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Carbonic Anhydrase Inhibitors: X-ray Crystallographic Structure of the Adduct of Human Isozyme II with a Bis-sulfonamide—Two Heads Are Better Than One?

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Abstract—The X-ray crystal structure for the adduct of human carbonic anhydrase II (hCA II) with 4-(4-sulfamoylphenylcarboxamidoethyl)benzenesulfonamide, a topically acting antiglaucoma sulfonamide has been resolved at a resolution of 1.8 Å. Its binding to the enzyme is similar with that of other sulfonamides, considering the interactions of the sulfonamide zinc anchoring group, but differs considerably when the organic part of the inhibitor is analyzed. This part of the inhibitor interacts only within the hydrophobic half of the CA active site, leaving the hydrophilic half able to accomodate several water molecules not present in the uncomplexed enzyme. Furthermore, the second head (sulfonamide moiety) participates in two strong hydrogen bonds with amino acid residues (Gly 132 and Gln 136) situated on the rim of the entrance to the active site cleft. Thus, the answer to the question in the title of this paper is that two heads are better than one, since the two sulfamoyl moieties of the inhibitor allow its proper orientation within the active site, with only one head binding in ionized form to the zinc ion, the organic part lying within the hydrophobic half of the active site, and the terminal, carboxamido containing phenylsulfamoyl head participating in strong hydrogen bonds with amino acid residues located at the entrance of it. All these findings are important for the design of better carboxamido CA inhibitors with applications in clinical medicine.

Introduction

4-(4-Sulfamoylphenylcarboxamidoethyl)benzenesulfonamide **1** has recently been shown to act as an efficient topical antiglaucoma sulfonamide with carbonic anhydrase (CA, EC 4.2.1.1) inhibitory acivity.[1] Indeed, this class of pharmacological agents have been used for more than 45 years as pressure lowering systemic drugs in the treatment of open-angle glaucoma as well as other diseases associated with acid base/secretory disequilibria.[2–6] Recently, they started to show increasing interest as potential agents for the treatment of macular edema, a condition for which no effective therapy was known up to now.[2–5] Among the many types of topically acting sulfonamides reported ultimately,[2] the compounds incorporating 4-sulfamoylphenylcarboxamido tails among others,[1] were particularly interesting for the following reasons: (i) they generally showed very strong in vitro affinity (in the low nanomolar range) for the critically relevant isozymes involved in aqueous humor secretion within the eye, that is, CA II and CA IV (for example **1** shows an inhibition constant of 5 nM against hCA II and of 13 nM against bCA IV, respectively)[1]; (ii) the hydrophilicity/lipophilicity balance of such compounds is good (for example **1** has a logP of 1.433, of the same order of magnitude with the clinically used drug dorzolamide **2**),[1] and as a consequence, their penetrability through the cornea for reaching ciliary processes CAs is excellent (for example **1** shows a rate of corneal penetration (k_{in}) of 6.3 10^{-3} h^{-1}, slightly better than that of **2**, with a k_{in} of 5.0×10^{-3} h^{-1}).[1] As a consequence, such compounds are easily formulated as eye drops, either in solution, or as suspensions, at physiological pH values, and their in vivo efficacy, in animal models of glaucoma, was much higher than that of the clinically available topically acting antiglaucoma sulfonamides dorzolamide **2** and brinzolamide **3**.[1] Indeed, **1** formulated as a 2% suspension in water at pH 7, produced a very potent and prolonged IOP lowering (of 9.5–12 mm Hg, for 5–6 h) in glaucomatous rabbits,

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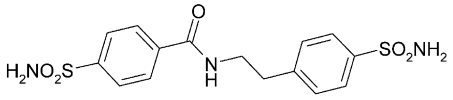
being much more effective than 2% dorzolamide **2**, which produced an IOP lowering of 3.6–6.7 mm Hg, lasting only 3 h, in the same animal model of glaucoma.[1]



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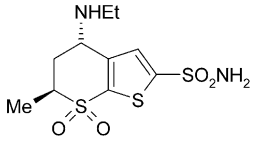

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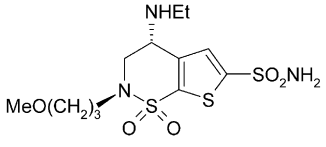

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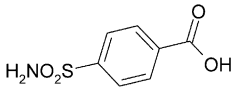

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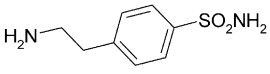

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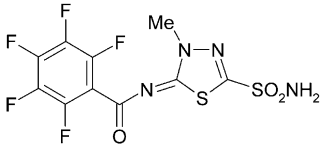

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In order to understand at molecular level the factors that may explain the high affinity of this CA inhibitor for the most abundant isozyme within the eye, CA II,[7,8] and also for allowing us to develop better topically acting enzyme inhibitors incorporating sulfamoylcarboxamido tails, an X-ray crystallographic study for the hCA II-**1** adduct has been performed, and is reported here. The structure of this adduct evidenced unprecedented interactions between the inhibitor and the enzyme active site and may be useful for the drug design of more active agents belonging to this class of pharmacological agents.

