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Carbonic Anhydrase Inhibitors: Binding of Indanesulfonamides to the Human Isoform II

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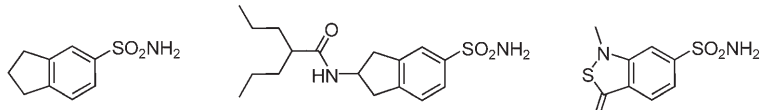
Indanesulfonamides are interesting lead compounds for designing selective inhibitors of the different isoforms of the zinc enzyme Carbonic Anhydrase (CA). Herein, we report for the first time the X-ray crystal structure of two such derivatives, namely indane-5-sulfonamide and indane-2-valproylamido-5-sulfonamide, in complex with the physiologically dominant human isoform II. The structural analysis reveals that, although these two inhibitors have quite similar chemical structures, the arrangement of their indane ring within the enzyme active site is significantly diverse. Thus, our findings suggest that the introduction of bulky substituents on the indane-sulfonamide ring may alter the binding mode of this potent class of CA inhibitors, although retaining good inhibitory properties. Accordingly, the introduction of bulky tail moieties on the indane-sulfonamide scaffold may represent a powerful strategy to induce a desired physicochemical property to an aromatic sulfonamide or to obtain inhibitors with diverse inhibition profiles and selectivity for various mammalian CAs.

Introduction

Known for decades, the carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes present in prokaryotes and eukaryotes and encoded by three distinct evolutionarily nonrelated gene families: α-CAs (in vertebrates, bacteria, algae, and cytoplasm of green plants), β-CAs (predominantly in bacteria, algae, and chloroplasts), and γ-CAs (mainly in archea and some bacteria).[1–3] In mammals, 16 isozymes are presently known, 13 of which are catalytically active (CAs I-VA, CA VB, CA VI, CA VII, CA IX, and CAs XII-XV), whereas the CA-related proteins (CARPs VIII, X, and XI) are devoid of catalytic activity.

In the last years, it has emerged that in addition to their well-known role for the development of diuretics and topically acting antiglaucoma drugs,[4,5] inhibitors of these enzymes may lead to novel applications, mainly as antiobesity and/or anticancer therapies.[6–10] In fact, two mitochondrial isoforms (CA VA and CA VB) are involved in lipogenesis and their inhibition leads to diminished fatty acid biosynthesis,[6,7] whereas at least two transmembrane, tumor associated isozymes (CA IX and CA XII) are highly overexpressed and involved in signaling/pH regulation processes within many types of hypoxic tumors.[8–10] Specific inhibitors for both type of such isoforms have been developed and some of them are in clinical evaluation.[11,12] In addition, many CAs isolated from other organisms were shown to be targets for the drug design, such as the α-CAs from *Plasmodium falciparum* and *Helicobacter pylori*,[13,14] the β-CAs from *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Candida albicans*, *Cryptococcus neoformans*, etc.[15–18] Work is in progress in several laboratories for developing specific inhibitors targeting these enzymes, which would lead to conceptually novel therapies.

Indanesulfonamides[19,20] were primarily obtained to better understand the drug design of anticonvulsant CA inhibitors (CAIs).[19–22] Indeed, Chazalette et al.[19] reported a series of such derivatives, starting with the parent, unsubstituted compound **1** (Figure 1), indane-5-sulfonamide, and some of its 1- and 2-amino derivatives. The amino group of these sulfonamides was then derivatized by means of acylation reactions with alkyl- and/or aryl-carboxylic acid chlorides.[19] In this way, starting from the medium-potency hCA II inhibitor **1** (K_i of 52 nM),[19] much more effective CAIs were produced, among which the



1 (K_i = 52 nM)[19] **2** (K_i = 3.4 nM)[19] **3** (IC_{50} = 245 nM)[25]

Figure 1. Structures of CA inhibitors.

