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Carbonic Anhydrase Inhibitors: X-ray Crystallographic Structure of the Adduct of Human Isozyme II with the Perfluorobenzoyl Analogue of Methazolamide. Implications for the Drug Design of Fluorinated Inhibitors

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The X-ray crystal structure for the adduct of human carbonic anhydrase (hCA) II with 4-methyl-5-perfluorophenylcarboximido-δ^2-1,3,4-thiadiazoline-2-sulfonamide (PFMZ), a topically acting antiglaucoma sulfonamide, has been resolved at a resolution of 1.8 Å. This compound is almost 10 times more effective as a hCA II inhibitor (K_I of 1.5 nM) compared to the lead molecule, methazolamide, a clinically used drug (K_I of 14 nM). Its binding to the enzyme active site is similar to that of other sulfonamide inhibitors, considering the interactions of the sulfonamide zinc anchoring group and thiadiazoline ring contacts, but differs considerably when the perfluorobenzoylimino fragment of the molecule is analyzed. Indeed, several unprecedented strong hydrogen bonds involving the imino nitrogen, carbonyl oxygen, a fluorine atom in the *ortho* position of the inhibitor, and two water molecules, as well as Gln 92 of the enzyme active site were seen. A stacking interaction of the perfluorophenyl ring of the inhibitor and the aromatic ring of Phe 131 was also observed for the first time in a CA–sulfonamide adduct. All these findings prove that more potent CA inhibitors incorporating perfluoroaryl/alkyl tails may be designed, with potentially improved antiglaucoma properties, in view of the new types of interactions seen here between the enzyme and the perfluorobenzoylated analogue of methazolamide.

Keywords: Perfluorobenzoyl; Carbonic anhydrase; Glaucoma; Methazolamide; Sulfonamide; X-ray crystallography

INTRODUCTION

4-Methyl-5-perfluorophenylcarboximido-δ^2-1,3,4-thiadiazoline-2-sulfonamide (PFMZ, **1**), the pentafluorobenzoyl analogue of methazolamide **2**, 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, there has been increased interest in them as potential agents for the treatment of macular edema, a condition for which no effective therapy is known up to now.[2–5] Among the many types of topically acting sulfonamides reported to date,[2] the compounds incorporating fluorinated tails, such as perfluorophenylsulfonyl-, perfluorobenzoyl-, or perfluorobutylsulfonyl- 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, i.e., CA II and CA IV (for example **1** shows an inhibition constant of 1.5 nM against hCA II and of 8 nM against bCA IV);[1] (ii) the water solubility of these derivatives is good (in the range of 1–2%) but at the same type they possess a rather high lipophilicity, due to the presence of the fluorinated tails in their molecules.[1] As a consequence, such compounds are easily formulated as eye drops at physiological pH values and their *in vivo* efficacy, in animal models of glaucoma, was much higher than that of the clinically available

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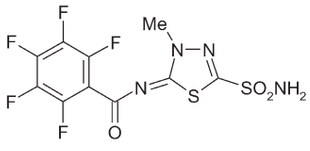
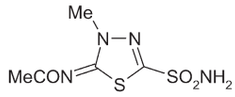
topically acting antiglaucoma sulfonamides dorzolamide **3** and brinzolamide **4**.[1]



1 (PFMZ)



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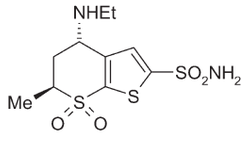

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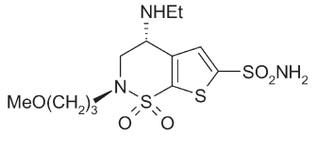

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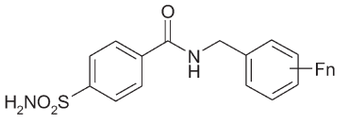

5. n = 1 - 5

In order to understand at the molecular level the factors that may explain the enhanced affinity of this type of fluorinated CA inhibitor for the most abundant isozyme within the eye, CA II, and also to allow us to develop better topically acting enzyme inhibitors incorporating fluorinated tails, an X-ray crystallographic study for the hCA II–**1** adduct has been performed, and is reported here. The structure of this adduct showed unprecedented interactions between the inhibitor and the enzyme active site and may be useful for the drug design of more active fluorinated CA inhibitors.

