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Carbonic anhydrase inhibitors: X-ray crystallographic structure of the adduct of human isozyme II with a topically acting antiglaucoma sulfonamide

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Abstract—The X-ray crystal structure for the adduct of human carbonic anhydrase (hCA) II with a topically acting antiglaucoma sulfonamide (the 2-*N*,*N*-diethylaminoethylamide of 5-(4-carboxybenzenesulfonamido-1,3,4-thiadiazole-2-sulfonamide), has been resolved at a resolution of 1.6 Å. This compound is a very potent inhibitor of the physiologically most relevant isozyme hCA II for the secretion of aqueous humor within the eye (K_I of 1.4 nM), and in animal models of glaucoma showed very effective intraocular pressure (IOP) lowering after topical administration. Surprisingly, the inhibitor bound within the enzyme active site is in the sulfonylimido-4H-δ^2-1,3,4-thiadiazoline tautomeric form. The inhibitor is directly bound to the Zn(II) ion of the enzyme through the deprotonated primary sulfonamide moiety, participating to the classical hydrogen bond network involving residues of the zinc-binding function and Thr 199 and Glu 106. The 1,3,4-thiadiazoline fragment of the inhibitor makes two hydrogen bonds with the active site residue Thr 200, the secondary sulfonamide moiety makes two hydrogen bonds involving a water molecule and the residue Gln 92, whereas the phenyl ring of the inhibitor participates to an edge-to-face interaction with the phenyl ring of Phe 131, the two cycles being almost perfectly perpendicular to each other. The tertiary amine fragment of the carboxamido tail and the carboxamido moiety itself make hydrogen bonds with water molecules present at the rim of the active site entrance and van der Waals contacts with His 4, Trp 5, and Phe 20. All these multiple interactions never evidenced previously in CA–sulfonamide complexes, explain the very high affinity of this inhibitor for the hCA II active site and may allow further optimization of this class of inhibitors.

1. Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs) such as acetazolamide AZA, methazolamide MZA, ethoxzolamide EZA, and dichlorophenamide DCP were and still are widely used systemic antiglaucoma drugs.[1–4] Their mechanism of action consists in inhibition of CA isozymes II and IV present in ciliary processes of the eye, with the consequent reduction of bicarbonate and aqueous humor secretion, and of elevated intraocular pressure (IOP), characteristic of this disease.[1–4] Since CA II/IV is present in many other tissues/organs, generally systemic CAIs possess undesired side effects such as numbness and tingling of extremities, metallic taste, depression, fatigue, malaise, weight loss, decreased libido, gastrointestinal irritation, metabolic acidosis, renal calculi, and transient myopia.[1–4] In order to avoid these undesired side effects, recently, topically effective CAIs have been developed. Two drugs are available clinically: dorzolamide DZA (since 1995)[5] and brinzolamide BRZ (since 1999).[6] Both drugs are applied topically as water solutions/suspensions, alone or in combination with other agents (such as β-blockers, prostaglandin derivatives, etc.) and produce a consistent and prolonged reduction of IOP.[1–4] Furthermore, recent reports show both the systemically as well as topically acting sulfonamide CAIs to be effective in the treatment of macular edema and other macular degeneration diseases, for which pharmacological treatment was unavailable up to now.[1–4] Much research is in fact in the search of even more effective topically acting CAIs, free of the inconveniences and side effects of the presently available drugs.[7–10]

