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Carbonic anhydrase inhibitors: X-ray crystallographic structure of the adduct of human isozyme II with EMATE, a dual inhibitor of carbonic anhydrases and steroid sulfatase

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Abstract—The X-ray crystal structure for the adduct of human carbonic anhydrase II (hCA II) with estrone-3-*O*-sulfamate (EMATE), an antiendocrine agent showing both CA and estrone sulfatase inhibitory properties, has been resolved at a resolution of 1.5 Å. Its binding to the enzyme is similar to that of other sulfamates/sulfonamides, considering the interactions of the zinc anchoring group, but differs considerably when the steroidal scaffold of the inhibitor is analyzed. This part of the inhibitor interacts only within the hydrophobic half of the CA active site, interacting with residues Val 121, Phe 131, Val 135 and Pro 202, and leaving the hydrophilic half able to accommodate several water molecules not present in the uncomplexed enzyme. In addition, a very short bond of 1.78 Å between the zinc ion and the coordinated nitrogen atom of the sulfamate moiety is observed, which may explain the high affinity of this inhibitor for hCA II (K_i of 10 nM).

1. Introduction

Estrone-3-*O*-sulfamate (EMATE) **1** has recently been shown[1] to act as an efficient inhibitor of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1).[2,3] Originally, this compound was designed as an inhibitor of steroid sulfatase, among which estrone sulfatase (ES) and dehydroepiandrosterone sulfatase (DHEAS), represent the key therapeutic targets for estrogen dependent tumors.[4,5] ES/DHEAS catalyze the hydrolysis of estrone sulfate (E1S) and DHEA-sulfates (DHEAS), respectively, releasing the corresponding unconjugated steroids.[4,5] Both, these classes of pharmacological agents, that is CA inhibitors and steroid sulfatase inhibitors, seem to represent promising alternatives for the management of different types of cancers,[6,7] since several CA isozymes (such as CA IX and CA XII among others) were shown to be predominantly present in tumors, lacking from their normal counterparts,[8,9] and their inhibition may lead to an effective therapeutic outcome.[10,11] Inhibition of ES/DHEAS on the other hand with sulfamates was also shown to constitute an useful therapeutic approach against breast cancer,[4,5] or for the design of immune modulators, as it was demonstrated that ES/DHEAS inhibition has a role in regulating T-helper cell function.[12] EMATE was, however, found to be estrogenic in rodents which may limit its anticancer applications in favour of non-steroidal sulfamates. EMATE, however, behaves as a very effective CA inhibitor against several isozymes, such as CA I, II and IX, exhibiting inhibition constants of 37 nM against hCA I, 10 nM against hCA II and 30 nM against the tumor associated isozyme hCA IX,[1a] being as effective an inhibitor as acetazolamide **2** or ethoxzolamide **3**, clinically used sulfonamide CA inhibitors.[2,3]

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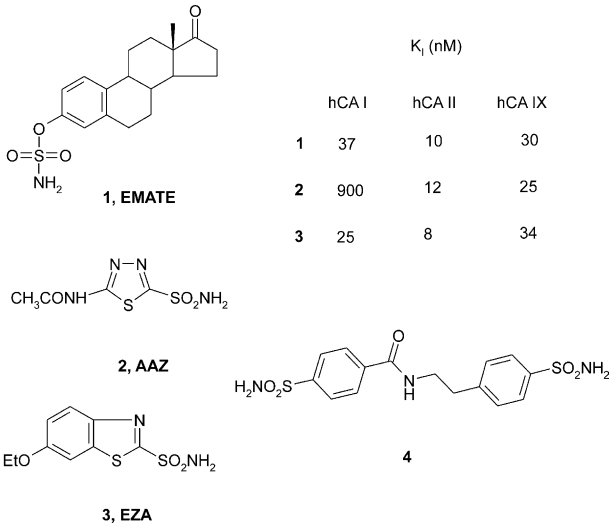

	K_I (nM)		
	hCA I	hCA II	hCA IX
1	37	10	30
2	900	12	25
3	25	8	34

In order to understand at a molecular level the factors that explain the high affinity of this sulfamate CA inhibitor (EMATE, **1**) for the most abundant isozyme in humans, hCA II,[2,3] and also to allow us to develop better enzyme inhibitors incorporating this new zinc-binding function,[13] an X-ray crystallographic study for the hCA II–**1** adduct has been performed, and is reported here. The structure of this adduct showed evidence of 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 agent.

2. Chemistry

Compound **1** was prepared as previously reported by our groups.[1,4c] As mentioned earlier, **1** is a very strong hCA II inhibitor (K_i of 10 nM). In contrast to the clinically used CA inhibitors (such as **2** and **3**, for example), EMATE contains a sulfamate zinc-binding group, and not a sulfonamide one. This type of compound has only recently started to be investigated for their CA inhibitory properties.[1,6,13]

3. Crystallography

The hCA II–**1** adduct obtained by co-crystallization, was subjected to detailed X-ray crystallography. The data were processed with MPSFLM,[14] and the programs SHELX97[15] and O[16] 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} of 0.23). The final number of water molecules was 241; the data collection parameters and the refinement statistics are reported in Table 1. A final refinement resolution of 1.5 Å has been achieved.[17]

The structure refinement allowed us determine the spatial arrangement of the inhibitor within the active site of the enzyme (Figs. 1 and 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.

