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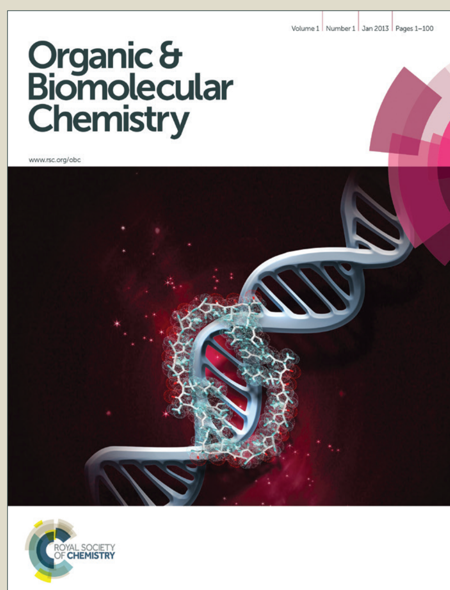

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X-ray crystallographic and kinetic investigations of 6-sulfamoyl-saccharin as a carbonic anhydrase inhibitor

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6-Sulfamoyl-saccharin was investigated as an inhibitor of 11 α-carbonic anhydrase (CA, EC 4.2.1.1) isoforms of human (h) origin, hCA I-XIV, and X-ray crystallographic data were obtained for its adduct with hCA II, the physiologically dominant isoform. This compound possesses two potential zinc-binding groups, the primary sulfamoyl one and the secondary, acylatedsulfonamide. Saccharin itself binds to the Zn(II) ion from the CA active site coordinating with this last group, in deprotonated (SO_2N^-CO) form. Here we explain why 6-sulfamoyl-saccharin, unlike saccharin, binds to the metal ion from the hCA II active site by its primary sulfonamide moiety and not the secondary one as saccharin itself. Our study is useful for shedding new light to the structure-based drug design of isoform-selective CA inhibitors of the sulfonamide type.

Introduction

The artificial sweetener saccharin (**SAC**, compound **1**)[1] (Figure 1), which incorporates a cyclic acylatedsulfonamide moiety (-SO_2NHCO-),[2] was reported by our group[1,3] to act as an inhibitor of the metalloenzyme Carbonic Anhydrase (CA, EC 4.2.1.1).[4-8] Furthermore, its mechanism of inhibition was demonstrated to be rather different from that of primary sulfonamides, the most investigated class of clinically used CA inhibitors (CAIs).[9-18] Indeed, even if **SAC**, like primary sulfonamides,[17-23] binds in deprotonated state to the metal ion of the CA active site, coordinating it through the nitrogen atom from the acylatedsulfamoyl moiety,[1] the presence of the CO group close to the metal ion and the lack of hydrogen atoms on the nitrogen, lead to rather different interactions of the acylated-sulfonamide and primary sulfonamides with enzyme active site.[19-25] These different interactions are also reflected in a diverse inhibition profile of **SAC** when compared to the primary sulfonamides such as for example acetazolamide (**AZM**, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide). In fact, **AZM** is a promiscuous highly efficient inhibitor of many of the 15 different human (h) CA isoforms (hCAs), whereas **SAC** is an efficient inhibitor only of the cytosolic isoform hCA VII and of the tumor-associated one hCA IX.[1,3,26-30] For this reason, **SAC** was frequently used in the last period for designing isoform-selective CAIs targeting the tumor-associated isoform CA IX.[2] Due to the fact there are six genetic families of CAs, the α-, β-, γ-, δ-, ζ- and η-classes,[31-36] as well as numerous isoforms of them in most organisms investigated so far,[4-8] the main challenge in designing CAIs is that of finding isoform-selective compounds.[9-19]

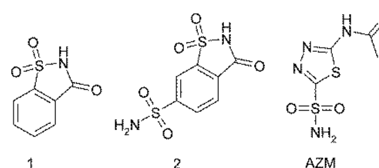

Figure 1 Chemical structures of compounds **1** (**SAC**), **2** and **AZM**.

