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Carbonic anhydrase inhibitors: X-ray crystal structure of a benzenesulfonamide strong CA II and CA IX inhibitor bearing a pentafluorophenylaminothioureido tail in complex with isozyme II

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Abstract—*N*-1-(4-Sulfamoylphenyl)-*N*-4-pentafluorophenyl-thiosemicarbazide was prepared by the reaction of 4-isothiocyanato-benzenesulfonamide with pentafluorophenyl hydrazine, and proved to be an effective inhibitor of several isozymes of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1), such as CA I, II, and IX. Against the physiologically relevant isozymes hCA II and hCA IX, the compound showed inhibition constants in the range of 15–19 nM, whereas it was less effective as a hCA I inhibitor (K_I of 78 nM). The high-resolution X-ray crystal structure of its adduct with hCA II showed the inhibitor to bind within the hydrophobic half of the enzyme active site, making extensive and strong van der Waals contacts with amino acid residues Gln92, Val121, Phe131, Leu198, Thr200, Pro202, in addition to the coordination of the sulfonamide nitrogen to the Zn(II) ion of the active site, and participation of the SO_2NH_2 group to a network of hydrogen bonds involving residues Thr199 and Glu106. These results are helpful for the design of better CA II or CA IX inhibitors based on the thioureido-benzenesulfonamide motif, with potential applications as anti-glaucoma or anti-cancer drugs.

1. Introduction

In previous contributions from these laboratories[1–8] we have investigated by means of X-ray crystallography the rational design of sulfonamide/sulfamate/sulfamide inhibitors of the metallo-enzyme carbonic anhydrase (CA, EC 4.2.1.1), which is involved in a multitude of physiological and pathological processes.[9,10] At least 14 different CA isozymes are presently known in humans,[9,10] and many of them are targets for the design of inhibitors with potential use as anti-glaucoma,[9,11] anti-obesity,[12,13] or anti-cancer drugs[14–16] among others.

Among the compounds previously investigated and characterized by means of X-ray crystallography in complex with the physiologically most important isozyme, that is, hCA II, are the topically acting anti-glaucoma sulfonamides **1**–**3**,[5,6,8] EMATE **4**,[3] a sulfamate also acting as steroid sulfatase inhibitor, which constituted the lead compound for the design of anti-tumor agents belonging to this class,[17] topiramate **5**,[1b] a widely used anti-epileptic, which is also the lead compound for the design of anti-obesity agents based on CA inhibitors (CAIs),[12,13] the anti-cancer sulfonamide in phase II clinical trials E7070 (indisulam) **6**,[2] the clinically used antipsychotic drug sulpiride **7**,[7] or the COX-2 'selective' inhibitor celecoxib **8**,[4] which is also a low nanomolar CAI, as recently shown by this group.[4,18] Resolving the high-resolution crystal structure of adducts of the derivatives **1**–**8** (or other such compounds)[1a] with hCA II allowed us a better understanding of the interaction of these enzymes with the sulfonamide/sulfamate inhibitors, and favored a more rational design of this type of pharmacological agents, with various applications for the treatment or prevention of serious diseases (such as glaucoma, obesity or cancer among others).[9–11]