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valproylamido compound **2** (Figure 1). In fact, the racemic mixture of this compound showed a K_i of 3.4 nM against hCA II and 1.6 nM against hCA I.[19] The pharmacological evaluation of this type of derivatives (initially tested only against hCA I and II)[19] was then extended to other isoforms with medicinal chemistry applications, with representatives of these sulfonamides acting as rather effective and also selective hCA IX and hCA VII inhibitors.[20,21] Indeed, isoform IX is a novel target for obtaining anticancer drugs,[8–12] whereas CA VII seems to be involved in important brain functions and might be the target (or one of the targets) of the anticonvulsant sulfonamides and sulfamates, together with the widespread isoform hCA II.[2,21–23]

Although molecular modeling studies for the interaction of some of these compounds with CA I, II, VII, IX, XII, and XIV have been recently published,[20,21] no X-ray crystal structure of any such derivative with a CA isozyme is available to date. Herein, we report for the first time such a study, as we obtained high resolution crystal structures for the adducts of two indanesulfonamide derivatives with the physiologically dominant human isoform II.

Results and Discussion

To understand at a molecular level the binding of two indanesulfonamides, that is, the simple, unsubstituted, medium potency inhibitor **1** and the valproylamido derivative **2** to hCA II, we investigated by means of X-ray crystallography the interactions of these compounds with this enzyme. Crystals of both enzyme–inhibitor complexes were isomorphous with those of the native protein,[24] allowing for the determination of their three-dimensional structures by difference Fourier techniques. The crystallographic R-factor/R-free values, calculated for the hCA II–**1** structure in the 20.00–1.60 Å resolution range and for the hCA II–**2** structure in the 20.00–1.40 Å resolution range were 0.177/0.196 and 0.185/0.199, respectively. The statistics for data collection and refinement for each complex are shown in Table 1.

Table 1. Crystal parameters, data collection, and refinement statistics for the hCA II–**1** and hCA II–**2** complexes.[a]

Crystal parameters	**1**	**2**
Space group	$P2_1$	$P2_1$
a [Å]	42.32	42.15
b [Å]	41.41	41.43
c [Å]	71.97	71.77
β [°]	104.43	104.22
Data collection statistics		
Resolution [Å]	(20.00–1.60 Å)	(20.00–1.40 Å)
Wavelength [Å]	1.200	1.000
Temperature [K]	100	100
Total reflections	110591	167683
Unique reflections	31132	46329
Completeness [%]	97.0 (92.0)	98.0 (91.6)
R-sym[b] [%]	6.3 (27.1)	5.9 (30.8)
Mean I/sigma(I)	18.0 (3.4)	17.4 (3.1)
Refinement statistics		
R-factor[c] [%]	17.7	18.5
R-free[c] [%]	19.6	19.9
rmsd from ideal geometry:		
Bond lengths [Å]	0.006	0.005
Bond angles [°]	1.8	1.8
Number of protein atoms	2048	2064
Number of inhibitor atoms	26	23
Number of water molecules	346	345
Average B factor [Å^2]	13.40	14.19

[a] Values in parentheses refer to the highest resolution shell (1.66–1.60 Å for **1**; 1.45–1.40 Å for **2**). [b] $R\text{-sym} = \Sigma|I_i - \langle I\rangle|/\Sigma I_i$; over all reflections. [c] $R\text{-factor} = \Sigma|F_o - F_c|/\Sigma F_o$; *R*-free calculated with 5% of data withheld from refinement.

The structural analysis of the hCA II–**1** adduct clearly showed the presence in the enzyme active site of a single inhibitor molecule, entirely defined by its electron density map (Figure 2). A second inhibitor molecule was found on the protein surface, far away from the active site and close to the enzyme N terminus. The presence of a second molecule having a much lower interaction affinity with the enzyme, as confirmed by its rather low occupation factor value (0.5), was considered as a crystallographic artifact because of the high ligand concentration used during crystallization set up. The inhibitor molecule located within the active site showed all the interactions generally observed in the complexes of hCA II with other sulfonamides, whose structure has been solved so far.[25–29] In particular, the ionized sulfonamide NH^- group coordinated to the catalytic Zn^{2+} ion (2.09 Å) with a tetrahedral geometry and donated a hydrogen bond to Thr199OG (2.70 Å). One sulfonamide oxygen accepted a hydrogen bond from the backbone NH group of Thr199 (2.88 Å), whereas the other one was at a distance of 2.95 Å from the Zn^{2+} ion. The indane ring of the inhibitor did not establish polar interactions within the enzyme active site, but made a large number of strong (< 4.5 Å) hydrophobic contacts

