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Carbonic anhydrase inhibitors. Comparison of chlorthalidone, indapamide, trichloromethiazide, and furosemide X-ray crystal structures in adducts with isozyme II, when several water molecules make the difference

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ABSTRACT

Thiazide and high ceiling diuretics were recently shown to inhibit all mammalian isoforms of carbonic anhydrase (CA, EC 4.2.1.1) with a very different profile as compared to classical inhibitors, such as acetazolamide, methazolamide, and ethoxzolamide. Some of these structurally related compounds have a very different behavior against the widespread isozyme CA II, with chlorthalidone, trichloromethiazide, and furosemide being efficient inhibitors against CA II (K_Is of 65–138 nM), whereas indapamide is a much weaker one (K_I of 2520 nM). Furthermore, some of these diuretics are quite efficient (low nanomolar) inhibitors of other isoforms, for example, chlorthalidone against hCA VB, VII, IX, and XIII; indapamide against CA VII, IX, XII, and XIII, trichloromethiazide against CA VII and IX, and furosemide against CA I and XIV. Examining the four X-ray crystal structures of their CA II adducts, we observed several (2–3) active site water molecules interacting with the chlorthalidone, trichloromethiazide, and furosemide scaffolds which may be responsible for this important difference of activity. Indeed, indapamide bound to CA II has no interactions with active site water molecules. Chlorthalidone bound within the CA II active site is in an enolic (lactimic) tautomeric form, with the enolic OH also participating in two strong hydrogen bonds with Asn67 and a water molecule. The newly evidenced binding modes of these diuretics may be exploited for designing better CA II inhibitors as well as compounds with selectivity/affinity for various isoforms with medicinal chemistry applications.

1. Introduction

Thiazide and high ceiling diuretics represent a class of pharmacological agents with a firm place in clinical medicine, and many representatives which share several common structural features such as the presence of free SO_2NH_2 moieties attached to a chlorobenzene scaffold, with the halogen atom in *ortho* to the sulfamoyl group, as well as various other usually bulky groups substituting the aromatic scaffold.[1] Drugs such as hydrochlorothiazide, chlorthalidone, indapamide, trichloromethiazide, or furosemide among others were the first well-tolerated and efficient antihypertensive drugs that significantly reduced cardiovascular disease (CVD) morbidity and mortality in placebo-controlled clinical studies.[2–6] Most of them are widely used in patients with CVD but are also used for those suffering from type II diabetes, obesity, and related metabolic complications, and are highly prescribed.[1–6] The mechanism of action of thiazide drugs is more complicated as initially thought, as apart from the diuretic/saluretic one,[1–6] it has recently been shown that many such sulfonamides exert a direct vasodilator effect by activating calcium-activated potassium channels (KCa),[4c] which in turn are dependent on the pH control of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) which catalyzes the interconversion between carbon dioxide and bicarbonate, with a release of a proton.[1,7] Furthermore, among all such sulfonamide diuretics, chlorthalidone seems to be the most effective agent,[2] together with indapamide.[2a] Recently, we[8,9] reinvestigated the CA inhibitory properties of this class of sulfonamides which have been launched in a period when only isoforms CA I and II were known, among the 16 mammalian CAs presently characterized in vertebrates.[7] Indeed, this family of enzymes comprises several cytosolic isoforms (CA I–III, CA VII, and CA XIII), five membrane-bound isozymes (CA IV, CA IX, CA XII, CA XIV, and CA XV), two mitochondrial forms (CA VA and VB), as well as one secreted CA isozyme, CA VI. Three acatalytic isozymes are also known, that is, CA VIII, CA X, and CA XI.[7,10–12] These enzymes are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and several other physiologic/pathologic processes.[7–13]

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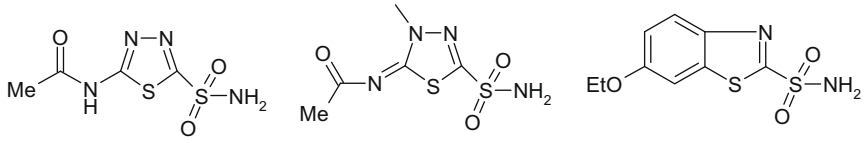

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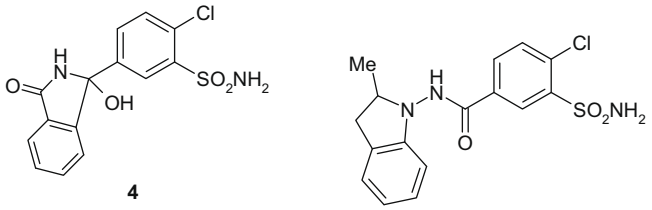

