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Carbonic anhydrase inhibitors: The X-ray crystal structure of the adduct of *N*-hydroxysulfamide with isozyme II explains why this new zinc binding function is effective in the design of potent inhibitors☆

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Abstract—*N*-Hydroxysulfamide is a 2000-fold more potent inhibitor of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) as compared to sulfamide. It also inhibits other physiologically relevant isoforms, such as the tumor-associated CA IX and XII (K_Is in the range of 0.865–1.34 μM). In order to understand the binding of this inhibitor to the enzyme active site, the X-ray crystal structure of the human hCA II–*N*-hydroxysulfamide adduct was resolved. The inhibitor coordinates to the active site zinc ion by the ionized primary amino group, participating in an extended network of hydrogen bonds with amino acid residues Thr199, Thr200 and two water molecules. The additional two hydrogen bonds in which *N*-hydroxysulfamide bound to hCA II is involved as compared to the corresponding adduct of sulfamide may explain its higher affinity for the enzyme, also providing hints for the design of tight-binding CA inhibitors possessing an organic moiety substituting the NH group in the *N*-hydroxysulfamide structure.

In the last years, all 12 catalytically active human isoforms of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1)[1,2] have been investigated in detail for their inhibition profile against the most important classes of inhibitors, the sulfonamides, sulfamates, and sulfamides.[3–10] Some of these agents have clinical applications for the treatment or prevention of several diseases, such as glaucoma, cancer, obesity, epilepsy, and some other neurological disorders.[1–5] Screening various classes of new inhibitors against many diverse isoforms is critically important in the search of isozyme-selective compounds. Such putative candidate drugs would possess reduced side effects as compared to the presently available derivatives, most of which indiscriminately inhibit many CA isoforms.[1–5] Indeed, physiologically relevant isozymes, such as hCA I, II, IV, VA, VB, VII, IX, XII, XIII, and XIV, have been investigated in detail for their inhibition with many types of sulfonamides as well as some sulfamates and sulfamides. Several drug design studies have also been reported in which compounds with selectivity for one or other such isozymes were obtained.[5–13] Furthermore, many novel representatives of CAs belonging to other gene families were isolated and characterized in organisms such as bacteria (e.g., *Helicobacter pylori*, *Neisseria gonorrhoeae*, *Escherichia coli*, *Mycobacterium tuberculosis*),[14–16] fungi (*Candida albicans*, *Cryptococcus neoformans*),[17,18] or in the protozoa causing malaria (*Plasmodium falciparum*).[19] Several inhibition studies of these enzymes are also available.[20,21] Inhibitors of such parasite enzymes may have important therapeutic applications for treating infections caused by these agents (some of which developed high level resistance to the classical antibiotics/antifungal agents),[22] in case that potent and selective inhibitors for the pathogenic over the host enzymes could be detected/designed.[23]

Keywords: Carbonic anhydrase; *N*-Hydroxysulfamide; Zinc binding group; Enzyme inhibitor; X-ray crystallography.

☆ The coordinates of the hCA II–*N*-hydroxysulfamide adduct have been deposited in PDB, ID code 2O4Z.