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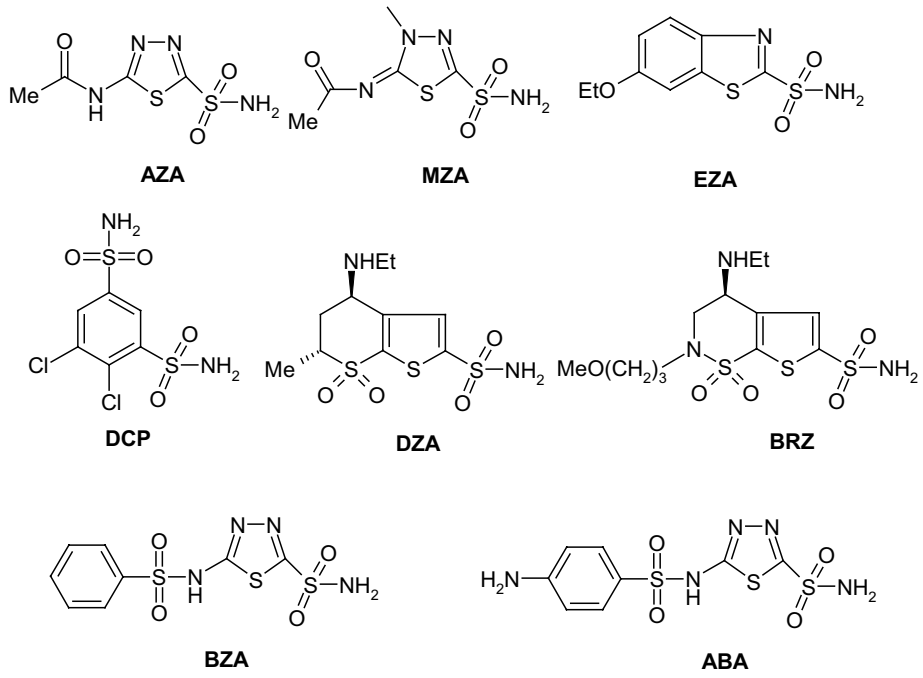
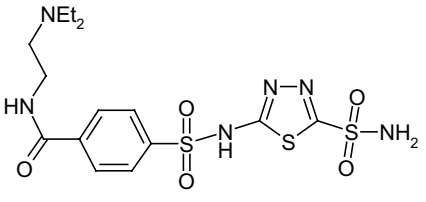


In addition to the six clinically used drugs mentioned above, benzolamide BZA, is an orphan drug belonging to this class of pharmacological agents.[11] This compound is a particularly potent inhibitor of several physiologically relevant isozymes (together with its close congener, aminobenzolamide ABA) and due to its very polar character crosses biological membranes much more difficultly as compared to other CAIs in clinical use.[7,11] Still recently, we have reported a new class of very potent and membrane permeant compounds derived from BZA as lead molecule, which were shown to potently lower intraocular pressure (IOP) in an animal model of glaucoma.[7] One of the best compounds (as in vitro enzyme inhibitor and in the in vivo model of glaucoma) in the above mentioned study was **1**, which is the 2-*N*,*N*-diethylaminoethylamide of 5-(4-carboxybenzenesulfonamido-1,3,4-thiadiazole-2-sulfonamide).[7] In order to understand at molecular level the interactions between this inhibitor and the enzyme active site, leading to the high affinity of **1** for many physiologically relevant CA isozymes, we performed a detailed X-ray crystal analysis for the adduct of this inhibitor with isozyme hCA II, the main responsible enzyme for aqueous humor secretion within the eye. Very interesting interactions have been evidenced in this way, both for the interaction of the enzyme with the inhibitor bound within its active site, but also an unexpected tautomeric form of the bound inhibitor has been evidenced for the first time here.



	K_I (nM)
hCA I	10
hCA II	1.4
bCA IV	8

1

2. Chemistry

Compound **1** has been prepared as previously reported by our group.[7] As mentioned earlier, **1** is a very strong hCA II inhibitor (K_I of 1.4 nM). The compound also efficiently inhibits other CA isozymes, such as, for example, CA I (K_I of 10 nM) and CA IV (K_I of 8 nM). In order to understand at molecular levels why the presence of the carboxamido tail of **1** leads to a dramatic increase (6.4 times) of its affinity for hCA II as compared to benzolamide (K_I of 9 nM for hCA II),[7] we decided to perform a high resolution X-ray crystallographic study of the adduct of **1** with this isozyme.