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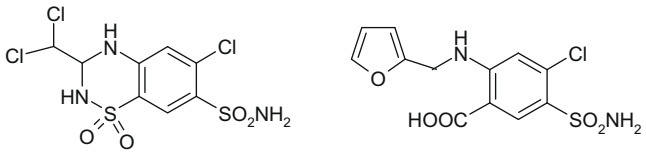

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Sulfonamide CA inhibitors (CAIs), such as acetazolamide **1**, methazolamide **2,** or ethoxzolamide **3** among others, are clinically used agents for the management of a variety of disorders connected to CA disbalances, such as glaucoma; in the treatment of edema due to congestive heart failure, or for drug-induced edema; such as mountain sickness drugs,[7–13] whereas other agents of this pharmacological class show applications as anticonvulsants,[14] antiobesity[15], or antitumor drugs/tumor diagnostic agents.[7,11,16] Sulfonamides such as chlorthalidone **4**, indapamide **5**, trichloromethiazide **6,** and furosemide **7** are widely used as diuretics,[1,8,9] as mentioned above, whereas many classes of new CAIs are constantly being reported due to at least two reasons: (i) a large number of catalytically active isoforms are known (12 in primates, 13 in other mammals)[7], which are drug targets for many types of applications, as mentioned above.[7–16] Indeed, for example, the antiglaucoma sulfonamide drugs target CA II and IV, the antiobesity CAIs target CA VA and CA VB, the anticonvulsant ones probably target CA VII and XIV, whereas the antitumor drugs/diagnostic agents target the transmembrane isoforms overexpressed in tumors, CA IX and XII;[7–16] and (ii) the lack of isozyme selectivity of the presently available, clinically used compounds.[7]

In recent reports, we have reinvestigated the inhibition activity of sulfonamide diuretics against all catalytically active mammalian CAs, that is, isoforms CA I–CA XIV, of human (h) or murine (m) origin.[8,9] Several interesting facts emerged from this study, among which the potent inhibition observed with some of these drugs against isozymes involved in important pathologies, such as cancer and obesity. For example, low nanomolar (or even subnanomolar) inhibitors were detected, for example, chlorthalidone **4** against CA VB, VII, IX, XII, and XIII, indapamide **5** against CA VII, IX, XII, and XIII, furosemide **7** against CA I, II, and XIV.[8,9] We have also resolved the X-ray crystal structure of indapamide **5**, a modest CA II inhibitor, in complex with this ubiquitous, house-keeping isoform.[8] An unexplained issue of the preceding study[8] was, however, the important differences in the inhibition profiles of these CAs with the structurally related drugs such as chlorthalidone **4**, indapamide **5**, trichloromethiazide **6,** and furosemide **7**, with compounds **4, 6,** and **7** being generally much better CA II inhibitors (K_Is of 65–138 nM), compared to indapamide **5** (K_I of 2520 nM). Here, we discuss the X-ray crystal structures for the adducts of chlorthalidone **4,** indapamide **5**, trichloromethiazide **6,** and furosemide **7** with hCA II, and by comparing them we unravel new binding modes of these diuretics. Primarily, we evidence the interaction between several (between 2 and 3) water molecules present within the enzyme active site for the hCA II–chlorthalidone/trichloromethiazide/furosemide adducts, which lack in the corresponding hCA II–indapamide complex, and which may be used for designing better CA II inhibitors as well as compounds with potential selectivity for various isoforms with medicinal chemistry applications, among the 12 such targets presently known.

2. Results and discussion

2.1. Chemistry and CA inhibition

Chlorthalidone **4**, indapamide **5**, trichloromethiazide **6,** and furosemide **7** possess several common structural features. Thus, all these compounds incorporate a 2-chloro-benzenesulfonamide 'warhead', which is present in many CAIs,[7] since the deprotonated sulfonamide moiety constitutes the zinc binding group, which is coordinated to the catalytic Zn(II) ion present at the bottom of the enzyme active site.[6–13] In *meta* to the sulfamoyl moiety, these drugs (except **6**) possess a bulky moiety, constituted by a benzoannulated five-membered ring incorporating a nitrogen heterocycle for **4** and **5**, or a COOH moiety for furosemide **7**. Trichloromethiazide is obviously slightly different, as the 2-chloro-benzenesulfonamide ring is annulated to the thiadiazine-*S*,*S*-dioxide ring, incorporating the $CHCl_2$ substituent. Although there are so many structural similarities among compounds **4**–**7,** their CA inhibition profiles are very different (see later in the text).

Inhibition data of the clinically used sulfonamide CAIs such as acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, chlorthalidone **4**, indapamide **5,** trichloromethiazide **6,** and furosemide **7** against isoforms CA I–XIV are shown in Table 1.