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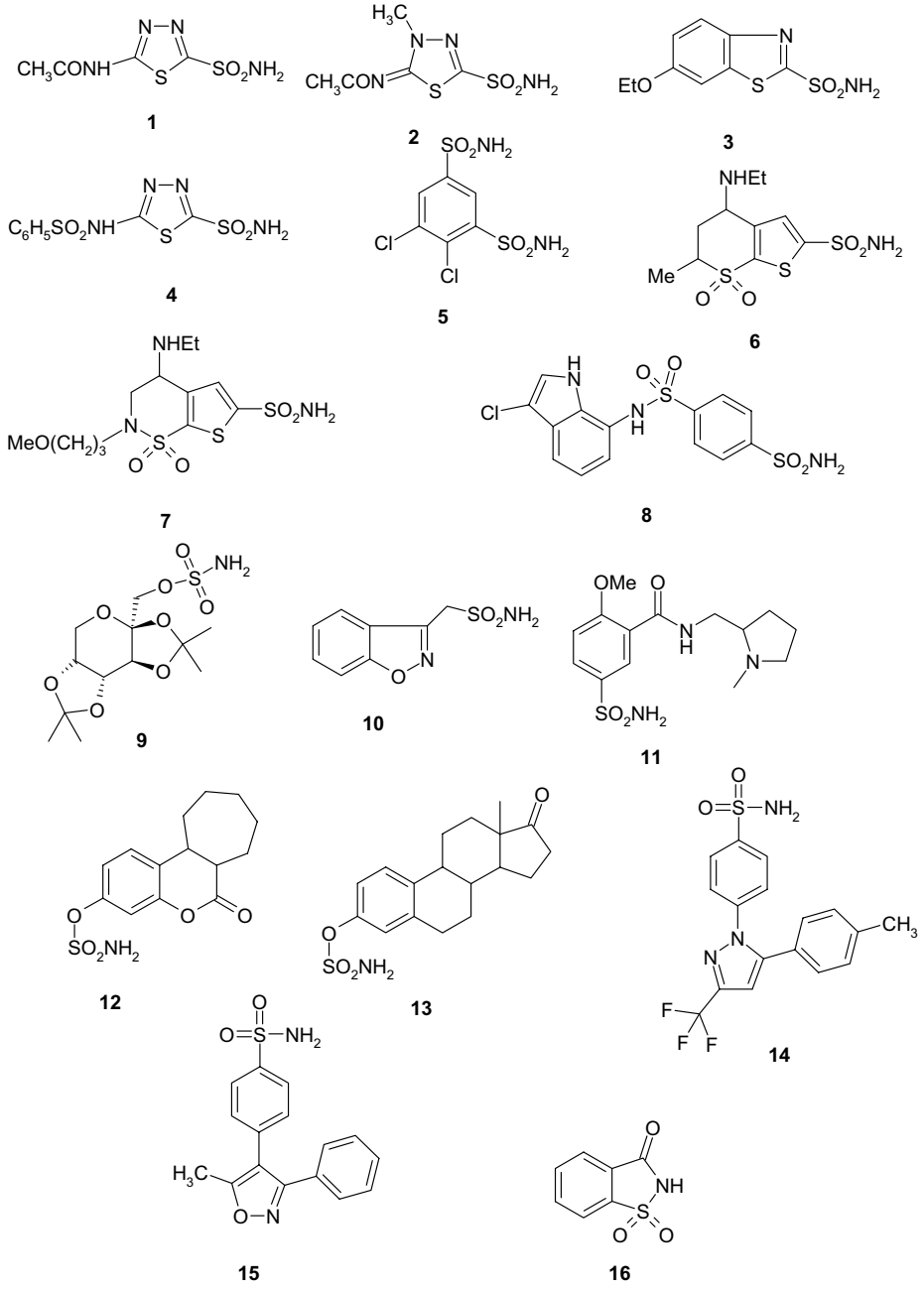


There are several clinically used drugs/orphan drugs reported to possess significant CA inhibitory properties (compounds **1**–**16**) and many other such derivatives belonging to the sulfonamide, sulfamate or sulfamide classes are constantly being reported.[1–12] Some of the clinically used compounds, such as acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, the orphan drug benzolamide **4** and dichlorophenamide **5**, are known for decades, and were initially developed in the search of novel diuretics, in the 1950s.[23] Although their diuretic use was not extensive, it has been observed that such enzyme inhibitors may be employed for the systemic treatment of glaucoma.[23] Thus, many such drugs (e.g., acetazolamide, methazolamide, and dichlorophenamide) are still presently used in ophthalmology, whereas two novel derivatives, dorzolamide **6** and brinzolamide **7**, have been developed in the late 1990s as topically acting antiglaucoma agents.[23] Other representatives of the class are the antitumor sulfonamide indisulam **8**, in Phase II clinical development for the treatment of solid tumors,[24] the antiepileptic sulfamate topiramate **9**[25] and sulfonamide zonisamide **10**,[26] which were recently shown to possess significant inhibitory properties against many physiologically relevant CA isozymes. The same is true for the antipsychotic sulpiride **11**.[27] The sulfamates possessing steroidal-like scaffold COUMATE-667 **12**[28] and EMATE **13**[29] were initially discovered as steroid sulfatase (STS) inhibitors and subsequently shown to be low nanomolar CA inhibitors (CAIs) too. These compounds are also in clinical development for the treatment of breast tumors in which both STS and some CA isozymes are overexpressed.[28] The sulfonamide COX-2 'selective' inhibitors celecoxib **14**[7b] and valdecoxib **15**[30] also act as potent inhibitors of

