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Combining the tail and the ring approaches for obtaining potent and isoform-selective carbonic anhydrase inhibitors: Solution and X-ray crystallographic studies ☆

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ABSTRACT

5-(3-Tosylureido)pyridine-2-sulfonamide and 4-tosylureido-benzenesulfonamide (**ts-SA**) only differ by the substitution of a CH by a nitrogen atom, but they have very different inhibitory properties against the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). By means of X-ray crystallography on the human CA II adducts of the two compounds these differences have been rationalized. As all sulfonamides, the two compounds bind in deprotonated form to the Zn(II) ion from the enzyme active site and their organic scaffolds extend throughout the cavity, participating in many interactions with amino acid residues and water molecules. However the pyridine derivative undergoes a tilt of the heterocyclic ring compared to the benzene analog, which leads to a very different orientation of the two scaffolds when bound to the enzyme. This tilt also leads to a clash between a carbon atom from the pyridine ring of the first inhibitor and the OH moiety of Thr200, leading to less effective inhibitory properties of the pyridine versus the benzene sulfonamide derivative. Indeed, **ts-SA** is a promiscuous, low nanomolar inhibitor of 7 out of 10 human (h) CA isoforms, whereas the pyridine sulfonamide is a low nanomolar inhibitor only of the tumor-associated hCA IX and XII, being less effective against other 9 isoforms. Thus, a difference of one atom (N vs CH) in two isostructural sulfonamides leads to drastic differences of activity, phenomenon understood at the atomic level through the high resolution crystallographic structure and kinetic measurements reported in the paper. Combining the tail and the ring approaches in the same chemotype leads to isoform-selective, highly effective sulfonamide CA inhibitors.

1. Introduction

There are two main approaches for designing sulfonamide carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs), the ring and the tail approaches.[1] The first one consists in exploring various aromatic/heterocyclic scaffolds on which the sulfonamide zinc binding group (ZBG) is attached, whereas the second one is focused on appending one or more 'tails' to a scaffold (usually an aromatic or heterocyclic ring system, already incorporating a ZBG of the sulfonamide, sulfamate or sulfamide type).[2–5] Examples of the ring approach are the clinically used drugs sulfanilamide **SA** (the compound for which the CA inhibition was originally reported),[6] acetazolamide **AAZ**, ethoxzolamide **EZA** or dichlorophenamide **DCP**, whereas the tosylureido derivative of **SA**, ts-SA, is a representative of the tail approach.[7] It may be observed that using the scaffold of **SA**, the tosylureido tail has been appended which led to a highly effective inhibitor of isoforms CA I, II and IV.[7] In this last approach, the tail moieties is considered as being highly relevant for inducing the desired physico-chemical properties as well as the good affinity for the target enzyme, and, more importantly, isoform-selectivity to the obtained CAIs.[2–8] The tail functionality interacts with amino acid residues towards the entrance of the CA active site, unlike the ring which is buried deeply in the active site, nearby the Zn(II) ion.[1–5] As the highest variability of amino acid residues in the many CA isoforms known to date is observed towards the rim of the active site cavity, these interactions of the tail moiety may lead to CAIs with a better selectivity profile compared to the clinically used drugs mentioned above. Indeed, this is an important issue for a family of enzymes with 15 diverse isoforms in humans (and many phylogenetically related orthologs in organisms all over the phylogenetic tree, starting from bacteria

☆ Coordinates and structure factors have been deposited in the Protein Data Bank as entry 4KV0.

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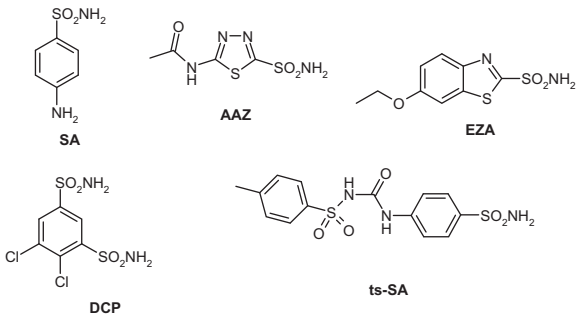
and plants and ending with fungi, protozoa, vertebrates, etc.).[9,10] In fact many of the clinically used, first generations sulfonamide CAIs, although effective as antiglaucoma, diuretic, antiobesity or antiepileptic agents,[1–5] also possess a large number of side effects due to the inhibition of off-target isoforms.[2]