It may be observed that the classical sulfonamide CAIs **1–3** are promiscuous inhibitors of all isozymes except CA III that is known to possess a low affinity for this class of inhibitors.[18] In fact, all isozymes are generally inhibited by these compounds with inhibition constants <100 nM (except CA I inhibition with acetazolamide **1** and CA IV inhibition with methazolamide **2**), but with many low

Table 1
Inhibition data with some of the clinically used sulfonamides **1–7** against isozymes I–XIV (the isoforms CA VIII, X, and XI are devoid of catalytic activity and probably do not bind sulfonamides as they do not contain Zn(II) ions)[1,7]

Isozyme*	K_I** (nM)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
hCA I[a]	250	50	25	348	51,900	345	62
hCA II[a]	12	14	8	138	2520	91	65
hCA III[a]	2.0×10^5	7.0×10^5	1.1×10^6	1.1×10^4	2.3×10^5	5.6×10^5	3.2×10^6
hCA IV[a]	74	6200	93	196	213	449	564
hCA VA[a]	63	65	25	917	890	763	499
hCA VB[a]	54	62	19	9	274	134	322
hCA VI[a]	11	10	43	1347	1606	2459	245
hCA VII[a]	2.5	2.1	0.8	2.8	0.23	7.9	513
hCA IX[b]	25	27	34	23	36	87	420
hCA XII[b]	5.7	3.4	22	4.5	10	312	261
mCA XIII[a]	17	19	50	15	13	645	550
hCA XIV[a]	41	43	2.5	4130	4950	3450	52

[a] Full length enzyme.
[b] Catalytic domain.
* h = human; m = murine isozyme. nt = not tested, data not available.
** Mean value from at least three different measurements.[14] Errors were in the range of ±5% of the obtained value (data not shown).

nanomolar or even subnanomolar inhibition patterns being present in the data of Table 1. It is thus obvious that the simple, classical CAIs **1–3** are not good leads for designing isozyme-selective CAIs. However, data of Table 1 also show that compounds **4–6**, possessing more complicated scaffolds as compared to **1–3**, show a much more interesting inhibition profile, with some isoforms being very well inhibited and others being weakly inhibited by these sulfonamides. For example, chlorthalidone **4** is a medium potency inhibitor of CA I, II, and IV (K_Is in the range of 138–348 nM), a very effective inhibitor of CA VB, CA VII, CA IX, CA XII, and CA XIII (K_Is in the range of 2.8–23 nM), and an ineffective inhibitor of CA III, VA, VI, and XIV (K_Is in the range of 917–1.1×10^4 nM). Structurally related to **4,** indapamide **5** is a quite ineffective inhibitor of CA I, II, III, VA, VI, and XIV (K_Is in the range of 890–2.3×10^5 nM), a medium potency inhibitor of CA IV and CA VB (K_Is in the range of 213–274 nM), and a very effective inhibitor of CA VII, IX, XII, and XIII (K_Is in the range of 0.23–36 nM). Inhibition data for trichloromethiazide **6** against various CA isoforms are not available in the literature and they are reported here for the first time (the hydrochlorothiazide (with a H instead of the $CHCl_2$ moiety present in **6**) inhibition data were recently released, see Ref. 7). It may be observed that **6** is an efficient inhibitor of CA II and IX (K_I of 87–91 nM) a very potent CA VII inhibitor (K_I of 7.9 nM). Trichlorometiazide is then a medium potency inhibitor of isoforms hCA I, IV, VB, and XII (K_Is in the range of 134–449 nM), being a much less effective inhibitor for hCA III, VA, VI, mCA XIII, and hCA XIV (K_Is in the range of 645–5.6×10^5 nM, Table 1). Furosemide **7** is a potent inhibitor of hCA I, II, and XIV (K_Is in the range of 52–65 nM), moderately inhibiting all other isoforms except hCA III (K_Is in the range of245–564 nM against hCA IV–mCA XIII). As all other sulfonamides known to date, furosemide is a weak hCA III inhibitor (K_I of 3.2 μM, Table 1).

2.2. X-ray crystallography

The overall hCA II-**4** complex is shown in Figure 1, whereas Figures 2 and 3 as well as Table 2 show the detailed interactions of the inhibitors **4–7** when bound within the enzyme active site. Figure 4 shows the superposition of the hCA II-**4**, hCA II-**5**, hCA II-**6,** and hCA II-**7** adducts.