3. Crystallography

The hCA II–**1** adduct obtained by co-crystallization, was subjected to detailed X-ray crystallography. The data were processed with MOSFLM[12] and all refinement calculations were done. SHELX97[13] and XtalView[14] were used to build the model and to compute the Fourier maps. The last refinement cycle yielded a final R factor of 0.20 (R_{free} of 0.22). The final number of water molecules was 320 and the final rmsd's from ideal geometry for bond lengths and angles were 0.02 Å and 0.5°, respectively. The statistics of data collection and refinement are shown in Table 1. A final refinement resolution of 1.6 Å has been achieved.[15]

The structure refinement allowed us evidencing the spatial arrangement of the inhibitor within the active site of the enzyme (Fig. 1). The electronic density around the Zn(II) ion for the hCA II–**1** complex is shown in Figure 2. The schematic, detailed representation of the interactions of **1** with the metal ion and

Table 1. Statistics of data collection and refinement for the hCA II–**1** adduct

	1–hCA II complex
Resolution range (Å)	30–1.6
Space group	$P2_1$
Unit cell (Å, ° for β)	$a = 41.57$, $b = 40.85$, $c = 71.18$, $\beta = 104.2$
Highest resolution shell (Å)	1.75–1.60
No. of unit reflections	25,059
Completeness (%)	97.4 (94.9)[a]
R_{sym} (%)	7.9
Refined residues	261
Refined water molecules	320
Resolution range in refinement (Å)	30–1.7
R_{cryst} ($F_o > 4\sigma F_o$, F_o)	19.7, 18.3
R_{free} ($F_o > 4\sigma F_o$)	23.8
Rms deviations	
Bond lengths (Å)	0.02
Bond angles (°)	0.5
Average B value (Å^2)	24.8
Ramachandran plot	
Most favored (%)	89.4
Additionally allowed (%)	10.1
Generously allowed (%)	0.5
Disallowed (%)	0.0

[a] Values in brackets are statistics for the highest resolution shell. The lower completeness in the outer shell is caused by the squared format of the R-axis 4 detector and not by any anisotropic diffraction of the crystal. We decided to include these reflections in the refinement due to their good statistics.

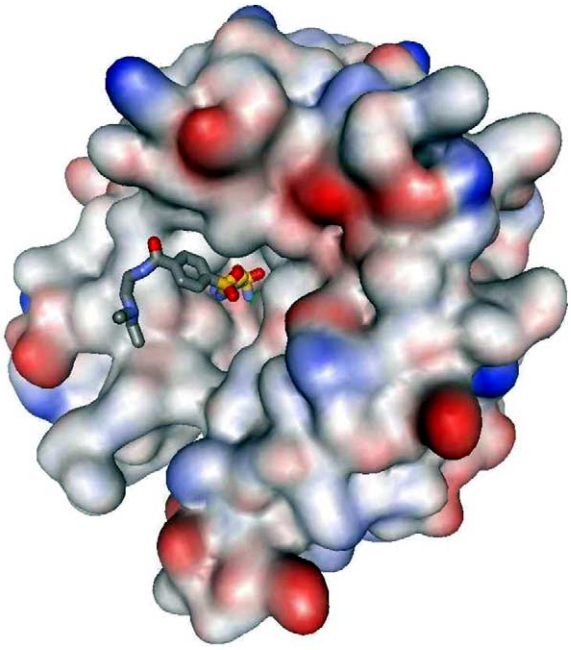


Figure 1. Binding of **1** within the hCA II active site. The surfaces are colored according to the hydropathy: blue—hydrophylic, red—hydrophobic region.

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

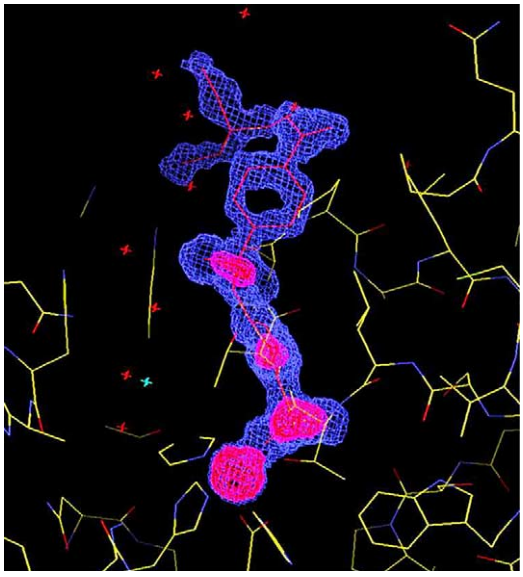


Figure 2. Electron density map around the Zn(II) ion of compound **1** bound within hCA II active site.