Both these strategies, the ring and the tail ones, were extensively used for obtaining diverse classes of sulfonamide and nonsulfonamide CAIs,[8–11] most of which incorporated benzenesulfonamide (as shown above for **ts-SA**), 1,3,4-thiadiazole-sulfonamide, thiophene-2-sulfonamide or other such five-membered heterocyclic ring systems.[2,3,12–18] However, they have rarely been combined for drug design purposes for an entirely new class of such pharmacological agents. Here we report an interesting example of combining the ring and tail approaches for obtaining a sulfonamide from a new class, 5-(3-tosylureido)pyridine-2-sulfonamide (**ts-PySA**, **5**), as well as its inhibition profile against all hCA isoforms. An X-ray crystallographic study on the hCA II adduct of this new sulfonamide allowed us to rationalize the interesting selectivity profile of this compound for the inhibition of pharmacologically relevant CA isoforms.

2. Results and discussion

2.1. Chemistry

The rationale for obtaining the novel sulfonamide reported here consisted in replacing one CH group in the sulfanilamide (**SA**) scaffold by a nitrogen atom, leading thus to an amino-pyridine sulfonamide, more precisely 5-aminopyridine-2-sulfonamide (**PySA**, **4**), a compound already reported in the '40s in the search of antibacterial sulfadrugs.[19] Our interest in this compound was double: (i) **PySA** is isostructural with **SA** (a medium potency, promiscuous inhibitor of most mammalian CA isoforms)[20] but the presence of the nitrogen atom may lead to a protonation of the compound and an enhanced water solubility, presumably correlated with diverse CA inhibitory properties compared to the lead compound **SA**. It is in fact well-known that one of the main pharmacological problems of the sulfonamide CAIs is their scarce water solubility and hence possible pharmacokinetic problems;[21] (ii) the amino group of **PySA**, similar to that of **SA** can be easily derivatized by the tail approach leading to the possibility to explore a chemical space difficultly accessible otherwise for sulfonamide CAIs. In fact, although the pyridine is one of the most investigated heterocycles, being found in a huge number of natural and synthetic products, no CAIs based on this ring system have been investigated so far.[22] Thus, we combine the ring approach (by using the pyridine scaffold on which a sulfonamide moiety was incorporated in *ortho* to the pyridine nitrogen atom) with the tail one, by derivatizing the 5-amino group of **PySA** by means of arylsulfonyl isocyanate chemistry, widely employed for obtaining inhibitors/activators of various enzymes, such as the CAs, matrix metalloproteinases (MMPs), serine proteases, etc,[7] in order to obtain a new type of CAI.

In order to prepare **PySA** we used the reported chemistry[19] shown in Scheme 1. Commercially available 2-mercapto-5-nitropyridine was reduced with sodium hydrosulfite and the obtained amine acetylated with acetic anhydride, leading to the acetamido derivative **1**. Compound **1** was treated with sodium hypochlorite in the presence of concentrated sulfuric acid, leading to the sulfonyl chloride **2**. This key intermediate was converted to the sulfonamide **3** by treatment with aqueous ammonia, and the acetyl group was removed with concentrated hydrochloric acid, leading thus to the pyridine analog of sulfanilamide, compound **4** (**PySA**). The amino group of **4** was then derivatized with tosyl isocyanate, leading to the toluenesulfonylurea **5** (**ts-PySA**), which is the analog of **ts-SA** reported earlier by us.[7a] The compounds prepared here were characterized by physico-chemical and spectroscopic techniques which confirmed their structures (see Section 4).