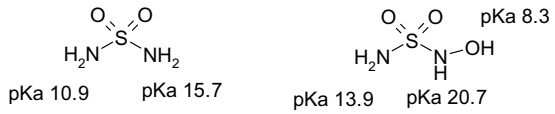
many CA isozymes, and some of their clinical applications (such as, e.g., the prevention of some gastrointestinal tumors) are correlated with the strong inhibition of some CAs.[7b,30] The sulfimine artificial sweetener saccharin **16** is also a very potent inhibitor of several physiologically relevant mammalian CA isoforms.[31]

It may be observed that all the clinically used compounds are sulfonamides or sulfamates. However, among the various classes of CAIs reported in the last years, there are also several classes of compounds incorporating modified sulfonamide/sulfamate moieties, such as *N*-hydroxysulfamides,[32] phosphorylated and N-hydroxylated sulfonamides,[33] or phosphorylated sulfamates[34] among others. Recently, it has been claimed[35] that sulfamides are ineffective as zinc binding groups (ZBGs) for the design of CAIs, based on two examples of sugar sulfamates and their corresponding sulfamides (topiramate **9**, its cyclic sulfate analogue RWJ-37947 and their corresponding sulfamide derivatives), and two simple bicyclic compounds possessing the general formula $RCH_2–XSO_2NH_2$ where X=O or NH, and R = benzofuran and benzodioxolan. We recently proved[13] that this claim[35] is not true, as we have demonstrated by means of X-ray crystallography that the diminished binding of the topiramate sulfamide analogue to the hCA II active site (as compared to the corresponding sulfamate **9**) is due to a clash between one methyl group of the inhibitor with the methyl of Ala65 from the enzyme active site.[13] At the same time, the sulfamide/sulfamate moieties of the two inhibitors bind superimposably to the active site metal ion, interacting also with residues Thr199 and Thr200.[13]

Here we examine another example of CAI recently reported by this group,[32] that is, *N*-hydroxysulfamide, which acts as a rather efficient inhibitor itself (although being such a simple molecule), but also leads to much more potent CAIs, when an organic scaffold is attached to this novel ZBG.[32] Here we report the X-ray crystal structure of the adduct of the dominant cytosolic human isozyme hCA II with *N*-hydroxysulfamide. These data may be useful for the design of novel classes of CAIs, with inhibition profiles and selectivity features distinct from those of the clinically used sulfonamides/sulfamates, and possessing a much less investigated ZBG.

Inhibitors generally bind in ionized form, as anions, to the metal ion within the CA active site.[1–5] For example, the sulfate ion is a very ineffective anion inhibitor of most CA isozymes[36] (Table 1). Data of Table 1 show that sulfate has an affinity in the range of 0.77→300 mM against some of the physiologically relevant isoforms, such as CA I, II, IX, and XII (the last two being tumor-associated CAs).[8] However, the isostructural/isoelectronic sulfamic acid and sulfamide, in which one or both oxygen atoms of sulfate were replaced by NH_2 groups, show increased affinity for these CA isozymes, and their X-ray crystal structures in adduct with hCA II have been in fact reported.[4c] These weak inhibitors were successfully used as lead molecules, and afforded much more potent, low nanomolar inhibitors, when various organic scaffolds have been attached to the sulfamate/sulfamide ZBGs.[4c,13,28,29] Indeed, some of the clinically used drugs/compounds in clinical development, such as **9**, **12**, and **13** among others, contain sulfamate as ZBG, and not the classical sulfonamide moiety, these compounds being low nanomolar inhibitors of many CA isozymes.[4c,13,28,29] Thus, it is critical to explore new ZBGs for the design of novel classes of CAIs, in order to identify both new types of tight-binding derivatives, as well as compounds with a diverse inhibition profile as compared to the clinically used drugs, which generally indiscriminately inhibit many CA isoforms, leading thus to various side effects.[1–3,23,13] One such new ZBG was recently shown to be the *N*-hydroxysulfamide one.[32] Indeed, the simple *N*-hydroxysulfamide showed a highly enhanced CA inhibitory activity as compared to both sulfamide and sulfamic acid, compounds with which it is structurally related (Table 1). Thus, *N*-hydroxysulfamide was approximately a 75-fold better hCA I inhibitor as compared to sulfamide, an approximately 2000-fold better hCA II inhibitor as compared to sulfamide, whereas these factors were 11 for the inhibition of hCA IX, and 9.8 for the inhibition of hCA XII (Table 1). In order to understand the highly enhanced affinity of *N*-hydroxysulfamide as compared to sulfamide[4c] for hCA II, we resolved the 3D crystal structure

