistry

Compounds **1**–**5** used in this assay were previously reported by our group.[25–31] Most of them are sulfanilamide **SA**, homosulfanilamide **HSA** and 4-aminoethylbenzenesulfonamide **AEB** derivatives (compounds **1a**–**s**), to which tails that should induce diverse physicochemical properties have been attached at the amino moiety. However, several of these compounds are derived from metanilamide (**2**), benzene-1,3-disulfonamide (**3**), or the thiadiazole/thiadiazoline-2-sulfonamides (**4a**, **b**, and **5**). The compounds tested were selected to cover a rather wide range of compounds to which acyl-, alkyl-/arylsulfonyl-, ureido-, and thioureido- tails have been attached to modulate some of the physicochemical properties important for a potential drug, such as hydrophilicity and lipophilicity among others.

3. Carbonic anhydrase inhibition

Inhibition data of three physiologically relevant CA isozymes, that is, hCA IX, XII and XIV with sulfonamides **1**–**5** and standard, clinically used inhibitors are shown in Table 1.[32–34] The CA IX and XII inhibition data with these sulfonamides have recently been reported[32] and are included here only for comparison reasons, because these three transmembrane isozymes are quite similar from the structural point of view (the structures of the catalytic domains of hCA XII and mCA XIV were reported)[8] and philogenetically.[1]

The following should be noted regarding inhibition of hCA XIV with this series of sulfonamides: (i) the three aromatic lead molecules, sulfanilamide **SA**, homosulfanilamide **HSA**, and 4-aminoethyl-benzenesulfonamide **AEB** show rather weak hCA XIV inhibitory activity, with inhibition constants in the range of 2.9–5.4 μM, and an increased inhibitory power with the length of the spacer between the sulfamoylphenyl- and the amino moiety from 0 to 2 methylene groups. Noteworthy, the hCA IX and XII inhibitory properties of these three compounds are much better (K_I's in the range of 33–294 nM against hCA IX, and 0.3–37 nM against hCA XII, respectively). A first group of the investigated sulfonamides, including **1a**–**i** and **2**, also showed modest hCA XIV inhibitory effects, with K_I's in the range of 1250–4200 nM. It may be observed that all of them (except the metanilamide derivative **2**) are sulfanilamides acylated or alkylsulfonylated at the 4-amino group. Thus, all these compounds showed enhanced hCA XIV inhibitory properties as compared to the lead **SA**, and generally, their inhibitory properties increased with the increase of the 4-acylating moiety from acetamido to BuCONH and benzoylamido moiety. This increase is not always linear; for example, the trifluoroacetamido derivative **1b** and the benzoyl derivative **1s** showed quite similar inhibition constants. Furthermore, it may be observed that the secondary sulfonamide **1i** is 3.36 times more potent as hCA XIV inhibitor as compared to the corresponding carboxamide **1a** (Table 1); (ii) a second group of derivatives, including **1j**–**q** and **3** act as more

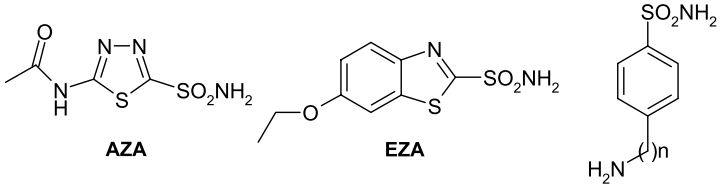
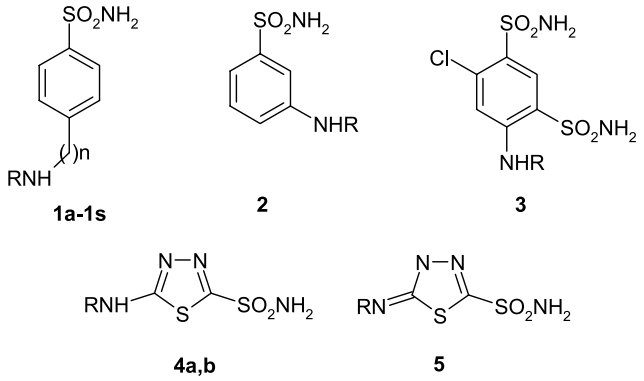