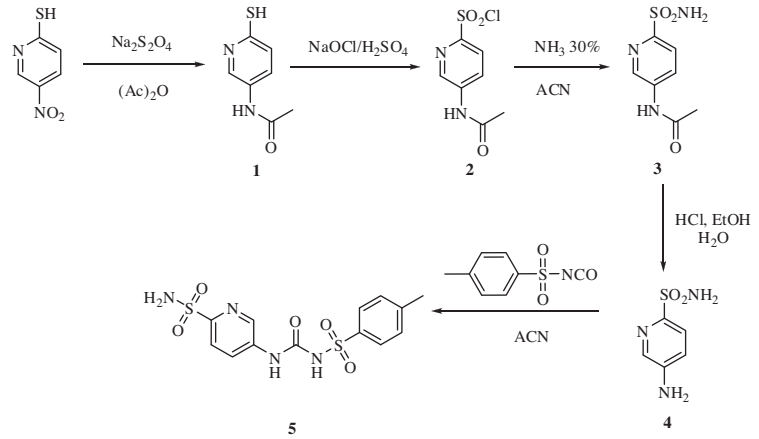
2.2. CA inhibition

The inhibition of eleven hCA isoforms has been investigated with derivatives **SA**, **PySA**, **ts-SA** and **ts-PySA** as well as the clinically used, standard CAI acetazolamide (**AAZ**).[23] The isoforms included in the study were cytosolic (hCA I–III, VII and XIII), membrane-associated or trans-membrane (hCA IV, IX, XII and XIV), mitochondrial (hCA VA) and secreted (in saliva and milk, hCA VI) enzymes (Table 1). All of them are involved in a host of important physiological and pathological functions in humans.[3–10]

The following structure activity relationship (SAR) can be observed from data of Table 1, for the inhibition of the CA isoforms investigated here with the sulfonamides mentioned above:

(i) Sulfanilamide **SA** and its pyridine analog **PySA** were generally ineffective or medium-potency inhibitors of most hCAs investigated here. For example, hCA I, hCA III, hCA IV, hCA VA, hCA VI and hCA XIV were poorly inhibited by these compounds, with K_Is in the range of 941 nM–>100 μM. However, the cytosolic (hCA II, VII and XIII) and transmembrane (hCA IX and XII) isoforms were better inhibited by these compounds. **SA** showed inhibition constants of 238–240 nM against hCA II and IX, whereas the other isoforms were inhibited with K_Is of 35–70 nM (Table 1). The inhibition profile of **PySA** was rather similar to that of **SA**, but the heterocyclic sulfonamide was less effective as CAIs compared to the aromatic one. Thus, all these isoforms (hCA II, VII, IX, XII and XIII) were inhibited with K_Is in the range of 114–279 nM.

(ii) The tosylated sulfanilamide **ts-SA** was ineffective as hCA III inhibitor (as most sulfonamides), due to the presence of the bulky Phe198 in the middle of the active site cavity of this isoform.[23] However, seven of the remaining 10 investigated CA isoforms were inhibited in the low nanomolar range by this compound, which showed K_Is in the range of 1.3–12 nM against hCA I, II, VII, IX, XII, XIII and XIV (Table 1). Only hCA IV, hCA VA and hCA VI were slightly less inhibited by this compound, with K_Is of 95–146 nM. The most sensitive isoform to this compound was hCA IX (K_I of 1.3 nM) and the least sensitive one was hCA IV (K_I of 146 nM). It may be observed that derivatization of **SA** by means of the tosylureido tail present in **ts-SA** leads to a drastic enhancement of the CA inhibitory properties against all isoforms (except CA III), sometimes by a factor of 1317 (hCA XIV)–3570 (hCA I). However, similar to the clinically used **AAZ** (see discussion later in the text), **ts-SA** is a rather promiscuous CAI, as it has a quite high affinity for 7 isoforms (some of which are quite diffuse, such as hCA I and II), and for the remaining three ones the inhibition profile is that of a medium-potency inhibitor. In fact, comparing this compound with **AAZ**, it may be observed that the clinically used drug inhibits 9 out of 10 isoforms with K_Is in the range of 2.5–74 nM. Only hCA I is inhibited less (K_I of 250 nM), whereas hCA III is the least inhibited



Scheme 1. Synthesis of sulfonamides **4** (**PySA**) and **5** (**ts-PySA**) from 5-nitro-2-mercapto-pyridine.

Table 1
Inhibition profile of human (h) isoforms hCA I–XIV with sulfanilamide **SA**, **PySA**, and their tosylureido derivatives. **AAZ** inhibition data (as standard compound) are also included for comparison. The assay was done by means of a stopped-flow method[23b]

Enzyme	K_I* (nM)				
	SA	**ts-SA**	**PySA**	**ts-PySA**	**AAZ**
hCA I	25,000	7	24,700	295	250
hCA II	240	12	253	21	12
hCA III	>100,000	>100,000	>100,000	>100,000	20,000
hCA IV	9760	146	9540	627	74
hCA VA	32,000	95	30,650	7320	63
hCA VI	941	136	1230	857	11
hCA VII	70	6.4	135	30	2.5
hCA IX	238	1.3	279	9.8	25
hCA XII	37	1.5	114	6.8	5.7
hCA XIII	35	11	155	38	17
hCA XIV	5400	4.1	5320	340	41