Table 1. Inhibition of isozymes hCA I, II, IX, and XII with sulfate, sulfamic acid, sulfamide, and *N*-hydroxysulfamide, and pK_a values of the two compounds calculated by using the ACD Labs 8.0 pKa dB software[42]



Inhibitor	K_I[a] (nM)			
	hCA I[b]	hCA II[b]	hCA IX[c]	hCA XII[c]
SO_4^{2-}[d]	63. 10^6	$>300\times10^6$	$>300\times10^6$	7.7×10^5
H_2NSO_3H[d]	$0.21.10^5$	$0.39.\ 10^6$	9.2×10^3	10.7×10^3
$H_2NSO_2NH_2$	0.31×10^6	1.13×10^6	9.6×10^3	13.2×10^3
H_2NSO_2NHOH	4050	566	865	1340

[a] Errors in the range of 5–10% of the shown data, from three different assays.
[b] Human recombinant isozymes.
[c] Catalytic domain of the human recombinant isozyme, CO_2 hydrase assay method.[32]
[d] As sodium salt.

of the hCA II–*N*-hydroxysulfamide adduct (Table 2 and Figs. 1–3).

Analysis of the three-dimensional structure of the hCA II–*N*-hydroxysulfamide complex[37] revealed that the overall protein structure remained largely unchanged upon binding of the inhibitor. As a matter of fact, an rms deviation value of 0.26 Å was calculated over the entire Cα atoms of the enzyme–inhibitor complex, with respect to the unbound enzyme. The electron density of the inhibitor bound in the neighborhood of the zinc ion is very well defined (Fig. 1). Unexpectedly, as shown in Figures 1 and 2, the inhibitor is coordinated to the Zn(II) ion by the ionized, terminal H_2N moiety of the *N*-hydroxysulfamide molecule, although this is not the most acidic moiety present in *N*-hydroxysulfamide. Indeed, theoretic calculation of pK_a in this compound[42] afforded a pK_a value of 13.9 for the terminal amino moiety, a pK_a of 8.3 for the OH moiety (the most acidic one), and a pK_a of 20.7 for the NH group (Table 1). The amino group of sulfamide (which is coordinated to Zn(II) in the hCA II–sulfamide complex[4c]) gave on the other hand a pK_a of 10.9 by the same method (which is in excellent agreement with the experimental pK_a value reported earlier).[4c] Obviously, the preference of Zn(II) for binding nitrogen over oxygen in ligands

Table 2. Crystallographic parameters and refinement statistics for the hCA II–*N*-hydroxysulfamide adduct

Parameter	Value
Crystal parameter	
Space group	$P2_1$
Cell Parameters	a = 42.10Å b = 41.39 Å c = 72.30 Å β = 14.38 °
Data collection statistics (20.0–2.1 Å)	
No. of total reflections	64872
No. of unique reflections	14225
Completeness (%)[a]	99.0 (97.2)
*F*2/sig(*F*2)	8.0 (1.5)
R-sym (%)[b]	16.9 (42.9)
Refinement statistics (20.0–2.1 Å)	
R-factor (%)[c]	20.8
R-free (%)	26.6
Rmsd of bonds from ideality (Å)	0.012
Rmsd of angles from ideality (°)	1.48

[a] Values in parentheses relate to the highest resolution shell (2.17–2.10).
[b] $R\text{-sym} = \Sigma|I_i - \langle I \rangle|/\Sigma I_i$.
[c] $R\text{-factor} = \Sigma|F_o - F_c|/\Sigma F_o$; *R*-free calculated with 5% of data.