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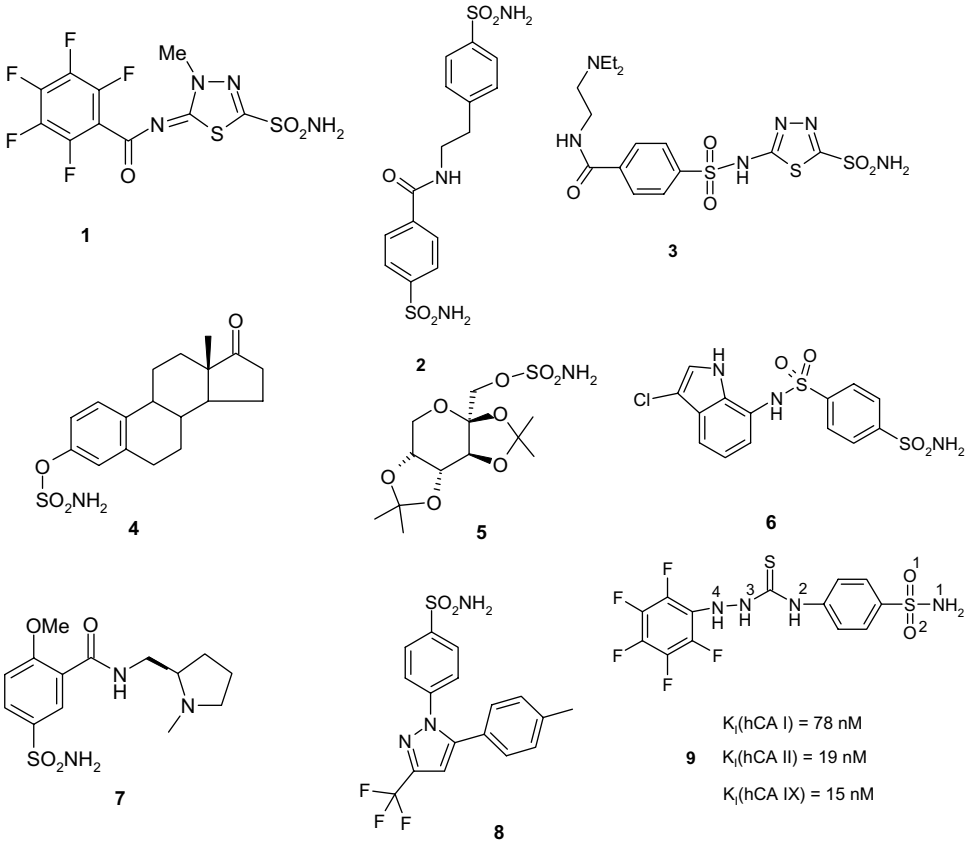

A class of derivatives which showed very promising applications among the various CAIs reported by our group in the last years, were the thioureas obtained from isothiocyanato sulfonamides (such as e.g., 4-isothiocyanatobenzenesulfonamide and its congeners) and amines, hydrazines or amino acids.[19–21] Such compounds generally showed potent inhibitory activity against the cytosolic isozyme hCA II as well as the transmembrane, tumor-associated isozyme hCA IX, being thus interesting candidates for developing anti-glaucoma/anti-tumor therapies based on them.[19–21] However, up to now no X-ray crystal structures of any such derivative in complex with a physiologically relevant isozyme (such as e.g., hCA II) is available. Here we report the first X-ray crystal structure of a thioureido-benzensulfonamide derivative (compound **9**) in complex with hCA II as well as its inhibitory properties against isozymes hCA I, II, and IX.

2. Chemistry and CA inhibition

Compound **9** (*N*-1-(4-sulfamoylphenyl)-*N*-4-pentafluorophenyl-thiosemicarbazide) has been prepared by the previously reported procedure,[19–21] by reacting 4-isothiocyanato-benzene-sulfonamide[19a] with the commercially available (Sigma–Aldrich) pentafluorophenylhydrazine. The new derivative has been tested for its interaction with three physiologically relevant CA isozymes, that is, the cytosolic isoforms hCA I and II, and the transmembrane, tumor-associated isozyme hCA IX (the catalytic domain of this multidomain protein[14a] has been employed in our experiments). Sulfonamide **9** proved to be an efficient hCA I inhibitor (with an inhibition constant of 78 nM) and a very good hCA II and hCA IX inhibitor, with K_I values of 19 and 15 nM, respectively. This is probably due to the rather high homology between the amino acid residues in the hCA II and hCA IX active sites, as previously shown by this group.[22] Based on such data, compound **9** is an attractive candidate to be studied in detail for its anti-tumor properties, since we have recently shown that sulfonamide CAIs interfere with tumor acidification produced by CA IX in hypoxic tumors.[15a]

3. Crystallography

To assess the molecular basis responsible for the inhibitory properties of **9** toward hCA II, we solved the crystal structure of the complex, which was prepared and crystallized as previously reported for other sulfonamide/sulfamate CA inhibitors.[1–8] This three-dimensional structure was analyzed by difference Fourier techniques, the crystals being isomorphous to those obtained for the native enzyme[23] and refined using the CNS program.[24] The statistics for data collection and refinement are summarized in Table 1.[25]

The structure refinement allowed us to evidence the spatial arrangement of the inhibitor within the enzyme active site (Fig. 1).