* Mean from 3 different assay, by a stopped flow technique (errors were in the range of ±5–10% of the reported values, data not shown).

isoform by this compound (K_I of 20 μM). Thus, both **AAZ** (as most CAIs belonging to the first and second generations of such pharmacological agents) as well as **ts-SA** are promiscuous sulfonamide CAIs. It should be also observed that **ts-SA** is equipotent or more potent than **AAZ** against many CA isoforms investigated here, such as hCA I, hCA II (equipotency of the two compounds), hCA IX, XII, XII and XIV.

(iii) The new sulfonamide reported here, **ts-PySA** (compound **5**) was a low nanomolar inhibitor of the tumor-associated isoforms hCA IX and XII (K_Is of 6.8–9.8 nM), effectively inhibited three cytosolic isoforms, hCA II, VII and XIII (K_I of 21–38 nM), and was much less effective as inhibitor of the remaining isoforms (hCA III was again not inhibited as for **SA**, **ts-SA** and **PySA**, Table 1). Thus, hCA I, hCA IV, hCA VA, hCA VI and hCA XIV were inhibited with K_Is in the range of 295–7320 nM. **ts-PySA** is thus a more effective inhibitor against all isoforms compared to the parent compound **PySA**, a feature in common with **ts-SA** when compared to **SA**. However, unlike **ts-SA**, **ts-PySA** is a much more isoform-selective CAI, as only two isoforms (the tumor-associated ones hCA IX and XII) were inhibited in the low nanomolar range by this compound, whereas three other cytosolic isoforms showed inhibition constants of 21–38 nM.

In order to understand the molecular features responsible for this interesting behaviour of CAI, we performed a crystallographic study for the adduct of hCA II with **ts-PySA**. It should be mentioned that the hCA II adduct of **ts-SA** has also been crystallized although not yet published (PDB code 1ZFK). However the structure is available in the Protein Database and was discussed in an earlier paper of this group.[4c]

2.3. X-ray crystallography

In order to rationalize inhibition of CAs with these compounds, X-ray crystallographic studies were performed on the adduct of hCA II with **ts-PySA** and the structure was compared with that of the hCA II–**ts-SA** adduct (K_Is of 21 and 12 nM, respectively, for these CAIs, see Table 1). The final model refinement statistics for the hCA II–**ts-PySA** adduct is shown in Table 2.

The electron density of all moieties of the inhibitor (except two carbon atoms from the terminal tolyl moiety) was clearly seen in the adduct (Fig. 1). In the refined model sulfonamide **5** binds to

Table 2
Summary of data collection and atomic model refinement statistics for the hCA II–**ts-PySA** (**5**) adduct*

	hCAII-ts-PySA
PDB ID	4KV0
Wavelength (Å)	1.542
Space group	$P2_1$
Unit cell (a,b,c,β) (Å,°)	42.24, 41.28, 72.05, 104.28
Limiting resolution (Å)	1.55 (1.64–1.55)
Unique reflections	32398 (4465)
R_{sym} (%)	3.9 (33.3)
Redundancy	1.9 (1.7)
Completeness overall (%)	91.9 (79.1)
$\langle I/\sigma(I)\rangle$	15.6 (2.2)
Refinement statistics	
Resolution range (Å)	30.0–1.55
Unique reflections, working/free	32386/1638
R_{factor} (%)	15.81
R_{free} (%)	17.87
No. of nonhydrogen atoms	2483
No. of water molecules	337
No. of compound atoms	24
r.m.s.d. bonds(Å)	0.006
r.m.s.d. angles (°)	1.30
Ramachandran statistics (%)	
Most favored	88.4
Additionally allowed	11.1
Generously allowed regions	0.5
Average B factor (Å^2)	
All atoms	12.2
compound	16.6
solvent	25.1

* Values in parentheses are for the highest resolution shell.