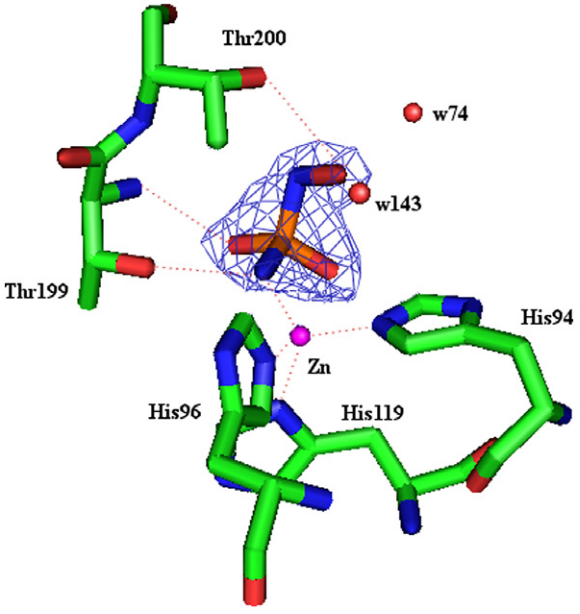


Figure 1. $2F_o - F_c$ electron density map contoured at 1σ level for *N*-hydroxysulfamide bound within the hCA II active site. The Zn(II) ion, its three protein ligands (His94, 96, and 119), residues Thr199 and Thr200, and water molecules w74 and w143 which interact with the inhibitor are also shown.

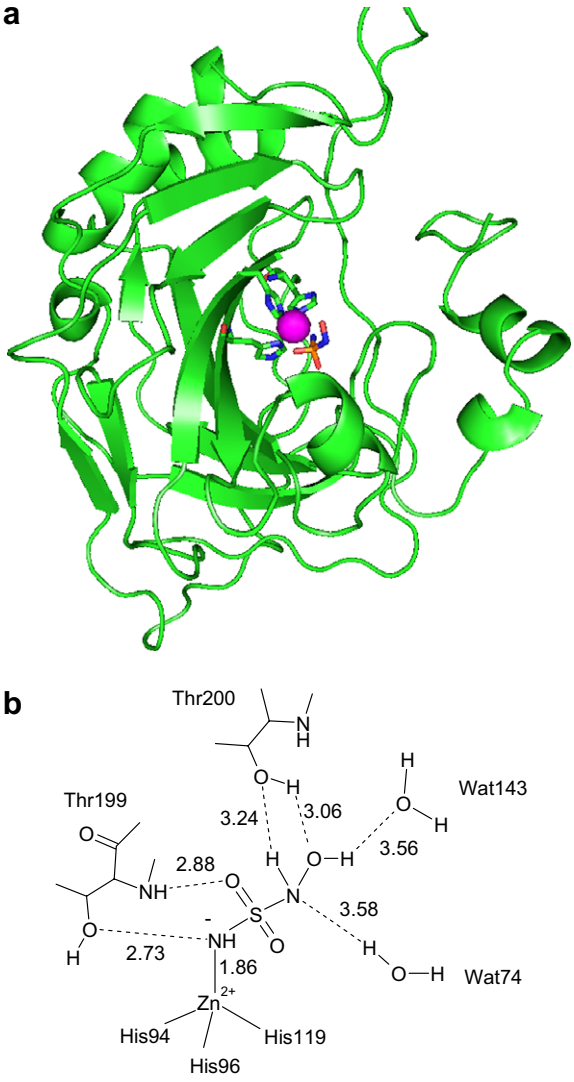


Figure 2. (a) Overall view of the hCA II–*N*-hydroxysulfamide adduct. The Zn(II) ion (pink sphere), its three ligands (His94, 96, and 119, in green), and the inhibitor molecule (yellow) are shown. (b) Schematic representation of the interactions and hydrogen bonds in which *N*-hydroxysulfamide participates when bound to the hCA II active site. Figures represent distances (in Å).