Human CAs belong to the α-class and exist in 15 different isoforms.[4-8] Some of them are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), others are membrane bound (CA IV, CA IX, CA XII and CA XIV), two are mitochondrial (CA VA, CA VB) and one is secreted in saliva and milk (CA VI). Moreover, three of these proteins are acatalytic (CA VIII, X and XI).[4-8] Many of these hCAs are therapeutic targets with the potential to be inhibited or activated, which elicits pharmacologic effects,[4-8,26-28] and this explains the

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dynamic research in the field, which may lead to pharmacological agents with applications as diuretic, antiglaucoma, antiobesity, antiepileptic and antitumor drugs.[9-28] Recently a sulfonamide CAI developed by this group entered Phase I clinical trials for the management of metastatic solid tumors.[37]

X-ray crystallography is a very useful tool for understanding the detailed interactions between CAIs and the different CA isoforms,[19-25] and also for designing inhibitors with enhanced selectivity or efficacy for inhibiting various CA isoforms.[38-48] In this context, we investigate here a primary sulfonamide containing a saccharin moiety (6-sulfonamido-1,2-benz-isothiazole-3-one-1,1-dioxide) (compound **2**)[49] by means of kinetic and X-ray crystallographic studies, in order to understand which are the consequences of the concomitant presence of two different ZBGs in the same compound for its binding to the enzyme as well as for the selectivity/inhibition profile against the catalytically active hCA isoforms. The comparison of compound **2** with **SAC1** is also reported.

Results and Discussion

Chemistry and carbonic anhydrase inhibition

Synthesis of compound **2** was reported earlier by one of our groups.[49] This compound was assayed as inhibitor of all physiologically important CA isoforms, i.e., hCA I-XIV, by a stopped-flow CO_2 hydrase assay method (Table 1). For comparison, data on compounds **1**[1]and **AZM** were also included in Table 1.

Data of Table 1 show that unlike **SAC 1**, which is an efficient inhibitor only of two isoforms, hCA VII (K_I of 10 nM) and hCA IX (K_I of 103 nM),sulfonamide**2** inhibits efficiently 7 out of 11 CA isoforms. Indeed, **2** is low nanomolar hCA VII and XIV inhibitor (K_Is of 6.7 – 10 nM), but also efficiently inhibits hCA II, hCA VI, hCA IX, hCA XII and hCA XIII, with K_Is ranging between 64.8 and 113 nM. Compound **2** acts as a weak hCA IV and VA inhibitor (K_Is of 271-405 nM), a very weak hCA I (K_I of 3406 nM) inhibitor and is highly inefficient against hCA III (Table 1). However, it should be also observed that the inhibition profile of **2** is very different from that of **AZM** which indiscriminately inhibits very well 9 out of 11 CA isoforms, with K_Is < 74 nM (Table 1). Thus, sulfonamide**2** maintains some selectivity typical of the saccharin type of derivatives, but the affinity for many CA isoforms is highly increased compared to **SAC 1**.

X-ray crystallography

To provide structural information on the binding mode of the saccharin derivative **2** to hCAs, the crystal structure of this molecule in complex with the cytosolic dominant isoform hCA II has been solved. Crystals of the hCA II/**2** adduct were isomorphous with those of the native protein, allowing for the determination of the crystallographic structure by difference Fourier techniques. Data collection and refinement statistics are summarized in Table 2.

Table 1 Inhibition data of the catalytically active isoforms hCA I-XIV with compounds **1, 2** and **AZM** as standard, by a stopped-flow CO_2 hydrase assay.[51]

	K_I (nM)*		
Isoform/Compound	**1****	**2**	**AZM**
hCA I	18540	3406	250
hCA II	5950	77.3	12
hCA III	$> 10^6$	7×10^4	2×10^5
hCA IV	7920	271	74
hCA VA	10060	405	63
hCA VI	935	70	11
hCA VII	10	6.7	2.5
hCA IX	103	113	25
hCA XII	633	64.8	5.7
hCA XIII	12100	82	17
hCA XIV	773	10	41

* Errors within the range of ± 5-10 % of the reported values, from 3 different assays (data not shown). **From ref.[1]

The analysis of the electron density maps showed the presence of one inhibitor molecule within the enzyme active site (Figure 2). Inhibitor binding did not generate significant changes in the hCA II active site nor in the overall structure; indeed, the rmsd for the superposition of the corresponding Cα atoms between the native enzyme and the enzyme-inhibitor adduct was only 0.4 Å. Even if compound **2** has two potential ZBGs, electron density maps clearly indicate that the binding occurs only by means of the primary sulfonamide. Classical interactions usually occurring between primary sulfonamides and hCA II active site are present also in this case (see Figure 3A for details).[19] The saccharin moiety further stabilizes the binding, filling the active site channel and establishing numerous van der Waals interactions (distance < 4 Å) with the side chains of Val121, Phe131, Leu141, Leu 198, and Thr200. A direct hydrogen bond interaction between one of the secondary

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sulfonamide oxygens and Gln92NE2 atom is also present (Figure 3A).