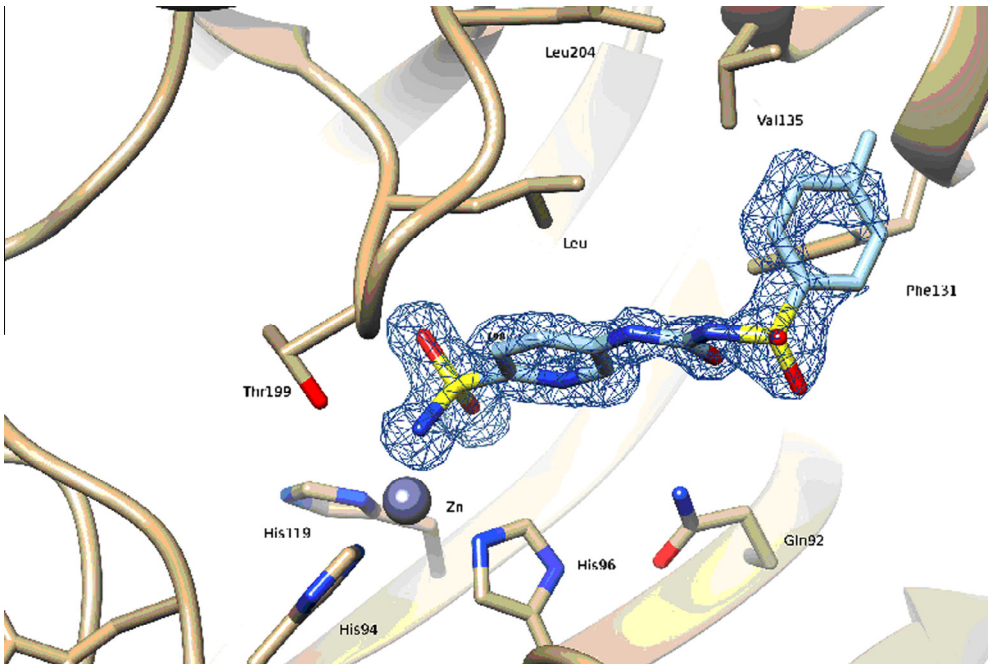


Figure 1. $|F_0 - F_c|$ difference electron density map contoured at 2σ for the sulfonamide **5** (**ts-PySA**) bound within the hCA II active site.

the zinc ion in the active site through the N1 atom of the deprotonated sulfonamide moiety at a distance of 2.02 Å. As in all other published hCA II-sulfonamide complexes,[24–27] additional hydrogen bonds from Thr199 N and OG to the sulfonamide oxygen atoms are present (OG1 Thr199—N4 atom of the sulfonamide moiety, of 2.81 Å, and N Thr199—O3 of the same sulfamoyl moiety from the inhibitor, of 3.02 Å), which further stabilize the enzyme–inhibitor adduct. Thus, as in many other adducts with sulfonamide inhibitors, the ZBG binds directly to the active site zinc ion along with the side chains of His94, His96 and His119, the metal ion being in a slightly distorted tetrahedral geometry (Fig. 1). The scaffold of the inhibitor extends throughout the active site cavity, with the sulfonylurea functionality forming strong hydrogen bond interactions with two solvent molecules (Wat 214—N12 of the inhibitor molecule of 2.94 Å; Wat 214—N9 of the inhibitor, of 2.68 Å; and Wat 99—O11 of the inhibitor, of 2.76 Å). The terminal six-membered hydrophobic ring resides in a hydrophobic pocket formed by the residues Phe131, Val135, Leu198 and Pro202 at the rim of the active site of hCA II and forms a T-shaped π-stacking with the aromatic ring of Phe131 (Fig. 1). However, there is a clash between one amino acid residue and the inhibitor molecule, as the distance between the OH moiety of Thr200 and the carbon atom in *para* to the pyridine nitrogen atom in the heterocyclic ring of the sulfonamide **5**, is of only 2.96 Å (OG1 Thr200—C7 from **5**) (Fig. 2).