Table 1. hCA IX, XII, and XIV inhibition data with sulfonamides **1**–**5**, acetazolamide (**AZA**), ethoxzolamide (**EZA**), sulfanilamide (**SA**), homosulfanilamide (**HSA**) and 4-aminoethyl-benzenesulfonamide (**AEB**)



Compound	*n*	R	K_I (nM)*		
			hCA IX[a]	hCA XII[a]	hCA XIV[b]
1a	0	CH_3CO	135	49	4200
1b	0	CF_3CO	112	31	1430
1c	0	EtCO	106	56	3700
1d	0	*n*-PrCO	83	85	2350
1e	0	*i*-PrCO	139	138	2400
1f	0	*n*-BuCO	79	147	2300
1g	0	*t*-BuCO	136	249	2650
1h	0	PhCO	73	21	1345
1i	0	$MeSO_2$	113	33	1250
1j	0	$PhSO_2$	52	68	935
1k	0	4-$AcNHC_6H_4SO_2$	37	76	880
1m	1	$PhSO_2$	40	83	465
1n	1	PhNH–C(=S)	26	18	237
1p	2	PhNH–C(=S)	21	24	203
1q	2	PhNH–C(=O)	18	13	260
1r	2	4-$H_2NO_2SC_6H_4NH$–C(=S)	3	79	85
1s	2	4-$H_2NO_2SC_6H_4CO$	12	13	79
2	—	PhNH–C(=O)	14	10	1450
3	—	PhNH–C(=O)	146	348	430
4a	—	4-$BrC_6H_4SO_2$	21	3.3	13
4b	—	4-$O_2NC_6H_4SO_2$	16	1.9	10
5	—	Furan-2-yl–CO	13	2.4	23
AZA	—	—	25	5.7	41
EZA	—	—	50	22	25
SA	—	—	294	37	5400
HSA	—	—	103	0.3	3200
AEB	—	—	33	3.2	2900

Data of hCA IX and XII are from Ref. 32.
[a] Catalytic domain of the human cloned isozyme.
[b] Human, full length recombinant enzyme.
* Mean from three assays (errors in the range of 5–10% of the reported value).

effective hCA XIV inhibitors, with K_I's in the range of 203–935 nM while at the same time being much more effective as hCA IX/XII inhibitors. These are either sulfanilamides arylsulfonylated at the 4-amino group (**1j** and **k**), or acylated/arylsulfonylated and ureido/thioureido derivatives of **HSA** and **AEB** (the only other structural class includes the benzene-1,3-disulfonamide derivative **3**). Thus, hCA XIV inhibitory properties are enhanced for derivatives showing these types of substitution patterns over the previously discussed ones. Furthermore, the **AEB** derivatives are better inhibitors than the corresponding **HSA** derivatives which in turn seem to be more effective inhibitors than the corresponding **SA** derivatives, clearly showing that the spacer between the benzenesulfonamide part and the tail part of the molecule critically influence the biologic activity; (iii) the most effective hCA XIV inhibitors in the series of investigated sulfonamides were **1r**, **s**, **4a**, **b**, and **5**, which showed inhibition constants in the range of 10–85 nM, of the same order of magnitude as the clinically used drugs acetazolamide and ethoxzolamide (K_I's of 25–41 nM). Thus, except the two bis-sulfonamides derived from **AEB**, **1r**, and **s**, the other potent hCA XIV inhibitors described here are heteroaromatic derivatives incorporating the 1,3,4-thiadiazole-2-sulfonamide or 1,3,4-thiadiazoline-2-sulfonamide moieties, present in

the clinically used CAIs. Particularly, potent inhibitors were the two sulfonylated derivatives **4a** and **b**, which are 3.15–4.10 times more effective than the lead compound acetazolamide. Thus, these substitution patterns may lead to quite effective hCA XIV inhibitors, but all these compounds also act as quite efficient inhibitors of isozymes IX and XII (Table 1).