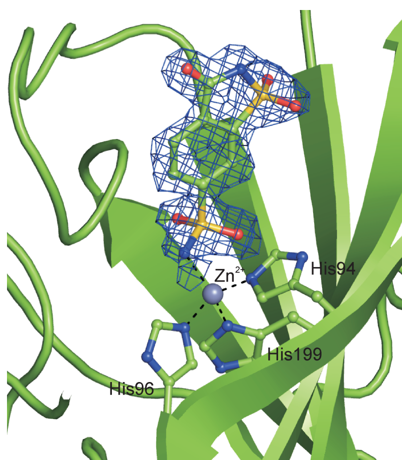

Figure 2 σA-weighted |2Fo-Fc| simulated-annealing OMIT map (contoured at 1.0 σ), relative to inhibitor molecule **2**. The catalytic zinc ion and its coordinating histidines are also shown.The enzyme is represented as ribbon diagram.

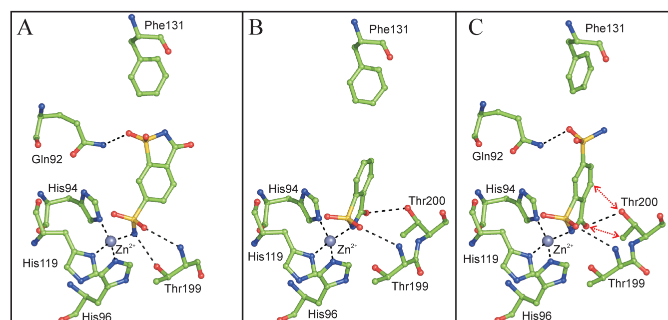

Figure 3 Schematic representation of the binding mode of inhibitors **2** (**A**) and **1** (**B**) (PDB codes 4XEI and 2Q1B)[1] to the hCA II active site cavity as obtained by x-ray crystallography. (**C**) Binding mode of inhibitor **2** to the hCA II active site cavity as obtained by a molecular modeling approach where the coordination of the secondary sulfonamide group to the catalytic zinc ion has been forced. The two red arrows indicate short distances between the inhibitor and Thr200 residue.

Interestingly, even if compounds **1** and **2** present a very similar chemical structure (Figure 1) their binding mode to hCA II active site is completely different. Indeed, while **1** coordinates to the zinc ion through the nitrogen atom of the secondary sulfonamide moiety (the only sulfonamide present in this molecule) (Figure 3B), **2** prefers to utilize the primary sulfonamide for the coordination. This is a quite surprising finding, considering that the secondary sulfonamide group of **2** has a lowerpKa value (2.8) with respect to that of the primary sulfonamide (9.7). Indeed, it should be reasonable to expect that the Zn^{2+} ion would be coordinated more favorably by the most acidic group. Thus, to understand the structural reasons of such behavior, we simulated the binding of compound **2** to the Zn^{2+} catalytic ion through the secondary sulfonamide nitrogen. In particular, the saccharin ring of **2** was structurally superimposed to the saccharin ring of **1,** when bound to the hCA II active site (PDB code 2Q1B), and the resulting adduct was subjected to energy minimization as described in the material and method section. Analysis of the obtained model, shown in Figure 3C, can plausibly explain why the coordination through the primary sulfonamide is preferred. Indeed, when compound **2** coordinates the zinc ion through the secondary sulfonamide nitrogen (Figure 3C), in order to avoid a clash of the primary sulfonamide with Phe131, the saccharin ring is forced to move with respect to its position in the hCA II/**1** adduct (Figure 3B). Moreover, also the Phe131 side chain conformation has to change. The conformation of this residue is conserved in many x-ray structures of hCA II/inhibitor adducts, and its movement may be energetically unfavorable, making thus less convenient the metal coordination through the secondary sulfonamide nitrogen. Finally, even if the minimized model of hCA II/**2** complex (Figure 3C) contains the same number of polar interactions of the corresponding x-ray structure (Figure 3A), two too short distances with Thr200 further contribute to destabilize the binding pattern through the cyclic sulfonamide moiety. Thus, altogether these data indicate that a movement of Phe131 side chain together with clashes with Thr200 could be the structural reasons that drive coordination of the zinc ion through the primary sulfonamide nitrogen.

The 1,2-benz-isothiazole-3-one-1,1-dioxide fragment of **2** was observed bound within the middle of the CA II active site cavity, interacting with amino acid residues in positions 92 and 131 (Fig. 3A), which are variable among the diverse CA isoforms.[7,9,19] This may also explain the inhibition pattern observed for this compound (Table 1), compared to those of **SAC 1** or **AZM**. In fact the last two derivatives are more compact compared to **2**, and bind at the bottom of the active site cavity, being thus unable to interact with the residues which differ among the various isoforms, unlike **2** which makes the contacts discussed above.