MATERIALS AND METHODS

Chemistry

PFMZ **1** was prepared as reported previously, from the imino derivative of methazolamide and perfluorobenzoyl chloride under Schotten–Baumann conditions.[1] hCA II, buffers and other reagents were from Sigma-Aldrich.

Crystallography

Crystals of hCAII (from Sigma-Aldrich) were obtained by the hanging drop technique, using 0.3 mM solution of protein in 100 mM TRIS–HCl buffer (pH 8.2).[7] The drops consisted of 6 μL of the enzyme solution and 6 μL of the precipitant solution containing 2.5 M $(NH_4)_2SO_4$ in 100 mM TRIS–HCl (pH 8.2) and 5 mM 4-(hydroxymercury)benzoate to promote the growth of highly oriented crystals. The drops were equilibrated by vapor diffusion against the precipitant solution at 4°C and crystals appeared after 10–12 days. The complex of hCAII with PFMZ was obtained by soaking the crystals of the protein for 5 days in a solution of 2.5 M $(NH_4)_2SO_4$ in 100 mM TRIS–HCl (pH 8.2) and about 50 μM inhibitor at 4°C. The soaked crystal was isomorphous to the native enzyme, being mono-clinic $P2_1$ with the following cell parameters: $a = 42.01$ Å, $b = 40.91$ Å, $c = 70.55$ Å, and $\beta = 104.0°$. The Fourier maps 2Fo–Fc and Fo–Fc were then calculated, where Fc and phases were obtained from the native hCA II model[8] from which all the water molecules had been omitted. The difference Fourier maps were inspected to detect evidence for inhibitor binding before the assignment of water molecules. The hCA II-PFMZ complex data were collected at Elettra Synchrotron in Trieste (Italy) on a 165 mm MarCCD detector at 70 mm from the crystal, using radiation of 1.00 Å wavelength and 20 s exposure and 100 K. All calculations were done with SHELX97 and XtalView.[9,10] These programs were used to build the model, to perform its refinement and to compute the Fourier maps. The last refinement cycle yielded a final *R* factor of 0.19 (Rfree of 0.27). The final number of water molecules was 217; the data collection parameters and the refinement statistics are reported in Table I.

RESULTS AND DISCUSSION

Chemistry

Fluorinated CA inhibitors have attracted much interest recently due to their strong antiglaucoma properties when administered topically.[1] In addition to the compounds reported by this group, which incorporate perfluorophenylsulfonyl-, perfluorobenzoyl-, perfluorooctylsulfonyl-, trifluoromethylsulfonyl- or perfluorobutylsulfonyl- tails among others, and aromatic/heterocyclic sulfonamides as heads for anchoring to the CA active site,[1] Christianson's group reported simple amides of type **5**, incorporating from one to five fluorine atoms, as well as their inhibition data against wild type and mutated hCA II.[11] Furthermore, the X-ray crystal structure of inhibitors **5** in complex with a mutant of hCA II, i.e., the Phe131Val mutant, have also been investigated by means of X-ray crystallography, showing interesting interactions between the inhibitors, the active site and the crystal lattice (actually in solid phase, generally two molecules of **5** bind to the enzyme, one within the active site, the other within the scaffolding of the crystal lattice of the CA II mutant).[11,12] Although such studies are irrelevant for the drug design of antiglaucoma

sulfonamides, they are mentioned here since they provide evidence of interesting interactions between the fluorinated inhibitors when bound to the enzyme. In the present work we have investigated by means of high resolution X-ray crystallography the interaction between one of our best topically acting perfluorinated sulfonamides, compound **1** (PFMZ, 4-methyl-5-perfluorophenylcarboximido-δ^2-1,3,4-thiadiazoline-2-sulfonamide)[1] and the isozyme principally responsible for aqueous humor secretion within the eye, hCA II. PFMZ is a very potent hCA II inhibitor (K_I of 1.5 nM) and although quite similar structurally to methazolamide **2**, it has an almost 10 times higher affinity for this isozyme as compared to the clinically used, systemically acting drug **2** (methazolamide has a K_I of 14 nM against hCA II under the same assay conditions as **1**).[1] Obviously the presence of the perfluorophenyl tail in the molecule of **1** explains the difference of activity between the two inhibitors discussed here, **1** and **2**. The reasons of the highly enhanced affinity of **1** for hCA II will be understood after discussing the structure of its adduct with the enzyme in the next section.