Interactions between the protein and Zn^{2+} ion are entirely preserved in the hCA II adducts with inhibitors **4–7** (Table 2, Figs. 1–3), as in all other hCA II–sulfonamide/sulfamate/sulfamide complexes investigated so far.[8,9,13,17] A careful analysis of the three-dimensional structure of the complex revealed a compact binding between the inhibitor and the enzyme active site, similar to that observed earlier for other such complexes, with the tetrahedral geometry of the Zn^{2+} binding site and the key hydrogen bonds between the SO_2NH_2 moiety of the inhibitors and enzyme active site all retained (Figs. 2 and 3, and Table 2).[8,9,13,17] In particular, the ionized nitrogen atoms of the sulfonamides group of **4–7** are coordinated to the zinc ion at a distance of 2.00–2.15 Å, (Table 2). This nitrogen is also hydrogen bonded to the hydroxyl group of Thr199 (N···Thr199OG = 2.77–2.95 Å), which in turn interacts with the Glu106OE1 atom (2.50 Å, data not shown). One oxygen atom of the sulfonamide moiety is 2.89–3.06 Å away from the catalytic Zn^{2+} ion, which is considered as 'semi'-coordinated to the metal ion, whereas the second one participates in a hydrogen bond

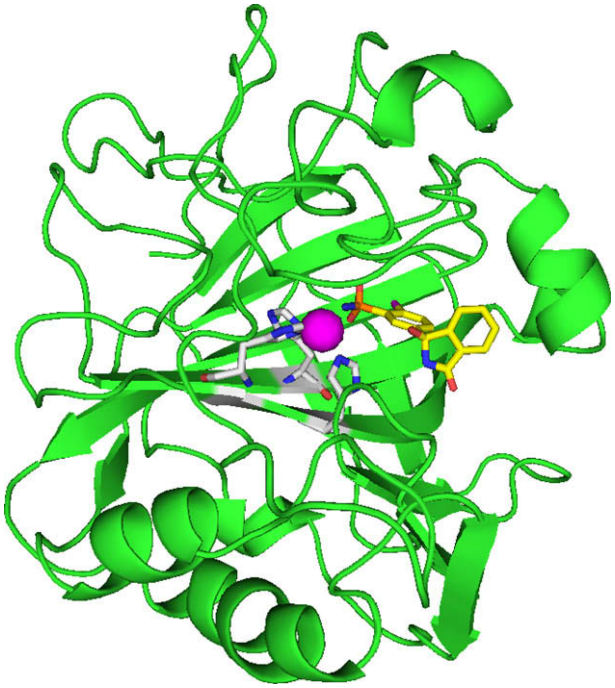

Figure 1. The hCA II–chlorthalidone **4** complex. The protein backbone is shown in green, the catalytic Zn(II) ion as violet sphere, its three histidine ligands (His94, 96, and 119) as CPK color, and the inhibitor **4** in yellow.