By looking at the crystallographic structure of the hCA II/**1** adduct, it is also possible to hypothesize that a derivatization of the saccharin ring in position 4 or 5, that would avoid the clash with Phe131, could still allow the binding through the secondary sulfonamide nitrogen, but this hypothesis should be verified experimentally.

Experimental

Chemistry and CA activity measurements and inhibition studies

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Synthesis of compound **2** was reported earlier.[49] An SLX-Applied Photophysics stopped-flow instrument has been used for measuring the catalytic activity and inhibition with a CO_2 hydration assay method.[50] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mMHepes (pH 7.4) and 20 mM $NaClO_4$ (for maintaining constant the ionic strength). The initial rates of the CA-catalyzed CO_2 hydration reaction were followed for a period of 10-100 s. The concentrations of substrate (CO_2) ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants, with at least six traces of the initial 5-10% of the reaction being used for determining the initial velocity, for each inhibitor. The uncatalyzed rates were determined and subtracted from the total observed rates. Stock solutions of inhibitors (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done with the assay buffer. Enzyme and inhibitor solutions were preincubated prior to assay for 15 min (at room temperature), 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 and the Cheng-Prusoff equation as reported earlier by our groups.[51-53] The CAs were recombinant proteins obtained in-house as reported earlier.[51-53]The concentrations of enzyme used in the assay ranged between 9.0 nM and 13.6 nM.

X-ray crystallography

Crystals of the hCA II/**2** complex were obtained by soaking enzyme crystals(monoclinic crystal form, space group $P2_1$) in a solution containing 20 mM inhibitor in the crystallization buffer (2.6 M $(NH_4)_2SO_4$, 0.3 MNaCl, 100 mM Tris-HCl (pH 8.2) and 5 mM 4-hydroxymercurybenzoate). After 1 hour crystals were flash cryo-cooled in the reservoir solution with the addition of 15% (v/v) glycerol as cryoprotector. A complete data set was collected using a copper rotating-anode generator developed by Rigaku equipped with a Rigaku Saturn CCD detector. Data were integrated and scaled using HKL2000.[54] Details of data collection statistics are reported in Table 2.

The structure of hCA II/**2** adduct has been analyzed by difference Fourier techniques, using the PDB file 1CA2[44] as starting model for refinement after deletion of non-protein atoms. Structure refinement (in the 20.0–1.8 Å resolution range) was carried out with the program CNS[55] and model building was performed with O.[56] In particular, an initial round of rigid body refinement was followed by simulated annealing and isotropic thermal factor (B-factor) refinement. The inspection of electron density maps in correspondence of the active site region after this single round of refinement revealed the presence of an inhibitor molecule in the active site of hCA II, which was easily built into the model. Restraints on inhibitor bond angles and distances were taken from similar structures in the Cambridge Structural Database[57] whereas standard restraints were used on protein bond angles and distances throughout refinement. Several alternating cycles of energy minimization, individual temperature factor refinement and manual model building gave the final models with R_{factor}/R_{free} values of 16.8/21.4. The correctness of stereochemistry was finally checked using PROCHECK.[58] Final refinement statistics are presented in Table 2. The atomic coordinates of hCA II/**2** complex were deposited in the Protein Data Bank, accession code 4XE1.

Crystal parameters	
Space group	*$P2_1$*
a (Å)	*42.2*
b (Å)	*41.7*
c (Å)	*71.7*
γ (°)	*104.3*
Number of independent molecules	*1*
Data collection statistics	
Resolution (Å)	*20-1.8*
Wavelength (Å)	*1.54178*
Temperature (K)	*100*
R_{merge} (%)[a]	*4.7 (10.1)*
Mean I/σ(I)	*21.2 (7.4)*
Total reflections	*84675*
Unique reflections	*22414*
Redundancy (%)	*3.8 (2.1)*
Completeness (%)	*99.1 (92.9)*
Refinement statistics	
R_{factor} (%)[b]	*16.8*
R_{free} (%)[b]	*21.4*
RMSD from ideal geometry:	
Bond lengths (Å)	*0.010*
Bond angles (°)	*1.6*
Number of protein atoms	*2069*
Number of water molecules	*219*
Number of inhibitor atoms	*16*
Average B factor ($Å^2$):	
All atoms	*14.2*
Protein atoms	*13.3*
Inhibitor atoms	*10.2*
Water molecules	*22.8*
Ramachandran plot	
Residues in most favored regions (%)	*87.6*
Residues in additional allowed regions (%)	*12.0*
Residues in generously allowed regions (%)	*0.5*

Table 2 Crystal parameters, data collection and refinement statistics.