Crystallography

The refined structure of the hCA II–PFMZ complex does not show any conformational changes of the protein tertiary structure. The r.m.s. deviation of the alpha carbon atoms superimposed on the native structure was of 0.123 Å. The final $|F_{obs}| - |F_{calc}|$ map (Figure 1) shows well a defined electron density for all inhibitor atoms.

Figure 2 shows the details of the hCA II active site complexed with PFMZ, whereas in Figure 3 the schematic representations of all the interactions of this inhibitor with relevant active site residues are presented.

The ionized sulfonamide moiety of PFMZ has replaced the hydroxyl ion coordinated to Zn(II) in the native enzyme (Zn–N distance of 1.95 Å), as in other hCA II–sulfonamide complexes for which the X-ray structures have been reported (Figure 2).[7,11–14] The Zn(II) ion remains in its stable tetrahedral geometry, being coordinated in addition of the sulfonamidate nitrogen, by the imidazolic nitrogens of His 94, His 96 and His 119. The proton of the coordinated sulfonamidate nitrogen atom also makes a hydrogen bond with the hydroxyl group of Thr 199, which in turn accepts a hydrogen bond from the carboxylate of Glu 106. One of the oxygen atoms of the 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 3.01 Å). These interactions are generally seen in all complexes of hCA II with sulfonamides and sulfamides.[7,11–14] The thiadiazoline ring of the inhibitor lies in the hydrophobic part of the active site cleft, where its ring atoms make van der Waals interactions with the side chains of Leu 204, Pro 202, Leu 198 and Val 135 (Figures 2 and 3). The carbonyl oxygen of PFMZ makes a strong hydrogen bond with the backbone amide nitrogen of Gln 92 (of 2.87 Å), an interaction also seen for the acetazolamide- hCA II adduct[13] as well as for the recently reported topiramate–hCA II adduct.[15] It is

TABLE I Statistics of data collection and refinement for the hCAII-PFMZ adduct

	PFMZ–hCA II complex
Resolution Range (Å)	40–1.8
Space group	$P2_1$
Unit cell (Å, ° for β)	a = 42.0, b = 41.0, c = 70.5, β = 104.0
Highest resolution shell (Å)	1.85–1.80
No. of reflections	20188
Completeness (%)	92.8 [82.9]
Mean I/σ_I	24.3 [2.2]
R_{sym} (%)	8.4 [48.4]
Refined residues	261
Refined water molecules	217
Resolution range in refinement (Å)	30–1.8
R_{cryst} ($F_o > 4\sigma F_o$; F_o)	18.7, 19.6
R_{free} ($F_o > 4\sigma F_o$)	19.2
Rms deviations	
Bond lengths (Å)	0.005
Bond angles (Å)	0.021
Average B value (Å^2)	25.4
Ramachandran plot	
Most favored (%)	86.6
Additionally allowed (%)	12.0
Generously allowed (%)	1.4
Disallowed (%)	0.0