Table 1. Statistics of data collection and refinement for the hCAII–EMATE adduct

	hCA II–EMATE complex
Resolution range (Å)	30–1.5
Space group	$P2_1$
Unit cell (Å, ° for β)	$a=41.6$, $b=40.4$, $c=71.4$, $\beta=104.4$
Highest resolution shell (Å)	1.60–1.50
No. of reflections	36,774
Completeness (%)	99.2 (96.1)
R_{sym} (%)	11.3
Refined residues	261
Refined water molecules	241
Resolution range in refinement (Å)	30–1.5
R_{cryst} ($F_o > 4\sigma F_o$; F_o)	20.7, 19.5
R_{free} ($F_o > 4\sigma F_o$)	23.2
Rms deviations	
Bond lengths (Å)	0.01
Bond angles (Å)	0.02
Average B value (Å^2)	19.2
Ramachandran plot	
Most favored (%)	88.0
Additionally allowed (%)	11.5
Generously allowed (%)	0.5
Disallowed (%)	0.0

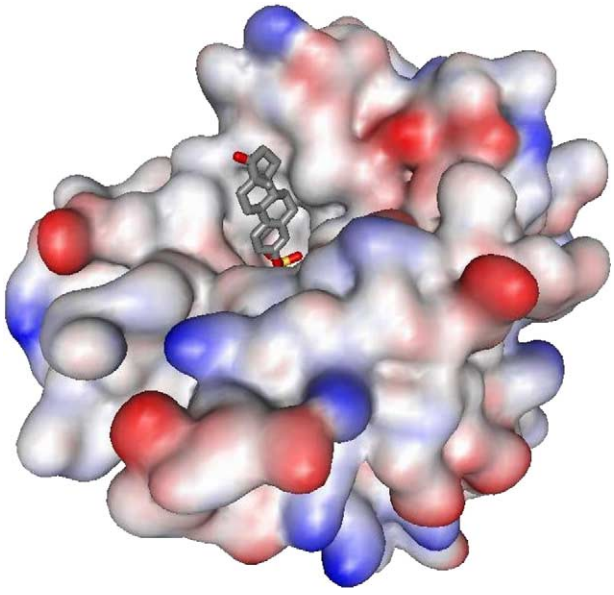

Figure 1. Binding of EMATE **1** within the hCA II active site.

As can be seen from these figures, the ionized sulfamate moiety of **1** replaces the hydroxyl ion/water molecule coordinated to Zn(II) in the native enzyme (Zn–N distance of 1.78 Å), as in other hCA II–sulfonamide/sulfamate complexes for which the X-ray structures have been reported (Figs. 1–3).[13,18–21] 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 one factor favoring the high affinity of EMATE for hCA II.[13,18–21] The Zn(II) ion remains in its stable tetrahedral geometry being coordinated, in addition to the sulfamate nitrogen, by the imidazolic nitrogens of His 94, His 96 and His 119. The proton of the coordinated sulfamate 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

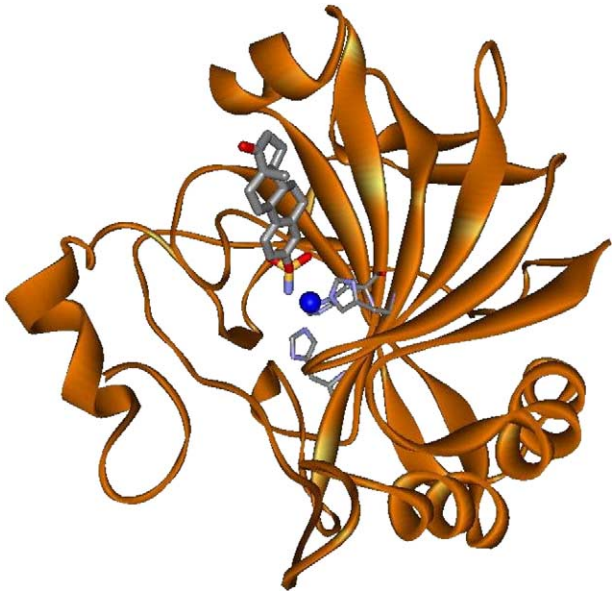

Figure 2. The hCA II–**1** adduct: the enzyme is shown as ribbon diagram, with the zinc ion (central blue sphere) and its protein ligands (His 94, 96 and 119) evidenced. The inhibitor molecule lies towards the hydrophobic half of the enzyme cavity.