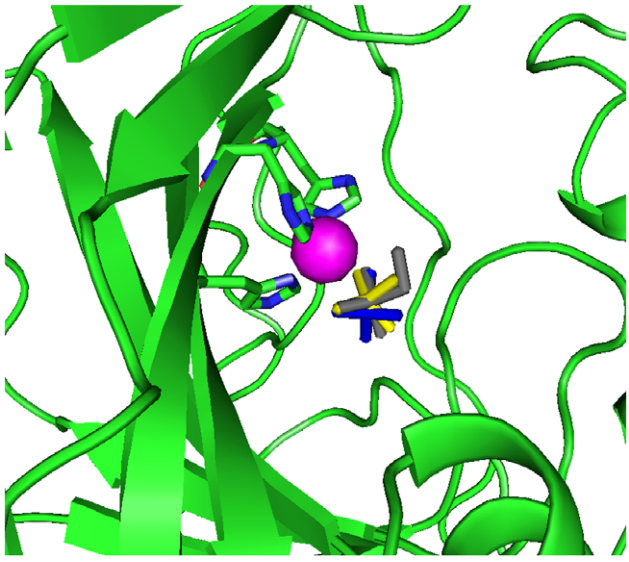


Figure 3. Superposition of the hCA II–*N*-hydroxysulfamide complex (gray) with the hCA II–sulfamate (blue)[4c] and hCA II–sulfamide[4c] (yellow) complexes. The three inhibitors are almost entirely superposable except for the extra OH moiety of *N*-hydroxysulfamide. The Zn(II) ion (violet sphere) and its three histidine ligands are also shown.

Table 3. Distances between various atoms in the hCA II–*N*-hydroxysulfamide adduct

N-Hydroxysulfamide	hCA II residue	Distance (Å)
N1	Zn	1.86
O1	Zn	3.19
N1	Oγ1Thr199	2.73
O2	N Thr199	2.88
O3	Oγ1Thr200	3.06
O3	w143	3.56
N2	w74	3.58
N2	Oγ1Thr200	3.24

possessing both these heteroatoms governs the interaction between the metal ion and the inhibitor in this complex and not merely the pK_a value and thus the ionization order of the various acidic groups present in the inhibitor. As a matter of fact, this is the first ever observation of an inhibitor which binds to CA by means of a less acidic functionality, in the presence of another more acidic moiety, which potentially would be able to bind easier to the metal ion due to its ionized character. Thus, *N*-hydroxysulfamide and sulfamide both bind the metal ion with the same functionality, the deprotonated H_2N group, although the last inhibitor is three pK_a units more acidic than the first one. In fact, as seen from Figure 3, the adducts of hCA II with *N*-hydroxysulfamide, sulfamide and sulfamate are quite superposable except for the extra OH moiety of the first inhibitor.

At this point, why is *N*-hydroxysulfamide a 2000 times better hCA II inhibitor than sulfamide, since it was postulated[23] for a long period that the pK_a of the sulfonamide group is one of the main determinants for the potency of such a compound as CAI (with more acidic sulfonamides better CA inhibitors as compared to the less acidic ones)? A reply to this question may be obtained by analyzing in more detail the structure (Table 3). Thus, the deprotonated primary amino group of the inhibitor is coordinated to the zinc ion, at a distance of 1.86 Å, intermediate between the very short distance evidenced for the sulfamide adduct (Zn–N of 1.76 Å)[4c] and the longer one evidenced for sulfamic acid (Zn–N of 2.07 Å).[4c] The NH moiety coordinated to Zn(II) also participates in a strong hydrogen bond with the OH moiety of Thr199 (of 2.73 Å), whereas one oxygen of the SO_2 moiety makes a second hydrogen bond (of 2.88 Å) with amide NH of the same amino acid residue. The other oxygen of the SO_2 moiety is at about 3.2 Å from the metal ion, as in many other sulfonamide–CA II adducts, including the sulfamide and sulfamic acid ones.[1–4] The NH moiety of the NHOH functionality also participates in two hydrogen bonds, similarly to the second H_2N moiety of sulfamide.[4c] Thus, a strong hydrogen bond with the OH moiety of Thr200 is observed (of 3.24 Å), and a second, weaker one with a water molecule (w74), of 3.58 Å. The OH group of the inhibitor molecule establishes again two hydrogen bonds, one with the same OH of Thr200 which participates in the hydrogen bond with the NH moiety of the inhibitor, this time of 3.06 Å, whereas the second one with another water molecule present within the active site, w143, of 3.56 Å. Thus, the presence of extra two hydrogen bonds in the hCA II–*N*-hydroxysulfamide adduct as compared to the hCA II–sulfamide adduct seems to be the main factor responsible for the enhanced affinity of the first inhibitor for the enzyme. In fact these supplementary interactions are due to the presence of the additional OH moiety in the molecule of the tight-binding inhibitor. In addition, *N*-hydroxysulfamide possesses a higher surface area and probably also a higher solvent accessible surface area as compared to sulfamide, which allows it to participate in better contacts with amino acid residues within the active site cavity. All these factors may explain its much better inhibitory activity against hCA II as compared to the structurally related sulfamide.