Thus, to better interpret these findings, we include in our analysis sulfonamide **ts-SA**,[7a] the tosylureido derivative of sulfanilamide (PDB file 1ZFK). From the superposition of the two structures from Figure 2, one may observe that the sulfanilamide-like fragments of the two inhibitors are not superimposable when the compounds are bound to the enzyme active site. The presence of the nitrogen atom in the ring of **5** leads to a tilting of the six-membered ring incorporating the sulfonamide ZBG compared to the corresponding fragment from **ts-SA**. This has as a consequence a totally different orientation of the two compounds when complexed to the enzyme, as neither their sulfonylureido nor their 4-tolyl fragments are superposable (Fig. 2). The tilt mentioned above leads to a shift of the scaffold of **ts-PySA** towards Thr200, which then leads to the clash with the OH moiety of this

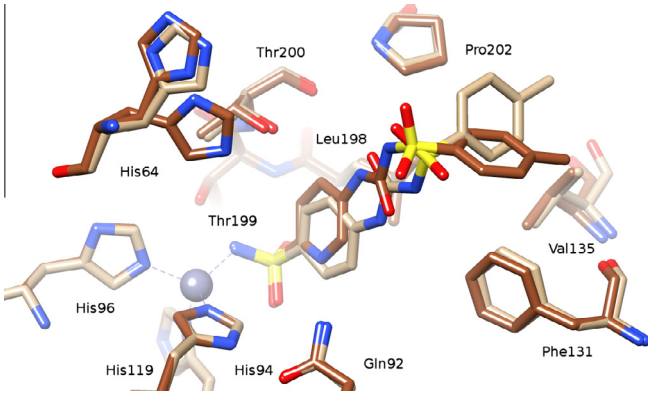


Figure 2. View of **ts-PySA 5** (brown) and **ts-SA** (gray, PDB file 1ZFK) superposed in the active site of hCA II. The two sulfonamides are represented as sticks, the Zn(II) ion is the grey sphere, its three ligands (His 94, 96 and 119) and residues in the binding of the inhibitors are also shown.

amino acid residue (a distance of 2.96 Å between the O atom of the amino acid and one carbon atom from the pyridine ring of the sulfonamide, as mentioned above). We have shown earlier for a sulfamide structurally related to topiramate (a clinically used antiepileptic/antiobesity agent) in complex with hCA II,[28] as well as for foscarnet, a phosphonate acting as weak CAI, in complex with hCA I,[29] that such clashes lead to a diminishing of the inhibitory power against the target isoform. In fact, this clash of the pyridine ring from compound **5** and Thr200 may explain the weaker CA inhibitory properties of **ts-PySA** compared to **ts-SA** (discussed above). On the other hand, the different orientation of the tail found in compound **5**, may explain its diverse selectivity profile compared to the structurally related inhibitor **ts-SA**. In fact the two compounds differ only by the substitution of a CH from **ts-SA** by a nitrogen atom in **ts-PySA**, but they differ significantly in their selectivity profiles for inhibition of the hCAs. In fact **ts-SA** (as discussed above) is a promiscuous, low nanomolar inhibitor of 7 CA isoforms, whereas **ts-PySA** inhibits only two isoforms in

the same range (hCA IX and XII, which are probably the most interesting drug targets in the human CA family of enzymes).[30] Thus, the ring was important in this case for orientating the inhibitor scaffold in a diverse manner compared to the classical benzenesulfonamide derivatives. The tail on the other hand led to an enhancement of the enzyme inhibitory activity compared to the parent compound **4**, and through its interactions at the rim of the active site, ensured a selectivity profile for compound **5**. This is thus a highly interesting example that combining the ring and the tail approach, interesting families of compounds can be obtained as inhibitors of various types of CAs. Work is in progress in our laboratories for obtaining a large series of such derivatives and to investigate in detail their properties.

3. Conclusion

5-(3-Tosylureido)pyridine-2-sulfonamide (**ts-PySA**) and 4-tosylureido-benzenesulfonamide (**ts-SA**) only differ by the substitution of a CH group from the benzene ring of the second compound by a nitrogen in the first one, but they have very different inhibitory properties against the many isoforms of CA found in humans, hCA I–XIV. By means of X-ray crystallography on the hCA II adducts of the two compounds these differences have been thoroughly understood and rationalized. As all sulfonamides, the two compounds bind in deprotonated form to the Zn(II) ion from the enzyme active site and their organic scaffolds extend throughout the cavity, participating in many interactions with amino acid residues and water molecules. However the pyridine derivative **ts-PySA** undergoes a tilt of the heterocyclic ring compared to the benzene analog, which leads to a very different orientation of the two compounds when bound within the enzyme active site. This tilt also leads to a clash between a carbon atom from the pyridine ring of **ts-PySA** and the OH moiety of Thr200, leading to less effective inhibitory properties of the pyridine versus the benzene sulfonamide derivative. Indeed, **ts-SA** is a promiscuous, low nanomolar inhibitor of 7 (hCA I, II, VII–XIV) out of 10 hCA isoforms, whereas the pyridine sulfonamide **ts-PySA** is a low nanomolar inhibitor only of the tumor-associated hCA IX and XII, being less effective against other 9 isoforms. Thus, a difference of one atom (N vs CH) in two isostructural sulfonamides leads to drastic differences of activity, phenomenon understood at the atomic level through the high resolution crystallographic structure and kinetic measurements reported in the paper. Combining the tail and the ring approaches in the same chemotype leads to isoform-selective, highly effective sulfonamide CA inhibitors.