distance of 1.76 Å), as in other hCA II–sulfonamide/sulfamate complexes for which the X-ray structures have been reported (Fig. 3).[16–23] 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.[16–23] In fact this is the shortest Zn–N bond ever evidenced up to now in a CA–sulfonamide adduct. The Zn(II) ion remained in its stable tetrahedral geometry, being coordinated in addition to the sulfonamidate nitrogen, by the imidazolic nitrogens of 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 (of 2.69 Å), which in turn accepts a hydrogen bond from the carboxylic group of Glu 106 (of 2.48 Å). One of the oxygen atoms of the coordinated sulfonamide moiety makes a hydrogen bond with the backbone amide of Thr 199 (2.90 Å), whereas the other one is semi-coordinated to the catalytic Zn(II) ion (O–Zn distance of 3.05 Å). These interactions are generally seen in all complexes of hCA II with sulfonamides and sulfamates.[16–23] The heterocyclic moiety of the inhibitor **1** is oriented toward the hydrophobic part of the active site cleft, similarly with that of acetazolamide[16] or the perfluorobenzoylimido analogue of methazolamide,[19] for which the X-ray structures in complex with hCA II are available. What is noteworthy (and has not been evidenced before in other CA–sulfonamide adducts) is the fact that Thr 200 participates to two hydrogen bonds with the two endocyclic nitrogen atoms of the heterocyclic ring. Clearly, in one of these bonds the OH moiety of Thr 200 is the donor of the hydrogen bond (of 2.79 Å, with N^3 of the heterocyclic ring), but in the second one, with N^4 (which is even shorter, of 2.50 Å), Thr 200 can only act as an acceptor of hydrogen bonds, through one lone pair of the oxygen atom (in fact, in the hCA II–acetazolamide complex, the OH of Thr 200 is not making any hydrogen bond with the thiadiazole ring, being situated at a

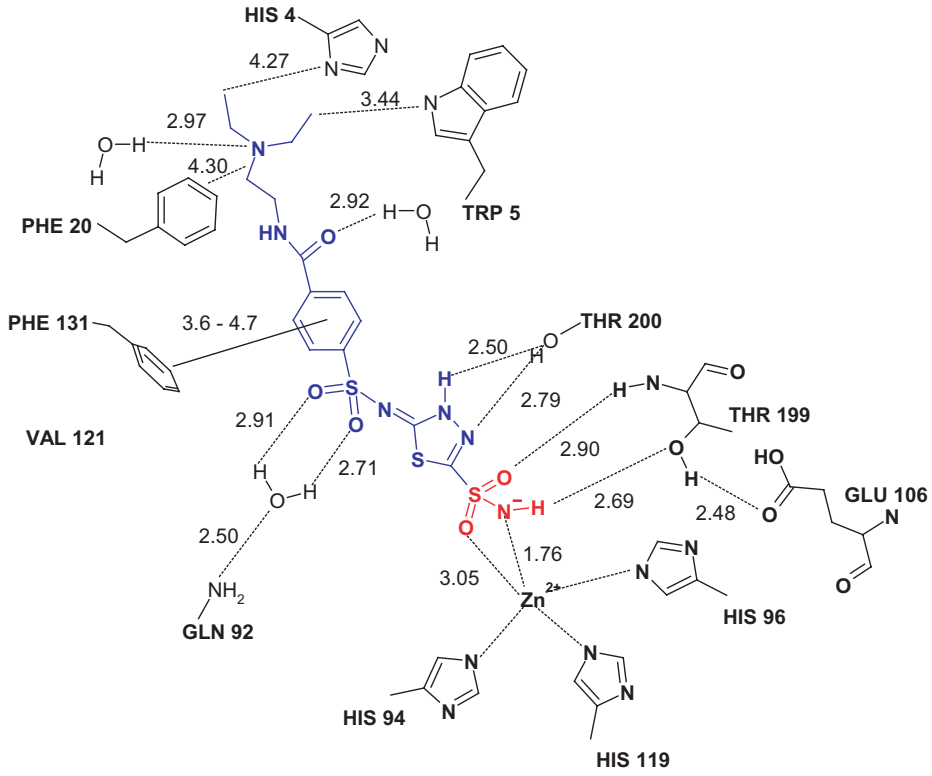


Figure 3. Detailed schematic representation of inhibitor **1** binding within the hCA II active site (figures represent distances in Å).