Table 1. Data collection and refinement statistics for the hCA II–**9** adduct

Data collection statistics (20.00–1.60 Å)	
Temperature (K)	100
Total reflections	108,382
Unique reflections	29,170
Completeness (%)	
Overall	95.8
Outermost data shell	92.6
R-sym[a]	
Overall	0.039
Outermost data shell	0.133
Mean *I*/sigma(*I*)	
Overall	28
Outermost data shell	9
Refinement statistics (20.00–1.60 Å)	
R-factor[b] (%)	18.9
R-free[b] (%)	21.6
rmsd from ideal geometry:	
Bond lengths (Å)	0.007
Bond angles (°)	1.90
Number of protein atoms	2070
Number of water molecules	201
Number of inhibitor atoms	26
Average *B*-factor (Å^2)	14.98

[a] $R\text{-sym} = \Sigma|I_i - \langle I\rangle|/\Sigma I_i$; over all reflections.
[b] $R\text{-factor} = \Sigma|F_o - F_c|/\Sigma F_o$; *R*-free calculated with 5% of data withheld from refinement.

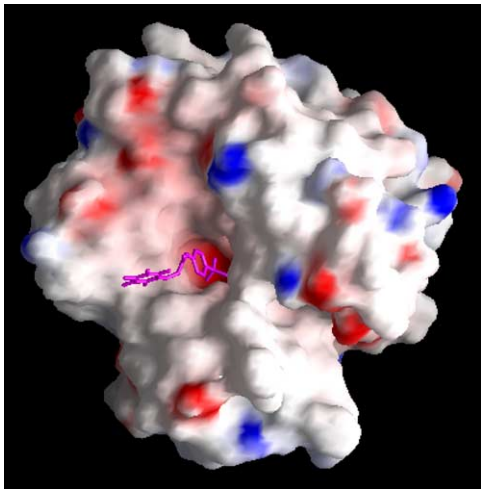

Figure 1. Electrostatic surface potential of hCA II in its complex with compound **9**. Polar atoms are colored in red (negatively charged) and blue (positively charged). The inhibitor molecule is shown in magenta.

Inspection of the electron density maps at various stages of the crystallographic refinement, showed features compatible with the presence of one inhibitor molecule bound to the active site, as clearly illustrated in Figure 2. These maps were well defined for the benzenesulfonamide-thioureido moiety of the inhibitor **9**. On the contrary, a rather poor definition was observed for the pentafluorophenylamino functionality, suggesting a greater flexibility of this group within the hCA II active site, a situation which has been observed earlier for the adduct of hCA II with indisulam **6**.[2] The binding of **9** to the enzyme active site did not significantly perturb the enzyme structure, even in close proximity of the ligand. As a matter of fact, the r.m.s. deviation, calculated over the entire Cα atoms of hCAII-**9** complex with respect to the unbound enzyme, was of 0.48 Å. Interactions between the protein and Zn(II) ion were entirely preserved in the adduct.

The main protein–inhibitor interactions are schematically depicted in Figure 3. According to this figure, **9** presents a spatial arrangement similar to that observed in other hCA II-sulfonamide/sulfamate complexes belonging to the benzensulfonamide type, such as **2**, **6**, **7**, and **8** among others, whose X-ray structure has been solved.[1–8] In particular, the N1 atom of the sulfonamide moiety replaces the hydroxyl ion/water molecule coordinated to Zn(II) in the native enzyme, with a Zn–N distance of 1.97 Å. The Zn(II) ion remained in a stable tetrahedral geometry, being also coordinated by the imidazolic nitrogens of His94, His96, and His119. The N1 atom of **9** is also hydrogen bonded to the hydroxyl group of Thr199 (ThrOG–N = 2.75 Å), which in turn interacts with the Glu106OE1 atom (2.46 Å). Furthermore, the inhibitor O1 atom is hydrogen bonded to the backbone amide of Thr199 (ThrN–O1 = 2.80 Å), whereas the O2 atom is at a distance of 2.97 Å from the catalytic Zn(II) ion. On the other hand, the phenylthioureido moiety of **9** is oriented toward the hydrophobic part of the active site cleft (Fig. 3), similarly with the orientation of the bis-sulfonamide **2** previously investigated (Fig. 4),[6] establishing a large number of strong van der Waals interactions (<4.5 Å) with residues Gln92, Val121, Phe131, Leu198, Thr200, Pro202, respectively. Some of these residues were already shown previously to be important for their interaction with benzenesulfonamide CAIs.[2–8] Finally, the pentafluorophenylamino moiety of **9** interacts poorly with the enzyme and is rather disordered. It is interesting to note that although the organic scaffold of compound **9** does not establish significant polar interactions with the enzyme (e.g., no hydrogen bonds were evidenced between the C=S group or the nitrogen atoms belonging to the thiosemicarbazide moiety of the inhibitor and amino acid residues of the active site), as otherwise observed for some other hCA II-sulfonamide/sulfamate complexes,[1–4,6] the large number of hydrophobic contacts mentioned above between the inhibitor and the enzyme can account for the good inhibitory properties of this molecule, which are of the same magnitude as those of clinically used CAIs[9,10] or of the compound in clinical trials as anti-tumor agent, indisulam **6**.[2]