4. Experimental protocols

4.1. Chemistry

Anhydrous solvents and all reagents were purchased from Sigma–Aldrich, Alfa Aesar and TCI. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes techniques to transfer solutions. NaH 60% in oil dispersion was washed with *n*-hexane until a homogeneous white solid was obtained, dried and stored under a nitrogen atmosphere prior to use. Infrared (IR) spectra were recorded as KBr plates and are expressed in ν (cm^{-1}). Nuclear magnetic resonance (^1H NMR, ^{13}C NMR, DEPT-135, DEPT-90, HSQC, HMBC) spectra were recorded using a Bruker Advance III 400 MHz spectrometer in $CDCl_3$, MeOH-d_4 or 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; br s, broad singlet; dd, double of doubles, appt, apparent triplet, appq, apparent quartet. The assignment of exchangeable protons (O*H* and N*H*) was confirmed by the addition of D_2O. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel F-254 plates. Flash chromatography purifications were performed on Merck Silica gel 60 (230–400 mesh ASTM) as the stationary phase and ethylacetate/*n*-hexane or MeOH/DCM were used as eluents. Melting points (mp) were carried out in open capillary tubes and are uncorrected.

4.1.1. 2-Thiol-5-acetaminopyridine (1)

2-Mercapto-5-nitropyridine (Bionet Research) (10 g, 64 mmol) was suspended in 100 mL of water and sodium hydrosulfite (39 g, 224 mmol) was added with stirring at 0 °C. Acetic anhydride (8.4 mL, 89.6 mmol) was then added. The mixture was stirred for 2 h, always kept in ice, and the yellow precipitate was filtered, washed with water, and dried to give **1** as a bright yellow solid (5.4 g, 50% yield). ^1H NMR (DMSO-d_6) δ: 2.01 (s, 3H); 7.22–7.36 (m, 2H); 8.22 (s, 1H); 10.07 (s, 1H).

4.1.2. 5-Acetamino-2-pyridinesulfonyl chloride (2)

The 2-thiol-5-acetaminopyridine **1** (5.16 g, 30.6 mmol) was added to 86 mL of H_2SO_4 concd and cooled to 0 °C. Sodium hypochlorite (13%, 146 mL, 306 mmol) was added dropwise. The reaction was stirred for 1 h at 0 °C. The resulting solution was diluted with water and ice and the precipitate was filtered under vacuum to obtain **2** as a white solid (4.97 g, 69% yield). ^1H NMR (DMSO-d_6) δ: 2.15 (s, 3H); 8.12 (d, J = 8.6 Hz, 1H); 8.46 (d, J = 8.6 Hz, 1H); 9.04 (s, 1H); 11.17 (s, 1H).

4.1.3. 5-Acetamino-2-pyridinesulfonamide (3)

To a cooled solution (0 °C) of **2** (3.5 g, 15 mmol) in acetonitrile (1.6 mL) aqueous ammonia (4.8 mL) was added dropwise. The reaction was stirred for 30 min at rt. The resulting solution was diluted with water and extracted with EtOAc. The aqueous phase was filtered to collect the precipitate, a white solid (1.06 g). The organic phase was evaporated, treated with acetone and filtered to collect **3** as a white solid (476 mg). The two solid fractions were reunited to give 1.53 g of the desired compound **3** (47% yield). ^1H NMR (DMSO-d_6) δ: 2.11 (s, 3H); 7.32 (s, 2H); 7.88 (d, J = 8.6 Hz, 1H); 8.23 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H); 8.80 (d, J = 2.3 Hz, 1H); 10.53 (s, 1H).