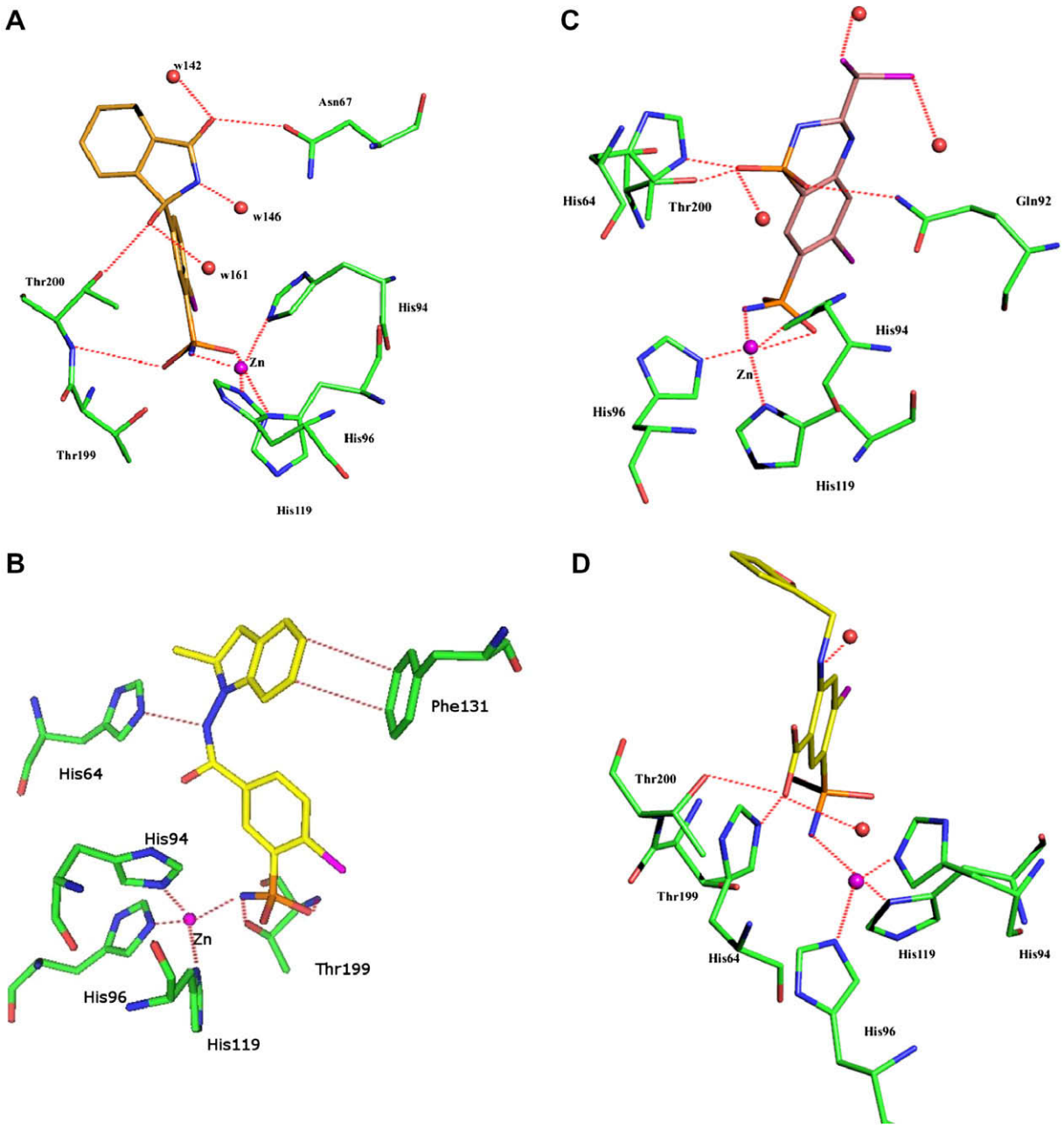

Figure 2. Detailed interactions in which chlorthalidone **4** (in gold) (A), indapamide **5** (in yellow) (B), trichloromethiazide **6** (in wheat) (C), and furosemide **7** (in yellow) (D) participate when bound within the hCA II active site. Active site residues coordinating the metal ion (His94, 96, 119) as well as those involved in the binding of inhibitors are also shown. Ordered water molecules critical for the binding of some of these inhibitors to the CA II active site are evidenced as red spheres.

(of 2.92–3.19 Å) with the backbone amide group of Thr199.[8,13,17] On the other hand, very interesting interactions have been evidenced between the OH moiety and NH–C=O fragments present in the five-membered cycle of chlorthalidone **4** and the hCA II active site amino acid residues/water molecules. Thus, the hydroxyl moiety participates in two hydrogen bonds, with the OH group of Thr200 (of 2.54 Å), and with a water molecule, W161, of 3.16 Å (Figs. 2 and 3A). The nitrogen atom of this fragment also makes a strong hydrogen bond (of 2.65 Å) with a second water molecule, W146 (Fig. 3A). Probably, the asymmetric center at the carbon atom bearing the OH and chlorobenzenesulfonamide moieties, coupled with the planarity of the isoindole ring, explains the very compact binding of chlorthalidone to the CA II active site. Indeed, most of the distances shown in Table 2 and Figure 3A–D are 0.10–0.17 Å shorter than the corresponding ones in other such adducts, for example, with the structurally related sulfonamides **5**–**7**.[8,9,17] However, the most interesting feature of chlorthalidone bound to CA II regards the endocyclic carboxamido moiety present in this sulfonamide diuretic. Indeed, the oxygen atom (OA3 in the crystallographic numbering of Table 2) of this moiety participates in two strong hydrogen bonds, one with a water molecule, W142, of 2.83 Å, and the second one with the oxygen present in the $CONH_2$ group of Asn67, of 2.70 Å (Figs. 2 and 3A). It is obvious that in order to participate in both these hydrogen bonds, chlorthalidone must adopt the enolic (lactimic) tautomeric form shown in Figure 3A, when bound to the CA II active site. This is, as far as we know, the first ever evidence showing that probably the biologically active form of this drug is the enolic one evidenced in this