Chemistry

Compound **1** has been prepared as previously reported by our group.[1] As mentioned earlier, **1** is a very strong hCA II inhibitor (K_i of 5 nM). The compound contains two sulfonamido moieties, one originating from the 4-carboxybenzenesulfonamide **4** (K_i of 260 nM),[9] and the other one from the 4-aminoethyl-benzenesulfonamide **5** (K_i of 160 nM),[10] the two starting materials from which **1** has been obtained by means of a coupling reaction in the presence of carbodiimides.[1] The important question at this point was: which is the responsible sulfonamide group(s) for binding to the Zn(II) ion within the enzyme active site? Obviously, theoretically it is possible that only one of the two sulfonamide moieties assures the zinc-anchoring within the enzyme active site, or it is also possible that two E–I complexes are formed, one in which the Zn(II) ion is complexed by the SO_2NH_2 moiety of **4**, and another one in which the metal ion is complexed by the SO_2NH_2 moiety of **5**. We were fortunately able to respond to these questions by resolving unambiguously the X-ray crystal structure for the adduct of **1** with hCA II.

Crystallography

The hCA II-**1** adduct obtained by co-crystallization, was subjected to detailed X-ray crystallography. The programs SHELX97[11] and O[12] were used to build the model and to compute the Fourier maps. The last refinement cycle yielded a final R factor of 0.21 (R_{free} = 0.25) with a final temperature factor of the inhibitor atoms ranging between 8.5 and 32.3 Å.[2] The final number of water molecules was 112 and the final rmsd's from ideal geometry for bond lengths and angles were 0.025 Å and 2.1°, respectively. The statistics of data collection and refinement are shown in Table 1. A final refinement resolution of 1.8 Å has been achieved.[13]

The structure refinement allowed us evidencing the spatial arrangement of the inhibitor within the active site of the enzyme (Fig. 1), whereas the electronic density for the binding of **1** to hCA II is shown in Figure 2. The schematic, detailed representation of the interactions of **1** with the metal ion and amino acid residues present in the hCA II active site are shown in Figure 3.

As seen from these figures, the ionized sulfonamide moiety of **1** has replaced the hydroxyl ion/water molecule coordinated to Zn(II) in the native enzyme (Zn–N distance of 1.86 Å), as in other hCA II-sulfonamide

Table 1. Statistics of data collection and refinement for the hCAII-**1** adduct

	hCA II- 1 complex
Resolution Range (Å)	40–1.8
Space group	$P2_1$
Unit cell (Å, ° for β)	a = 42.0, b = 41.20, c = 72.8, α = 90, β = 103.9, γ = 90
Highest resolution shell (Å)	1.85–1.80
No. of reflections	26204
Completeness (%)	99.5
Mean I/σ_I	24.3 [2.2]
R_{sym} (%)[&]	8.4 [48.4]
Refined residues	261
Refined water molecules	112
Resolution range in refinement (Å)	30–1.9
R_{cryst} ($F_o > 4\sigma F_o$; F_o)	21.0, 19.6
R_{free} ($F_o > 4\sigma F_o$)	25.0
Rms deviations	
Bond lengths (Å)	0.025
Bond angles (°)	2.1
Average B value (Å^2)	32.0
Ramachandran plot	
Most favored (%)	86.5
Additionally allowed (%)	13.0
Generously allowed (%)	0.5
Disallowed (%)	0.0

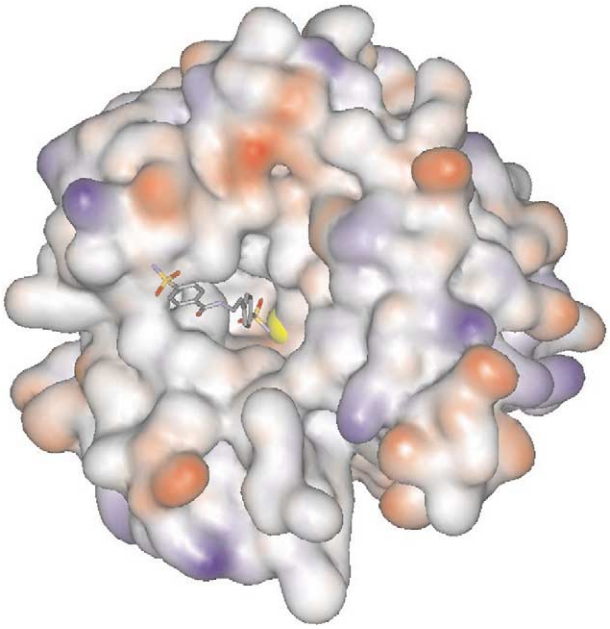

Figure 1. The hCA II-**1** adduct. The zinc ion (yellow sphere), and the inhibitor molecule are evidenced. The inhibitor occupies only the hydrophobic half of the active site, participating in a multitude of hydrophobic interactions with eight amino acid residues present there.