4.1.4. 5-Aminopyridine-2-sulfonamide (4)

Into a round-bottom flask equipped with a stir bar and N_2 were placed **3** (1.06 g, 4.9 mmol), H_2O (0.5 mL), and EtOH (11 mL). Concentrated HCl (4.4 mL) was added, and the mixture was refluxed for 3 h. The reaction mixture was cooled to rt, quenched with saturated $NaHCO_3$ solution and extracted with EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to afford **4** (583 mg, 68.5%) as a pale yellow solid. ^1H NMR (DMSO-d_6) δ: 6.05 (s, 2H); 6.96 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, 1H); 7.02 (s, 2H); 7.56 (d, J = 8.4 Hz, 1H); 7.93 (d, J = 2.2 Hz, 1H). Compound **4** was reported in the lit.[19]

4.1.5. 5-(3-Tosylureido)pyridine-2-sulfonamide 5

A suspension of 2-sulfamyl-5-aminopyridine **4** (0.2 g, 1.0 equiv) in dry acetonitrile (4 mL) was treated with 4-methylbenzenesulfonyl isocyanate (1.0 equiv) according to the general procedure previously reported.[7] The reaction was quenched with H_2O (1.0 mL) and the solvents were evaporated under vacuo to give a residue that was purified by silica gel column chromatography eluting with MeOH/DCM (10% v/v), to afford the titled compound **5** as a white solid.

4.1.6. 5-(3-Tosylureido)pyridine-2-sulfonamide 5

23% yield; mp >250 °C; silica gel TLC R_f 0.10 (MeOH/DCM 10% v/v); δ_H (400 MHz, DMSO-d_6) 2.36 (3H, s), 7.21 (2H, s, exchange with D_2O, SO_2NH_2), 7.23 (2H, d, J 8.4), 7.71 (3H, dd, J 6.8, 8.4), 8.06 (1H, dd, J 2.4, 9.0), 8.68 (1H, d, J 2.4), 9.09 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 21.8, 121.7, 124.4, 127.6, 129.2, 139.2, 140.6, 142.0, 144.2, 151.4, 158.7; Elemental analysis: calcd C 42.15, H 3.81, N 15.13, S 17.31; found: C 42.04, H 3.81, N 14.97, S 17.09; m/z (ESI negative) 369.08 $[M-H]^-$.

4.2. Co-crystallization and X-ray data collection

Crystals of hCA II complexed with **ts-PySA** (compound **5**) were obtained using the sitting drop vapor diffusion method as described earlier.[24–27] An equal volume of 0.8 mM solution of hCA II in Tris pH 8.0 and 0.8 mM of inhibitor in Hepes 20 mM pH 7.4 were mixed and incubated for 15 min. One microlitre drops of the complex solution were mixed with a solution 1.5 or 1.6 M sodium citrate, 50 mM Tris pH 8.0 and were equilibrated against the same solution at 296 K. Crystals of the complexes grew in a few days. The crystals were flash-frozen at 100 K using a solution obtained by adding 25% (v/v) glycerol to the mother liquor solution as cryoprotectant. A data set on a crystal of the complex hCA II-compound **5** was collected using synchrotron radiation at the XALOC beamline at ALBA (Barcelona, Spain) using a wavelength of 0.980 Å and a DECTRIS Pilatus 6M detector. Data were integrated and scaled using the program XDS.[31] Data processing statistics are showed in Table 2.

4.3. Structure determination

The crystal structure of hCA II (PDB accession code 3P58) without solvent molecules and other heteroatoms was used to obtain initial phases of the structure using Refmac5.[32] Five percentage of the unique reflections were selected randomly and excluded from the refinement data set for the purpose of R_{free} calculations. The initial $|F_0 - F_c|$ difference electron density maps unambiguously showed the inhibitor. Atomic model for the inhibitor was calculated and energy minimized using the program JLigand 1.0.39.[31] Refinement proceeded using normal protocols of positional, isotropic atomic displacement parameters alternating with manual building of the model using COOT.[33] Solvent molecules were introduced automatically using the program ARP.[34] Final rounds of refinement for the model included hydrogen at calculated positions and refined using a riding model. The quality of the final model was assessed with PROCHECK.[35] Crystal and refinement data are summarized in Table 2.

4.4. CA inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity.[23b] 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 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, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier,[4,8,9] and represent the mean from at least three different determinations. All CAs were recombinant proteins obtained as reported earlier by these groups.[8,8,25,26]

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