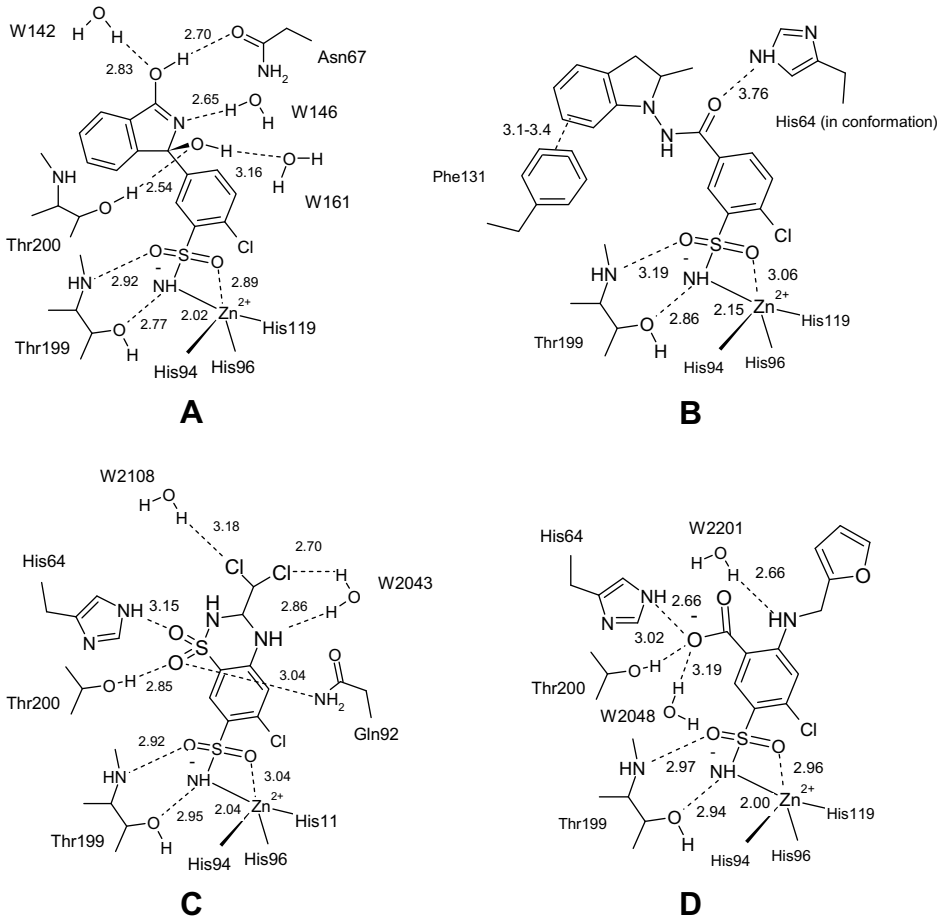

Figure 3. Comparison of interactions in which chlorthalidone **4** (**A**), indapamide **5** (**B**), trichloromethiazide **6** (C), and furosemide **7** (D) participate when bound to the hCA II active site.

study. For the hCA II–**5** adduct, only a stacking interaction of the benzoannulated ring of **5** with the phenyl group of Phe131 and a weak hydrogen bond (of 3.76 Å) with the imidazole of His64 (in its *in* conformation) were detected, when indapamide is bound within the active site (Fig. 3B). As mentioned above, the corresponding moieties of chlorthalidone participate in many more strong hydrogen bonds (of 2.54–3.16 Å) with Thr200, Asn67 as well as three water molecules (W142, 146, and 161), interactions which are absent in the hCA II–indapamide adduct (Fig. 3A). Thus, the particular enantiomeric form in which chlorthalidone **4** exists when bound to the hCA II active site, its distinct orientation and additional three ordered water molecules explain the higher affinity of this compound (18.3 times) for hCA II, as compared to indapamide **5**. The importance of active site-ordered water molecules is even more reinforced when examining the remaining two adducts of hCA II with trichloromethiazide **6** (Figs. 2 and 3C) and furosemide **7** (Figs. 2 and 3D), which contain two such water molecules each. These interactions between such water molecules, various moieties of the inhibitor, and amino acid residues may thus explain the higher stability of the enzyme inhibitor adducts for compounds **4, 6,** and **7**, which are by orders of magnitude better hCA II inhibitors as compared to the structurally related indapamide **5** (Table 1). In particular, for the hCA II–**6** complex, in addition to the Zn(II) coordination of the sulfamoyl moiety of the inhibitor and participation in the hydrogen bond network with Thr199/Glu106 (Table 2 and Fig. 3C), the following strong polar interactions have been evidenced: (i) three strong hydrogen bonds involving the inhibitor endocyclic SO_2 moiety, with the OH of Thr200, the imidazole of His64 and the terminal amide of Gln92, of 2.85–3.15 Å (Table 2 and Fig. 3C); and (ii) three hydrogen bonds involving two water molecules (W2043 and W2108) and the two chlorine atoms as well as one endocyclic NH moiety of the thiadiazine ring of the inhibitor molecule, of 2.70–3.18 Å (Fig. 3C). In the case of the hCA II–furosemide **7** adduct, again the benzenesulfonamide fragment of the inhibitor is anchored to the Zn(II) ion of the enzyme active site in a similar manner as for the previously discussed adducts of sulfonamides **4–6** (Table 2 and Fig. 3D), but the different substituents in *meta* and *para* to the sulfamoyl anchor lead to very different interactions with water molecules/amino acid residues from the enzyme active site (Figs. 2 and 3D). Thus, the secondary amine group makes a strong hydrogen bond with a water molecule (of 2.66 Å), whereas the COOH group of furosemide (presumably a carboxylate at the pH 7.5 of the crystallization buffer) makes three such strong hydrogen bonds, of 2.66–3.19 Å (confirming thus that it is in the form COO^-) with a water molecule (W2048), the OH of Thr200 and the imidazole of His64 (these two interactions were exactly the same in the hCA II–**6** adduct discussed earlier, demonstrating that the COOH moiety of furosemide **7** and the endocyclic SO_2 one of trichloromethiazide **6** have very similar conformations in the corresponding adducts with hCA II). This is in fact observed also in the superposition of Figure 4.