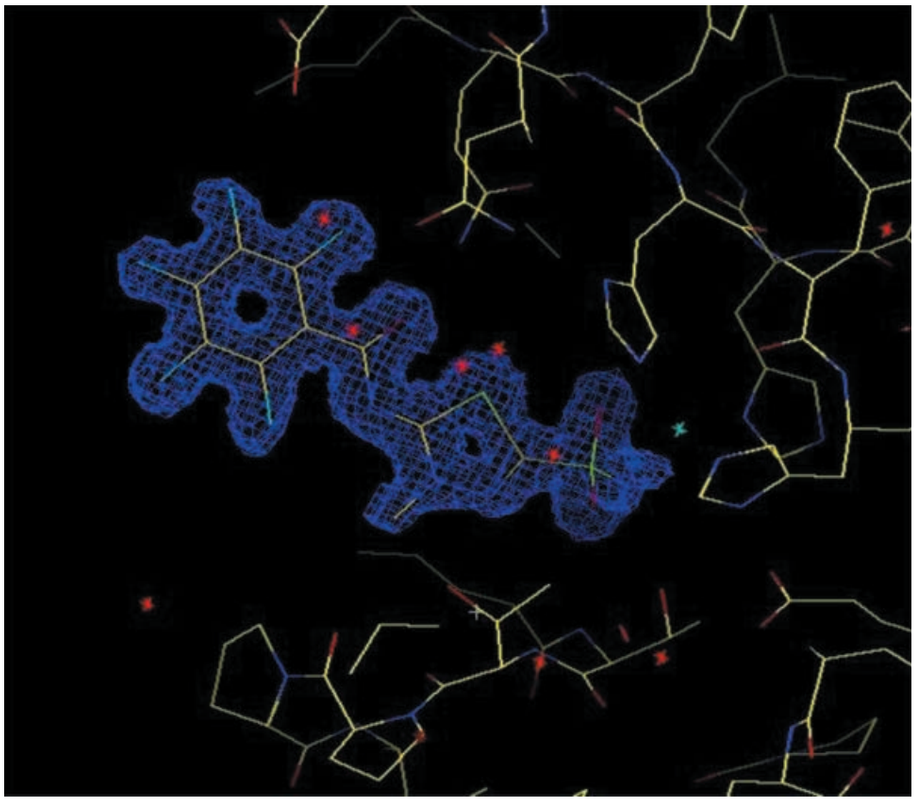

FIGURE 1 $|F_{obs}| - |F_{calc}|$ electron density map of the hCA II–PFMZ complex at 1.8 Å resolution.

clear that the presence of moieties in the inhibitor molecule able to participate in a strong hydrogen bond with this residue (Gln 92) situated at the entrance of the active sit cleft leads to enhanced affinity of the inhibitor for the CA active site. Besides Gln 92, two other residues situated in the hydrophilic half of the CA active site, i.e., Glu 69 and Asn 67, make van der Waals contacts with the PFMZ molecule complexed to hCA II (Figure 3). But the most notable and unprecedented interactions seen in this complex regard the hydrogen bond network involving the exocyclic nitrogen atom of the inhibitor, two water molecules (Wat 1194 and Wat 1199) and a *ortho* fluorine atom belonging to the perfluorobenzoyl tail of PFMZ (Figure 3). Thus, a strong hydrogen bond (of 2.97 Å) is seen between the imino nitrogen of PFMZ and Wat 1194, which in turn makes a hydrogen bond with a second water molecule of the active site, Wat 1199 (with a distance of 2.73 Å). The second hydrogen of Wat 1194 also participates in a weaker hydrogen bond (3.32 Å) with the carbonyl oxygen of PFMZ. The other hydrogen atom of Wat 1199 makes a weak hydrogen bond with the fluorine atom in ortho position of the perfluorobenzoyl tail of PFMZ (Figure 3). Finally, a very interesting interaction is observed between the perfluorophenyl ring of PFMZ and the phenyl moiety of Phe 131, a residue critical for the binding of inhibitors with long tails to hCA II.[16] Indeed, these two rings are almost perfectly parallel, being situated at a distance of 3.4–4.7 Å. This type of stacking interactions has never been observed in a hCA II–sulfonamide adduct (it should be mentioned that the fluorinated sulfonamides **5** previously investigated by X-ray crystallography were crystallized with a mutant lacking just this essential amino acid residue, i.e., the Phe131Val mutant).[11,12] As recently discussed by Christianson's group for inhibitors of type **5**,[11] it is probable that the perfluorination of the phenyl ring of PFMZ diminishes π electron density in this aromatic ring making it more suitable for participation in such stacking interactions with the unfluorinated aromatic ring of Phe 131. On the other hand, it is also possible that a charge-transfer complexation[11] may occur between these two structural elements in the E-I adduct discussed here. All these interesting and strong interactions between structural elements belonging to the perfluorobenzoylimino tail of the inhibitor **1** and amino acid residues situated at the entrance of the hCA II active site explain the almost 10 times increase in affinity of PFMZ for hCA II, as compared to the parent molecule, methazolamide, a systemic antiglaucoma sulfonamides still in clinical use.