4. Conclusions

The inhibition of the transmembrane isozymes hCA XIV has been investigated with a library of aromatic and heteroaromatic sulfonamides synthesized earlier by us. Most of them were sulfanilamide, homosulfanilamide and 4-aminoethyl-benzenesulfonamide derivatives, to which tails that should induce different physicochemical properties have been attached at the amino moiety, whereas several of these compounds were derived from metanilamide, benzene-1,3-disulfonamide or the 1,3,4-thiadiazole/thiadiazoline-2-sulfonamides. The tails were of the alkyl/aryl-carboxamido/sulfonamido-, ureido- or thioureido-types. The sulfanilamides acylated at the 4-amino group with short aliphatic/aromatic moieties incorporating 2–6 carbon atoms showed modest hCA XIV inhibitory activity (K_I's in the range of 1.25–4.2 μM) which were anyhow better than that of sulfanilamide itself (K_I of 5.4 μM). Better activity showed the homosulfanilamide and 4-aminoethyl-benzenesulfonamide derivatives bearing arylsulfonamido/ureido and thioureido moieties, with K_I's in the range of 203–935 nM. The best activity was observed for the heteroaromatic compounds incorporating 1,3,4-thiadiazole/thiadiazoline-2-sulfonamide and 5-arylcarboxamido/sulfonamido moieties, with K_I's in the range of 10–85 nM. All these compounds were generally much better inhibitors of the other two transmembrane CA isozyme, hCA IX and XII. Thus, highly potent hCA XIV inhibitors were detected, but isozyme-specific inhibitors were not discovered for the moment.

5. Experimental

5.1. Chemistry

Acetazolamide (**AZA**), ethoxzolamide (**EZA**), sulfanilamide (**SA**), homosulfanilamide (**HSA**) and 4-aminoethyl-benzenesulfonamide (**AEB**) were from Sigma-Aldrich (Milan, Italy). Compounds **1**–**5** used in this assay were previously reported by our group.[25–31]

5.2. The CA XIV-GST construct

A putative full-length cDNA of hCA XIV (accession No. AB025904) was obtained by RT-PCR with poly(A)-RNA from the human spinal cord (Clontech, Palo Alto, CA) and the 5′ and 3′ rapid amplification of cDNA ends (RACE) has been performed by the previously described method.[1,32] The cDNA fragment encoding the open reading frame of hCA XIV was amplified by PCR using adopter primers including *Eco*RI and *Sal*I recognition sequences (underlined in the following sequences, respectively): 5′-CCGAATTCGGACTG CATGTTGTTCTCCGCCCTCCT-3′ and 5′-TTTGTC GACTTATGCCTCAGTCGTGGCTT-3′. The PCR reaction was hot-started with incubation for 5 min at 94 °C and consisted of 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C. The PCR products were cleaved with the corresponding restriction enzymes, purified and cloned in-frame into a modified pGEX-4T2 vector using T_4-ligase (Promega). The proper cDNA sequence of the hCA XIV insert included in the vector was reconfirmed by DNA sequencing. The constructs were then transfected into *E. coli* strain BL21 for production of the hCA XIV protein, similar to the procedure already described for hCA IX.[33] The protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside, cells were harvested when the OD_{600} arrived at 1.00 and lysed by sonication in PBS. The cell homogenate was incubated at room temperature for 15 min and homogenized twice with a Polytron (Brinkmann) for 30 s each at 4 °C. Centrifugation at 30,000*g* for 30 min afforded the supernatant containing the soluble proteins. The obtained supernatant was then applied to a prepacked Glutathione Sepharose 4B column (Amersham). The column was extensively washed with buffer and then the fusion (GST–hCA XIV) protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris–HCl pH 8.0. Finally the GST part of the fusion protein was cleaved with thrombin. The advantage of this method is that hCA XIV is purified quite easily and the procedure is quite simple. The obtained hCA XIV was further purified by sulfonamide affinity chromatography, the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[34]

5.3. CA inhibition assay

An SX.18MV-R 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 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following 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 (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) 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 nonlinear least-squares methods using PRISM 3, from Lineweaver–Burk plots, as reported earlier,[16,25] and represent the mean from at

least three different determinations. The other cloned enzymes (hCA IX and hCA XII) were obtained as reported earlier by this group.[18–23]

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