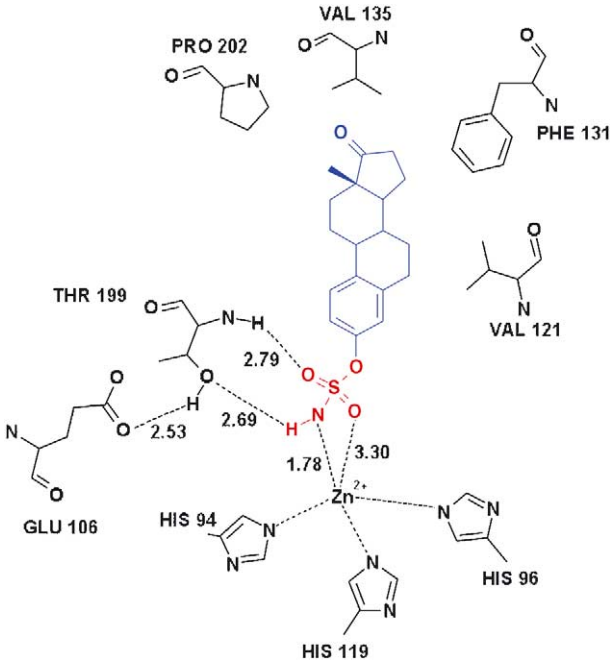

Figure 3. Detailed schematic representation of inhibitor **1** binding within the hCA II active site (figures represent distances in Å).

of Glu 106 (Fig. 3). One of the oxygen atoms of the coordinated sulfamate moiety makes a hydrogen bond with the backbone amide of Thr 199 (of 2.79 Å), whereas the other one is semi-coordinated to the catalytic Zn(II) ion (O–Zn distance of 3.30 Å). These interactions are generally seen in all complexes of hCA II with sulfonamides and sulfamates.[13,18–21] The steroidal moiety of the inhibitor **1** lies in the hydrophobic part of the active site cleft, making efficient van der Waals contacts with the side chains of Val 121, Phe 131, Val 135, Leu 204, and Pro 202 (Fig. 3). The carbonyl oxygen of EMATE does *not* make any hydrogen bond with amino acid residues within the active site, which was a rather unexpected finding. Thus, the most notable and unprecedented feature observed in this complex is 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 several amino acid residues; however, it makes practically no contacts with residues of the hydrophilic half of the active site, which have been shown to be critical for the binding of all other types of inhibitors investigated by means of X-ray crystallography.[13,18–21] Furthermore, in the particular complex discussed here, this part of the active site contains several water molecules which are not present in the structure of the uncomplexed CA II (data not shown). Only one other compound was recently shown to participate in this type of interaction when complexed to hCA II: the bis-sulfonamide **4**, the high resolution crystal structure of which, in complex with hCA II, was recently reported by us.[21] **4** Binds only in the hydrophobic half of the active site, interacting with the same amino acid residues as EMATE, and not making hydrogen bonds with the hydrophilic amino acid residues of it. The broad features of the EMATE interaction were demonstrated in a prior docking study by some of us.[1b] The binding mode in this case is similar to the present structure, albeit with the steroid system rotated by ca. 90° compared to the present structure and biased towards a different hydrophobic region, the C ring being close to Asn 60. The Zn–N distance in the docked model is 1.91 Å.[1b]

Thus, the present structural data allowed us to establish a very particular binding mode of **1** within the CA active site, with two interactions not previously observed for other hCA II–sulfamate complexes: (i) the appreciable shortening of the zinc–nitrogen sulfamate bond in the complex of EMATE with hCA II, together with the classical interactions of the zinc-binding group with amino acid residues Thr 199 and Glu 106; (ii) the binding of the organic part of the inhibitor only within the hydrophobic half of the active site, leaving the hydrophilic half able to accommodate several water molecules not present in the uncomplexed enzyme.[22]

By varying the different structural elements present in the molecule of **1** (such as for example the nature/substitution pattern of the steroidal ring; the nature of the zinc binding function, etc.) it can be envisaged that even more potent CA inhibitors with different types of application in biomedical sciences can be designed.

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17. The soaked crystal was isomorphous to the native enzyme, being mono-clinic $P2_1$ with the following cell parameters: $a=41.6$, $b=40.4$, $c=71.4$, $\beta=104.4^\circ$. The Fourier maps $2F_o-F_c$ and F_o-F_c were then calculated, where Fc and phases were obtained from the native hCA II model from which all the water molecules have been omitted. The difference Fourier maps after the first refinement cycle, before the assignment of water molecules, already showed clear evidence for the presence of the inhibitor molecule, whose occupancy was at the end of the refinement of 100%. The hCA II–EMATE complex data were collected at Elettra Synchrotron in Trieste (Italy) on a 165 mm MarCCD detector at 100 mm from the crystal, using radiation of 1.00 Å wavelength and about 15-s exposure and 110 K.
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