Another important aspect for drug design is related to the orientation of the inhibitor molecule when bound to the active site. In fact (Fig. 2a) the NHOH moiety is oriented toward the exit of the cavity, with both the groups interacting with the OH of Thr200, but with the NH exposed to the solvent. It is thus quite envisageable that a derivatization of this moiety, by means of a bulky aromatic/heterocyclic R moiety, in compounds of the type H_2N–$SO_2N(R)OH$ will afford a similar binding to the ^-HN–SO_2NOH fragment of the inhibitor to the enzyme, whereas the R groups will be able to participate in hydrophobic and polar interactions with the internal part of the active site extending after Thr200, in which the organic scaffolds of aromatic/heterocyclic sulfonamides bind.[1–4,43] This would undoubtedly lead to quite potent CAIs. Work is in progress to test this hypothesis.

In conclusion, we understand why *N*-hydroxysulfamide is a 2000-fold more potent CA inhibitor as compared to the structurally related sulfamide. The X-ray crystal structure of the hCA II–*N*-hydroxysulfamide adduct

showed the inhibitor to be coordinated to the active site zinc ion by the ionized primary amino group, also participating in an extended network of hydrogen bonds with amino acid residues Thr199, Thr200 and two water molecules. The additional two hydrogen bonds in which *N*-hydroxysulfamide bound to hCA II is involved as compared to the corresponding adduct of sulfamide may explain its higher affinity for the enzyme, also providing hints for the design of tight-binding CA inhibitors possessing an organic moiety substituting the NH group in the *N*-hydroxysulfamide structure.

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37. The hCA II–*N*-hydroxysulfamide complex was co-crystallized at 4 °C by the hanging drop vapor diffusion method. Drops containing 5 μl of 20 mg/ml recombinant hCA II in 50 mM Tris·HCl buffer, pH 7.8, were mixed with 5 μl of precipitant buffer (2.4 M $(NH_4)_2SO_4$ in 50 mM Tris·HCl pH 7.8, and 1 mM sodium 4-(hydroxymercury)benzoate) with added 5 mM *N*-hydroxysulfamide[32] and equilibrated over a reservoir of 1 ml of precipitant buffer. Diffraction data were collected under cryogenic conditions (100 K) on a CCD Detector KM4 CCD/Sapphire using CuKα radiation (1.5418 Å). The unit cell dimensions were determined to be: $a = 42.10$ Å, $b = 41.39$ Å, $c = 72.30$ Å and $\alpha = \gamma = 90$ °, $\beta = 104.38$ ° in the space group $P2_1$. Data were processed with CrysAlis RED (Oxford Diffraction 2006).[38] The structure was analyzed by difference Fourier technique, using the PDB file 1CA2 as starting model. The refinement was carried out with the program REFMAC5.[39] The model building and map inspections were performed using the COOT program.[40] The final model of the complex had an *R*-factor of 20.8% and *R*-free 26.6% in the resolution range 20.0–2.1 Å, with a rms deviation from standard geometry of 0.012 Å in bond lengths and 1.48° in angles. The correctness of stereochemistry was checked using PROCHECK.[41] Coordinates and structure factors have been deposited with the Protein Data Bank, PDB ID code 2O4Z. Crystallographic parameters and refinement statistics are summarized in Table 2.
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