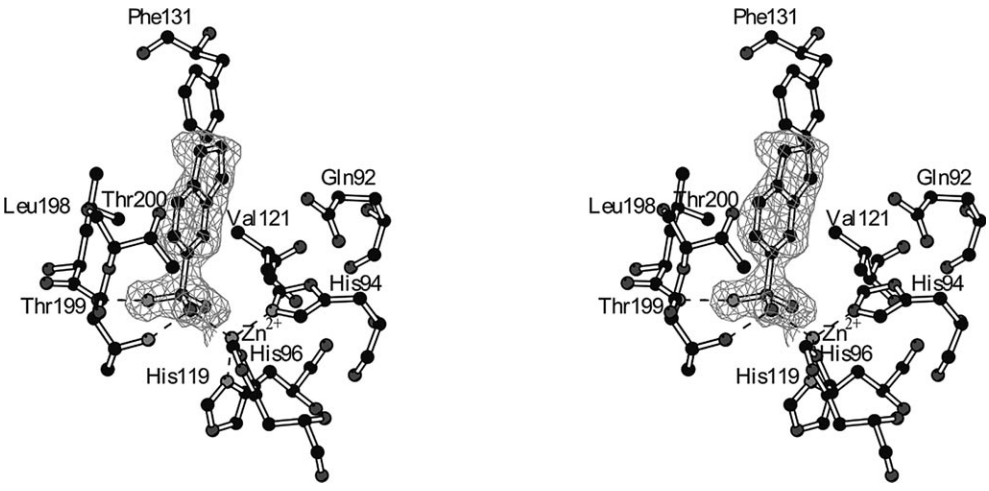

Figure 2. Stereoview of the active site region in the hCA II–**1** complex. The simulated annealing omit $|2F_o - F_c|$ electron density map relative to the inhibitor molecule is shown.

with residues Gln92, His94, Val121, Phe131, Leu198, Thr199, and Thr200.

The analysis of the electron density maps in the hCA II–**2** complex showed that, although the racemic mixture of compound **2** was present in the crystallization solutions, only the *R* enantiomer was observed bound to the active site (Figure 3).

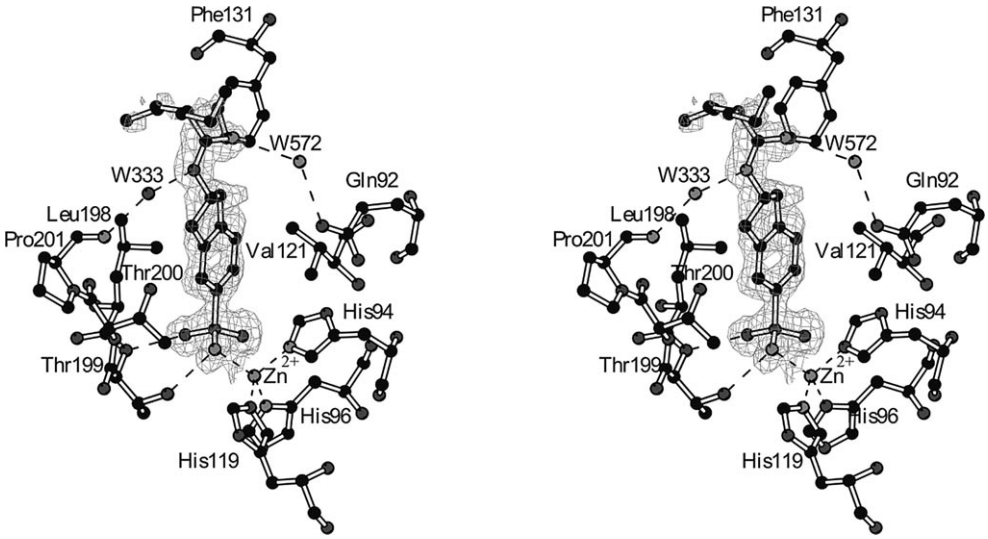

Figure 3. Stereoview of the active site region in the hCA II–**2** complex. The simulated annealing omit $|2F_o-F_c|$ electron density map relative to the inhibitor molecule is shown.