4. Conclusions

We report here the first X-ray crystal structure of a CAI belonging to the thioureido-benzenesulfonamide type of compounds, previously shown to possess potent hCA II and hCA IX inhibitory properties, and in consequence putative applications in the design of anti-glaucoma or anti-tumor therapies. *N*-1-(4-Sulfamoylphenyl)-*N*-4-pentafluorophenyl-thiosemicarbazide was prepared by reacting 4-isothiocyanato-benzenesulfonamide with pentafluorophenyl hydrazine, and proved to be an effective hCA I inhibitor, and a very good hCA II and hCA IX

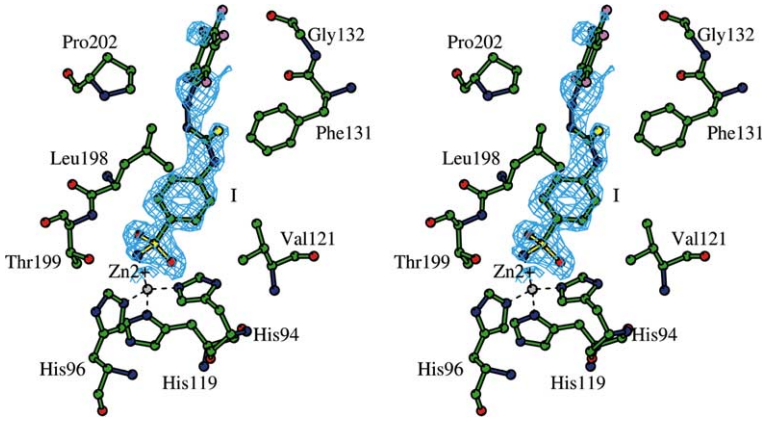

Figure 2. Stereo view of the active site region in the hCA II-**9** complex. The inhibitor (labeled I) is shown associated with simulated annealing omit $|2F_o - F_c|$ electron density map,[24] computed at 1.60 Å and contoured at 1.0σ.

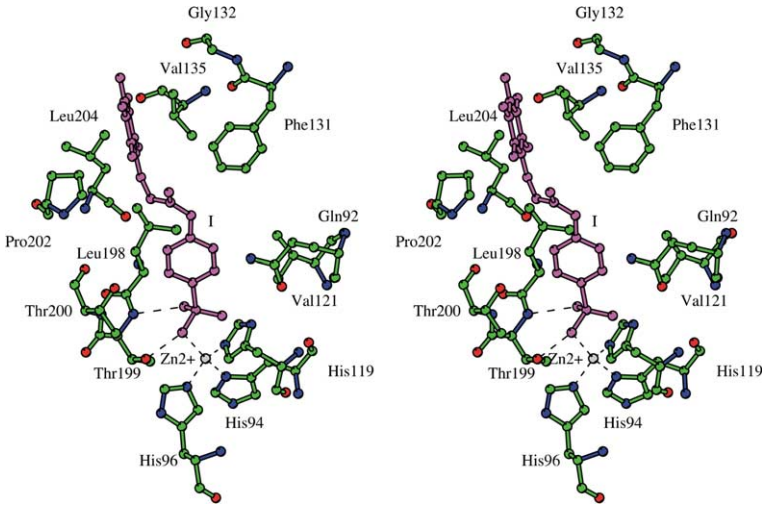

Figure 3. Stereo view of the active site region in the hCA II-**9** complex showing the residues participating in recognition of the inhibitor molecule, reported in magenta. Hydrogen bonds and the active site Zn(II) ion coordination are also shown (dotted lines).