By superposing the X-ray crystal structure of the chlorthalidone **4**-hCA II adduct with those of the adducts of the same isoform with the structurally related diuretics **5**–**7**, we can have some hints regarding the diverse inhibition profiles of these sulfonamides against various CAs, but also some insights for the de-

Table 2
Distances between atoms of chlorthalidone **4,** indapamide **5,** trichloromethiazide **6,** and furosemide **7,** and hCA II active site residues/metal ion, involved in the binding of the inhibitor within the enzyme active site in the four hCA II–inhibitor complexes

	hCA II residue	Distance (Å)
Chlorthalidone 4		
NA1	Zn	2.02
OA2	Zn	2.89
NA1	Oγ1 Thr199	2.77
OA1	N Thr199	2.92
OA3	Oδ1 Asn67	2.70
OA3	w 142	2.83
NA2	w 146	2.65
OA8	w 161	3.16
OA8	Oγ1 Thr200	2.54
Indapamide 5		
NAS	Zn	2.15
OAW	Zn	3.06
NAS	Oγ1 Thr199	2.86
OAV	N Thr199	3.19
NAR	Nδ2 His64	3.76
OAU	Nδ2 His64	3.95
CAG	Cε1 Phe131	3.41
CAH	Cε2 Phe131	3.46
Trichloromethiazide 6		
N15	Zn	2.04
O13	Zn	3.04
O16	Oγ1 Thr200	2.85
O17	Nε2 Gln92	3.04
O16	Nε2 His64	3.15
N8	w 2043	2.86
C119	w 2043	2.70
C120	w 2108	3.18
O17	w 2044	2.79
Furosemide 7		
N1	Zn	2.00
O1	Zn	2.96
O5	Oγ1 Thr200	3.02
O5	Nε2 His64	2.66
O5	w 2048	3.19
N2	w 2201	2.66

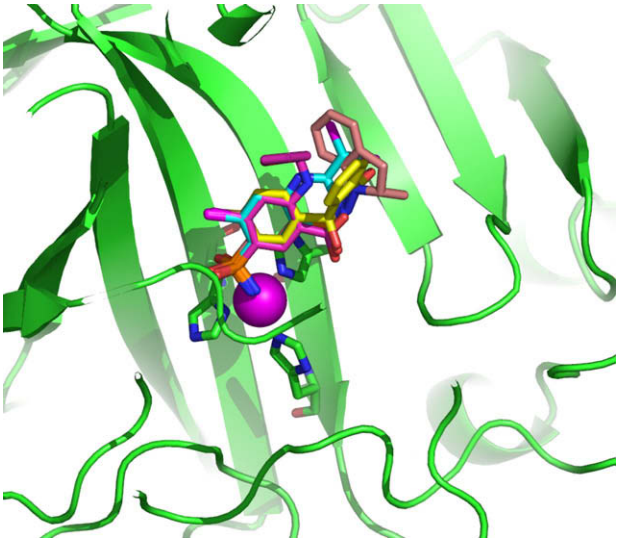

Figure 4. Superposition of the hCA II–chlortalidone **4** (yellow, PDB file 3F4X), hCA II–indapamide **5** (wheat, PDB file 3BL1), hCA II–triclorometiazide **6** (sky, PDB file 1ZGF), and hCA II–furosemide **7** (magenta, PDB file 1Z9Y) adducts. The His ligands coordinating the zinc ion (violet sphere) and protein backbones (green) of all four complexes are entirely superposable.