The maps were well defined for the amido-indane-sulfonamide moiety of the inhibitor, whereas they showed a poorer definition for the valproyl tail, thus suggesting a significant flexibility of the latter group within the enzyme active site, as expected for such a long aliphatic chain attached to a rigid ring system. The main protein–inhibitor interactions are schematically depicted in Figure 3. According to this figure, the deprotonated primary sulfonamide nitrogen atom replaces the hydroxyl ion/water molecule coordinated to Zn^{2+} in the native enzyme, with a Zn^{2+}–N distance of 2.11 Å. The Zn^{2+} ion remains in a stable tetrahedral geometry, being also coordinated by the imidazolic nitrogen atoms of His94, His96, and His119. On the other hand, the sulfonamide nitrogen is also involved in a hydrogen bond with the side-chain oxygen of Thr199 (2.80 Å), which in turn interacts with the Glu106OE1 atom (2.55 Å). One oxygen of the primary sulfonamide moiety accepts a hydrogen bond from the backbone NH of Thr199 (2.94 Å), whereas the other one is located 2.83 Å away from the catalytic Zn^{2+} ion. Several interactions stabilize the organic scaffold of the inhibitor within the active site cavity (Figure 3). In particular, the indane ring establishes a number of strong hydrophobic interactions (distance <4.5 Å) with residues Gln92, Val121, Phe131, Leu198, and Thr200, whereas the amide bond is stabilized by van der Waals contacts with Phe131 and by a hydrogen bond network involving water molecules W333 and W572 and residues Pro201 and Gln92 (see Figure 3). Finally, the valproyl tail is oriented toward a hydrophobic region of the active site, where it poorly interacts with the enzyme, and is partially exposed on protein surface.

Although **1** and **2** have quite similar structures, the arrangement of their indane moieties within the active site showed significant differences. In fact, the indane ring plane within the hCA II–**2** complex was rotated of about 180° with respect to that observed in hCA II–**1** counterpart. Even though in the first case this moiety established fewer van der Waals contacts with the enzyme (28 in number) with respect to those established by the same moiety in the hCA II–**1** complex (37 in number), this structural rearrangement seemed necessary to avoid a steric clash between the bulky valproylamide functionality of **2** and the residues present in the hydrophobic region within the active site. The hypothesis of a structural rearrangement of the inhibitor, caused by the insertion of a bulky substituent on the indane ring, found a confirmation in the crystal structure of the hCA II–**3** complex reported by Klebe's group (Figure 1).[25] This compound is a submicromolar hCA II inhibitor, which was developed using virtual screening of some compound libraries.[25] Compound **3** is structurally related to **1**, as it incorporates an unsubstituted thiazolinone ring. The binding mode of this compound to the hCA II active site was evidenced by the analysis of the enzyme–inhibitor adduct.[25] The structural overlay of **1**, **3**, and **2** bound to hCA II (Figure 4) showed a great

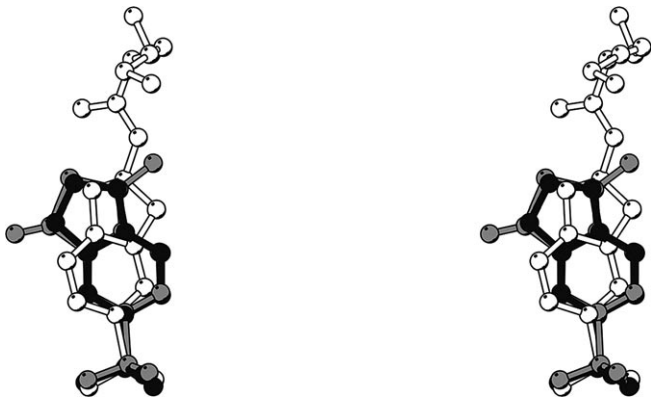

Figure 4. Stereoview of inhibitors **1** (colored in black), **2** (colored in white), and **3** (colored in gray) when bound to the hCA II active site.

level of similarity in the binding mode of the first two inhibitors, as compared to the latter one. In fact, the thiazolinone moiety of **3** was oriented similarly to that observed for the indane ring of **1**, both participating in a large number of hydrophobic contacts with the enzyme active site.