distance of 3.2 Å of N^3).[16b] This clearly demonstrates that there is a proton bound to N^4 of the heterocyclic ring and that the tautomeric form of the inhibitor **1** is not the 1,3,4-thiadiazole but the 4H-δ^2-1,3,4-thiadiazoline one (Fig. 3). Furthermore, the bond length between the nitrogen atom of the secondary sulfonamide moiety of **1** and the C-5 atom of the heterocyclic ring is of only 1.40 Å (data not shown), whereas in the hCA II–aminobenzolamide (ABA) complex this is of 1.70 Å,[16c] this being an additional proof that the tautomeric form of **1** is the 4H-δ^2-1,3,4-thiadiazoline one. This finding is really unexpected but it may explain in part both the enhanced affinity of **1** for hCA II (as compared to benzolamide or acetazolamide) as well as the increased liposolubility of this compound, which is much more membrane permeant as compared to benzolamide.[7] This last aspect was considered by us to be due to the presence of the carboxamido tail[7] (which may be true) but the lack of a proton to the secondary sulfonamide moiety (which in benzolamide has a pK_a of 3.7)[11] of **1** may account for the reduced polar character of this compound at physiological pH values.[7] Another interesting finding in the present work regards the two hydrogen bonds of the two oxygen atoms belonging to the secondary sulfonamide moiety of **1**, which through one water molecule are bridged by means of another hydrogen bond (of 2.50 Å) to Gln 92, an amino acid residue known to play an important role for the binding of inhibitors to hCA II.[16–23] Usually, the amino moiety of the carboxamido group of Gln 92 directly made hydrogen bonds with, for example, the CONH oxygen of acetazolamide,[16b] or the oxygen of the $C_6F_5CON=$ moiety of the perfluorobenzoylimido analogue of methazolamide.[19] In the specific case of this adduct, Gln 92 has the role of stabilizing the central part of the inhibitor molecule within the active site through this network of three hydrogen bonds involving a water molecule and the two oxygen atom of the secondary $SO_2N=$ moiety. An unprecedented interaction was also observed for the phenyl ring of **1** when bound to the hCA II active site: this structural element forms edge-to-face[24] attractive electrostatic interactions with the phenyl ring of Phe 131, another amino acid residue known to play critical roles for the binding of inhibitors to hCA II.[16–23] Practically the two aromatic rings are almost perfectly perpendicular to each other, a case never observed previously for sulfonamide CA inhibitors bound to the enzyme active site. Finally, the terminal moiety (tail) of the inhibitor also participates in other two hydrogen bonds with two water molecules: one involves the carboxamido oxygen atom (of 2.92 Å), the other one the nitrogen atom of the diethylaminoethyl moiety (of 2.97 Å). The tail of the inhibitor also makes extensive van der Waals contacts with amino acid residues situated on the rim of the entrance to the active site, such as His 4, Trp 5, and Phe 20, with distances between the triethylamino moiety of **1** and different atoms of these residues in the range of 3.44–4.27 Å (Fig. 3).

In conclusion, we report here a high resolution X-ray crystallographic study for the adduct of a topically acting antiglaucoma sulfonamide, the 2-*N*,*N*-diethylaminoethylamide of 5-(4-carboxybenzenesulfonamido)-

1,3,4-thiadiazole-2-sulfonamide, with the isozyme hCA II, responsible of aqueous humor secretion within the eye. All the structural elements of this inhibitor (except for the primary sulfonamide moiety, which binds to the Zn(II) ion of the enzyme in the usual way) show unprecedented interactions with different amino acid residues/water molecules present within the active site, explaining its very high affinity for hCA II, and allowing us to draw important conclusions for the design of better pharmacological agents of this type.[25]

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