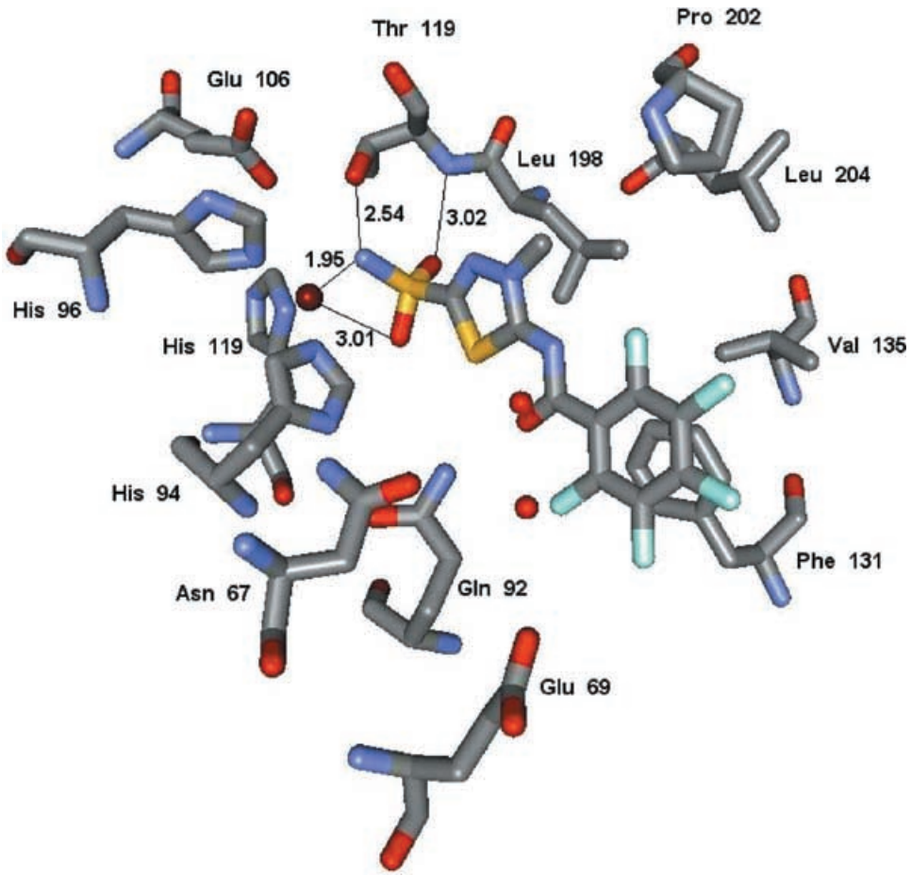

FIGURE 2 PFMZ **1** bound to hCA II active site. The zinc ion, its three histidine ligands (His 94, His 96, and His 119) as well as other amino acid residues involved in binding of the inhibitor are shown. Distances between different atoms are shown in Å.

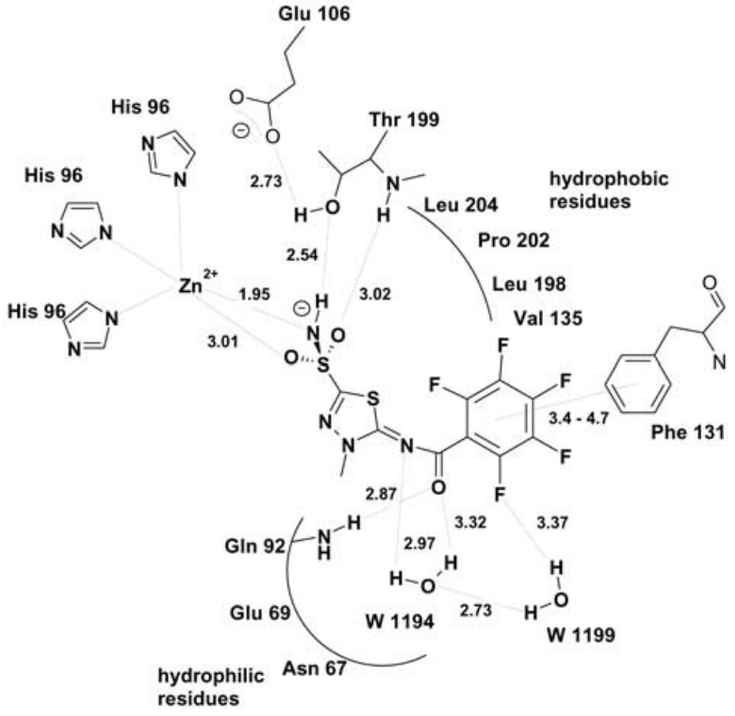

FIGURE 3 Schematic representation of PFMZ binding to the hCA II active site (numbers represent distances in Å).

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