[a] $R_{merge} = \Sigma_{hkl}\Sigma_i |I(hkl) - \langle I(hkl)\rangle| / \Sigma_{hkl}\Sigma_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an observation and $\langle I(hkl)\rangle$ is the mean value for itsunique reflection; summations are over all reflections.

[b] $R_{factor} = \Sigma_h ||F(h)| - |Fc(h)|| / \Sigma_h |Fo(h)|$, where Fo and Fc are the observedand calculated structure-factor amplitudes, respectively. R_{free} is calculatedin same manner as R_{factor}, except that it uses 5% of the data omitted fromrefinement.

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pKA calculation and molecular modeling

pKavalues were calculated at 298 K using the ChemAxon prediction method, by means of the MarvinSketch 14.11.24.0 graphical user interface (version 14.11.24.0).[59]

Athree-dimensional model for the hCA II/**2** complex with the inhibitor coordinating the zinc ion through the secondary sulfonamide nitrogen was generated by using the crystallographic structure of the hCA II/**1** complex as template and by superimposing the saccharin ring of compound **2** to that of compound **1**.[1] Several cycles of constrained energy minimization by using the Insight/Discover program package were performed. The CVVF force field and 10000 steps of conjugate gradient minimization were used. During minimization all protein atoms were kept fixed except those of residues delimiting the active site cavity.

Conclusions

Although a large number of CA inhibition mechanisms were described ultimately, some of which do not contemplate the participation of the metal ion in the interaction with the bound inhibitor, coordination of the deprotonated inhibitor molecule to the metal ion remains one of the most general processes through which low nanomolar, clinically relevant inhibitors can be obtained. Indeed, the primary sulfonamides, sulfamates and sulfamides, as well as some carboxylates and hydroxamates possess this mechanism of CA inhibition. However one of the aspects which has been poorly addressed until now was how the enzyme selects one of more potential ZBGs present in the same inhibitor molecule. In an earlier work from this group[60] we showed that a primary bis-sulfonamide possessing two slightly different sulfamoyl moieties (in the compound $4\text{-}H_2NO_2S\text{-}C_6H_4\text{-}CONHCH_2CH_2\text{-}C_6H_4\text{-}SO_2NH_2$ (*para*)) coordinated to the Zn(II) from the CA II active site forming only one adduct (not two), in which the deprotonated sulfamoyl moiety from the 4-aminoethylbenzenesulfonamide fragment of the inhibitor was coordinated to the metal ion. However we were unable to provide an explanation for this selection of just one of two structurally very similar ZBGs by the enzyme, and formation of one, not two enzyme-inhibitor adducts. The actual contribution considers two rather diverse but structurally related ZBGs: the primary versus secondary cyclic sulfonamides as fragments for obtaining isoform-selective, potent CAIs. In fact saccharin **1** binds Zn(II) through the SO_2N^-CO present also in its sulfamoylated derivative **2**, which additionally also contains a primary sulfamoyl group in position 6 of the phenyl ring. The enzyme discriminated again for the two diverse ZBGs present in **2**, and only the adduct in which the primary, deprotonated sulfonamide coordinates to Zn(II) has been formed, as for the bis-sulfonamide mentioned and investigated earlier.[60] In this case, by using a combination of X-ray crystallography and molecular modeling, we were able to explain why this discrimination occurs: binding of 2 through its secondary cyclic sulfonamide moiety would lead to clashes of the primary sulfamoyl moiety with Phe131, an amino acid residue crucial for the binding of inhibitors to the CA II active site, as well as with Thr200, an amino acid situated nearby Thr199, one of the two gate-keeper amino acid residues of this isoform. Thus, although the cyclic secondary sulfonamide from **2** is much more acidic than the primary sulfonamide group of this compound, which should favor coordination by the first moiety, the energy loss due to clashes with the two amino acids mentioned above are probably the governing factors leading to the net preference for coordination to the metal ion through the SO_2NH^- moiety and not the SO_2N^-CO one. The different inhibition mechanism of saccharin and its sulfamoylated derivative **2**, also lead to net differences of affinity for the various human CA isoforms (Table 1) which is relevant for obtaining isoform-selective inhibitors. Indeed, **2** is a low nM inhibitor of two out of 11 isoforms, and it has K_I values between 10 and 113 nM against other 5 isoforms, having thus a very different profile compared to acetazolamide **AZM** or saccharin itself. Our finding may be thus used for the rational, structure-based drug design of CAIs with diverse inhibition profiles.

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Notes and references

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