In agreement with the observed diverse binding mode, sulfonamides **1** and **2** presented a different inhibitory property, as

demonstrated by the inhibition constant values reported in this work. Indeed, the introduction in compound **2** of a bulky valproylamido moiety on the indane ring led to a 15-fold increase of the hCA II inhibitory properties, as compared to the unsubstituted sulfonamide **1**. This increase can be ascribed to the supplementary interactions to which the valproylamido fragment of compound **2** participates when the inhibitor is bound within the active site.

In conclusion, the structural analysis here reported clearly shows that the introduction of bulky substituents on the indane-sulfonamide ring may modify the binding mode of this potent class of CA inhibitors, although retaining good inhibitory properties. These findings are also in agreement with the recent investigations of indanesulfonamides CA IX and CA VII inhibitors, by means of docking studies.[20,21] Consequently, the introduction of bulky tails on indane-sulfonamides may represent a powerful strategy to induce the desired physicochemical properties (that is, enhanced liposolubility) to an aromatic sulfonamide scaffold or to obtain inhibitors with diverse inhibition profiles and selectivity for various mammalian CAs.

Experimental Section

Crystals of hCA II–**1** and hCA II–**2** complexes were obtained by co-crystallization experiments, adding a 5 molar excess of inhibitor to a 10 mg mL^{-1} protein solution in 100 mM Tris- HCl buffer, pH 8.5. The precipitant solution contained for both adducts 2.5 M $(NH_4)_2SO_4$, 0.3 M NaCl, 100 mM Tris-HCl (pH 8.2), and 5 mM 4-(hydroxymercurybenzoic) acid, to improve the crystal quality. Crystals grew within a few days and were isomorphous to those of the native enzyme.[24] X-ray diffraction data were collected at 100 K, at the Synchrotron source Elettra in Trieste, using a Mar CCD detector. The resolutions of the data collections were 1.60 Å and 1.40 Å for the hCA II–**1** and for hCA II–**2** complexes, respectively. Prior to cryogenic freezing, the crystals were transferred to the precipitant solution with the addition of 15% (*v*/*v*) glycerol. Diffracted data were processed using the HKL crystallographic data reduction package.[30] Diffraction data for each crystal were indexed in the $P2_1$ space group with one molecule in the asymmetric unit. Unit cell parameters and data reduction statistics are summarized in Table 1. The two enzyme–inhibitor complex structures were analyzed by difference Fourier techniques, using the atomic coordinates of the native hCA II (PDB entry 1CA2)[24] as starting model. The refinements were carried out with the program CNS,[31] whereas the model building and map inspections were performed using the O program.[32] Fourier maps calculated with $(3F_o-2F_c)$ and (F_o-F_c) coefficients showed prominent electron density features in the active site regions. After initial refinement limited to the enzyme, inhibitor molecules were gradually built into the models for further refinement. Restraints on inhibitor bond angles and distances were taken from similar structures in the Cambridge Structural Database and standard restraints were used on protein bond angles and distances throughout refinement. The ordered water molecules were added automatically and checked individually. Each peak contoured at 3σ in the $|F_o|-|F_c|$ maps was identified as a water molecule, provided that hydrogen bonds would be allowed between this site and the model. The correctness of stereochemistry was finally checked using PROCHECK.[33] Final refinement statistics for both structures are reported in Tables 1. Coordinates and structure factors have been deposited with the Protein Data Bank (accession codes 2QOA and 2QO8).

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Keywords: carbonic anhydrase II · enzyme–inhibitor complexes · medicinal chemistry · sulfonamide design · X-ray structure

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