complexes for which the X-ray structures have been reported (Fig. 2).[7,8,14–16] What is important to note is that the Zn–N bond is appreciably shortened in this complex, as usually this distance is around 1.95–2.10 Å, and this shortening may be considered as a first factor favoring the high affinity of such sulfonamides for CA.[7,8,14,15] The Zn(II) ion remains in its stable tetrahedral geometry, being coordinated in addition to the sulfonamidate nitrogen, by the imidazolic nitrogens of

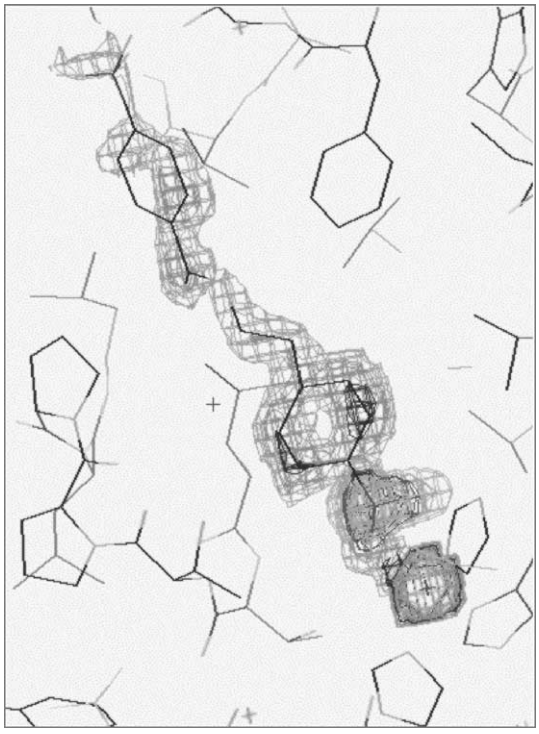

Figure 2. Electronic density map for the binding of **1** within the hCA II active site.

His 94, His 96 and His 119. The proton of the coordinated sulfonamidate nitrogen atom of the inhibitor also makes a hydrogen bond with the hydroxyl group of Thr 199, which in turn accepts a hydrogen bond from the carboxylic group of Glu 106 (Fig. 3). One of the oxygen atoms of the coordinated sulfonamide moiety makes a hydrogen bond with the backbone amide of Thr 199, whereas the other one is semi-coordinated to the catalytic Zn(II) ion (O–Zn distance of 3.15 Å). These interactions are generally seen in all complexes of hCA II with sulfonamides and sulfamates.[7,8,14,15] The phenethyl moiety and the phenylcarboxamido part of the inhibitor **1** lie in the hydrophobic part of the active site cleft, where they make van der Waals interactions with the side chains of Val 135, Phe 131, Leu 204, Pro 202, Trp 209, Val 121, Leu 198, and Thr 200 (Fig. 2). The carbonyl oxygen and the NH group of the carboxamido moiety do *not* make any hydrogen bond with amino acid residues within the active site, which was a rather unexpected finding, since for other phenylcarboxamide-substituted sulfonamides for which the X-ray structure has recently been reported, such as for example the pentafluorophenylcarboxamido analogue of methazolamide **6**,[16] the carboximido linker is involved in strong hydrogen bond networks involving residues Gln 92, Asn 67 and Glu 69. Thus, the most notable and unprecedented interaction evidenced in this complex regards the fact that the organic part of the inhibitor entirely lies in the hydrophobic half of the active site, where it participates in a multitude of favorable van der Waals interactions with eight amino acid residues, whereas it practically makes no contacts with residues of the hydrophilic half of the active site, which were shown to be critical for the binding of all other types of inhibitors investigated by means of X-ray crystallography.[8,14,15] Furthermore, in this particular complex discussed here, this part of the active site contains several water molecules which are not present in the structure