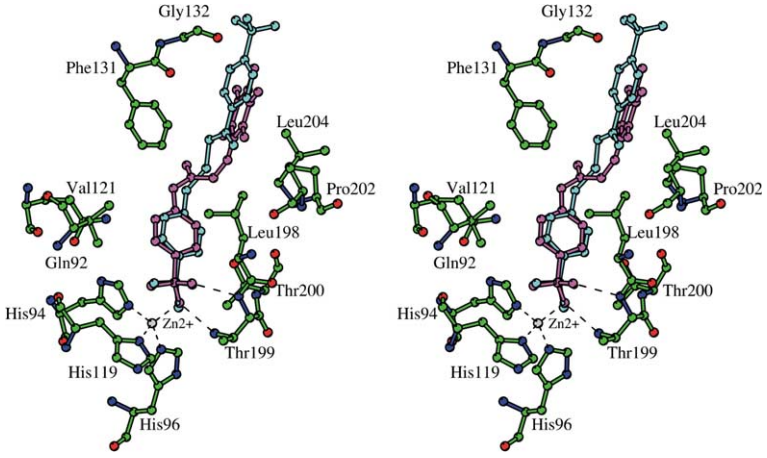

Figure 4. Stereo view of the superimposition of hCAII-inhibitor adducts: bis-sulfonamide **2** is reported in cyan while **9** is reported in magenta. Amino acid residues important for binding of these inhibitors, the Zn(II) ion and its three protein ligands are also shown.

inhibitor (inhibition constants in the range of 15–19 nM). The high-resolution X-ray crystal structure of its adduct with hCA II showed the inhibitor to bind within the hydrophobic half of the active site, making extensive and strong van der Waals contacts with amino acid residues Gln92, Val121, Phe131, Leu198, Thr200,

Pro202, in addition to the coordination of the sulfonamide nitrogen to the Zn(II) of the active site, and participation of the SO_2NH_2 group in a network of hydrogen bonds involving residues Thr199 and Glu106. These results are helpful for the design of better CA II or CA IX inhibitors based on the thioureido-benzenesulfonamide motif.

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25. Crystals of the hCA II-**9** complex were obtained by co-crystallization as previously described.[2–8] Crystals were isomorphous to those of the native enzyme belonging to the space group $P2_1$ with unit cell parameters of $a = 41.40$ Å, $b = 40.48$ Å, $c = 70.77$ Å and $\beta = 104.20°$. The X-ray data collection was performed at the ELETTRA Synchrotron (Trieste, Italy), using a Mar CCD detector. The crystal diffracted up to 1.60 Å resolution. Diffracted intensities were processed and scaled with the HKL package (Denzo/Scalepack).[26] The structure of hCAII-**9** complex was analyzed by difference Fourier techniques, using the PDB file 1CA2[23] as a starting model for refinement. Water molecules were removed from the starting model prior to structure factor and phase calculations. The crystallographic *R*-factor and *R*-free, calculated in the 20.00–1.60 Å resolution range, based on the starting model coordinates, were 0.343 and 0.366, respectively. The inspection of electron density maps in correspondence of the active site region clearly indicated the presence of an inhibitor molecule, which was easily built and introduced into the atomic coordinates set. The refinement, carried out with the program CNS,[24] proceeded to convergence by several runs of positional and *B*-factor refinement alternated with manual building using the O program.[27] Water molecules were then added to the model through the automatic protocol of CNS together with the contribution of the disordered solvent. The final crystallographic *R*-factor and *R*-free values calculated for

the 29,170 observed reflection were 0.189 and 0.216, respectively. The refined model included 2096 complex atoms, 26 atoms belonging to the inhibitor and 201 water molecules. The r.m.s. deviations from ideal value of bond lengths and bond angles[28] were 0.007 Å and 1.90°, respectively. The average temperature factor (*B*) for all atoms was 14.98 $Å^2$. The correctness of stereochemistry was finally checked using PROCHECK.[29].

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