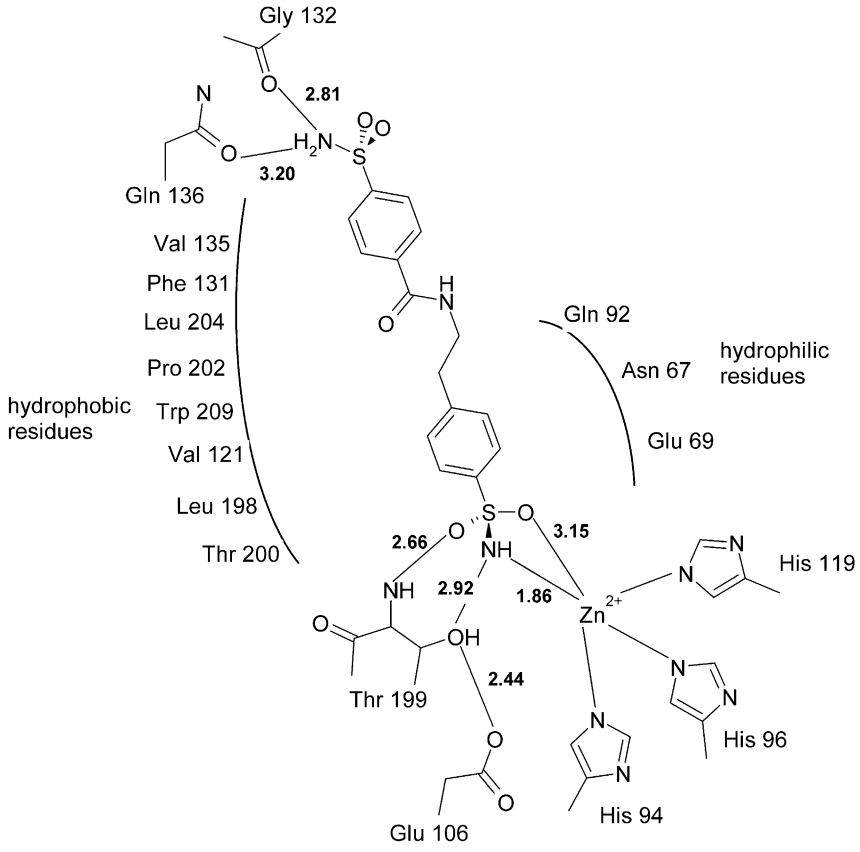

Figure 3. Schematic representation of inhibitor **1** binding within the hCA II active site (figures represent distances in Å).

of the uncomplexed CA II (data not shown). Another very interesting finding concerns the anchoring of the terminal sulfonamide moiety to two amino acid residues situated at the entrance of the active site, Gln 136 and Gly 132, never evidenced before for other hCA II-sulfonamide adducts. Thus, the amino group of this terminal sulfonamide moiety hydrogen bonds with the oxygen of the backbone amide of Gln 136 and with the corresponding oxygen of Gly 132, these two strong bonds being of 3.20, and 2.81 Å, respectively (Fig. 3).

Thus, unambiguously, the present structural data allowed us to establish that only one sulfonamide moiety of the two heads that **1** possesses, participates in interaction with the metal ion, when the inhibitor is bound within the hCA II active site. This is an interesting example of discrimination by the metal ion from an enzyme active site for two very closely related anchoring groups. In fact, the pK_a of both sulfamoyl moieties from **1** is in the same range, of around 10 pK_a units,[9] and thus, both sulfonamidates are formed in equal proportion in aqueous solution, at pH 7.5 at which the crystals were grown. A competition may thus arise for the binding to the Zn(II) ion, either by means of the 4-aminoethylbenzenesulfonamide part, or by the 4-carboxy-benzenesulfonamide part of the molecule of **1**, respectively. Still, no two different complexes were evidenced by means of high resolution X-ray crystallography, proving that only one of the two complexes is formed, as illustrated in Figures 2 and 3. Furthermore, the explanation of this phenomenon may be due to the very particular binding mode of **1** within the CA active site, with two interactions not previously observed for other hCA II–sulfonamide complexes: (i) the binding of the organic part of the inhibitor only within the hydrophobic half of the active site, leaving the hydrophilic half able to accomodate several water molecules not present in the uncomplexed enzyme; (ii) the second head (sulfonamide moiety) of the inhibitor participates in two strong hydrogen bonds with amino acid residues situated on the rim of the entrance to the active site cleft. Thus, the answer to the question in the title of this paper is yes, two heads are in this case better than one, but not for entropic reasons, as one should generally expect. In fact, the two sulfamoyl moieties of **1** allow a proper orientation of the inhibitor within the active site, with the first head binding in ionized form to the zinc ion, the organic part lying only in the hydrophobic part of the active site, and with the terminal, carboxamido containing phenylsulfamoyl head participating in strong hydrogen bonds with amino acid residues located at the entrance within the cavity.

By varying the different structural elements present in the molecule of **1** (such as for example the nature/substitution pattern of the two phenyl rings, the length and nature of the spacer between them; the nature of the terminal head, a sulfamoyl moiety in this case) it is possible to envision even more potent CA inhibitors with different types of application in biomedical sciences.

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