sign of CA II inhibitors with improved efficacy. Thus, data of Figs. 2 and 3 show that these structurally related sulfonamides (**4–7)** make many different interactions with amino acid residues and water molecules when bound within the hCA II active site. The main similarities for the binding of these CAIs to the enzyme active site regard the 2-chloro-benzenesulfonamide moiety present in all of them. Basically, the sulfonamidate zinc-binding groups are anchored in the same manner to the metal ion and Thr199 in all adducts (Fig. 3A–D), with the chlorophenyl-sulfamoyl fragments of **4–7** being entirely superposable, although there are small variations in the distance of the coordinative bond and the hydrogen bonds (Table 2 and Fig. 4). This fragment of the inhibitors occupies the channel leading from the bottom of the CA II active site toward the edge of the cavity, and making a host of favorable van der Waals interactions (data not shown) with many amino acid residues lining the active site. The presence of the chlorine atom in *ortho* to the sulfamoyl moiety of these inhibitors has no influence on the binding of the benzenesulfonamide warhead to the enzyme (Figs. 2–4). However, the conformation in which the remaining elements of the scaffolds of inhibitors **4–7** bind within the CA II active site and the interactions they make with amino acid residues and water molecules is very different for the four sulfonamide complexes examined here. Thus, the superposition of the four adducts shown in Figure 4 demonstrates that the protein scaffold is unchanged in these complexes, whereas the moiety substituting in the 3-position the benzenesulfonamide scaffolds of the inhibitors lies in very different active site regions extending toward the exit of the active site cavity. The benzoannulated heterocycle rings of **4** and **5** are in fact almost perpendicular to each other, and they make a host of favorable interactions with amino acids/water molecules in the case of the chlorthalidone adduct, and only two such contacts in the case of the indapamide adduct. For the trichloromethiazide adduct, the $CHCl_2$ fragment makes two strong hydrogen bonds with water molecules and is orientated in a part of the active site in which enough space is available to introduce various substituents, thus possibly leading to better CA II inhibitors. As far as we know, this possibility has not been explored synthetically up to now. On the other hand, the endocyclic SO_2 moiety of **6**, similar to the COOH moiety present in furosemide **7,** participates in three strong hydrogen bonds involving Thr200 and His64 in both adducts, as well as Gln92 for 6, and a water molecule for 7. It is also interesting to note that the aminomethylfuryl group present in furosemide does not make substantial contacts with amino acid residues, only participating in a hydrogen bond (the NH moiety) with a water molecule. Also these data allow us to propose that by changing the nature of substituents at this secondary amine, or by introducing various functionalities in the furane ring, it is probable that compounds with interesting CA inhibitory properties can be obtained easily. Work is in progress in this laboratory to exploit these structural data for obtaining CAIs with different inhibition profiles. As far as we know, this is also the first documented case in which ordered water molecules in the enzyme active site can lead to such a strong discrimination between structurally related inhibitors.

As the entrance of the active site cavity in the various CA isoforms is the region with least conserved amino acid residues among the investigated isozymes,[7–13] we may also explain the quite diverse inhibition profiles observed with sulfonamides **1–7** discussed here (Table 1). Indeed, compounds without a bulky tail orientated toward the exit of the active site cavity, such as **1–3**, indistinctly inhibit all CA isoforms with high efficacy (usually with inhibition constants <100 nM), leading to promiscuous, unselective CAIs. On the contrary, compound possessing such bulkier moieties, which bind toward the exit/edge of the active site cavity (such as **4–7**) and may thus interact differently with the various amino acid residues present in those regions, does indeed show some levels of selectivity or inhibiting certain CA isozymes, as exemplified here by the three discussed sulfonamides **4–7**. However, as the X-ray

crystal structure is not available yet for all CA isoforms (i.e., CA VB, VI, VII, IX, XIII, and XV)[7] the nature and number of these amino acid residues responsible for the selective inhibition of all CA isozymes are still unknown.

3. Conclusions

We reinvestigated the CA inhibitory activity of some thiazide, widely clinically used sulfonamide type diuretics. These compounds inhibit all mammalian CA isoforms but with a very different profile as compared to classical inhibitors, such as acetazolamide, methazolamide, and ethoxzolamide. Some of these diuretics have a very different behavior against the widespread isozyme CA II, with chlorthalidone, trichloromethiazide, and furosemide being efficient inhibitors against CA II (K_Is of 65–138 nM), whereas indapamide is a much weaker one (K_I of 2520 nM). Furthermore, some diuretics are quite efficient (low nanomolar) inhibitors of other isoforms, for example, chlorthalidone against hCA VB, VII, IX, and XIII; indapamide against CA VII, IX, XII, and XIII, trichloromethiazide against CA VII and IX, and furosemide against CA I and XIV. Examining the four X-ray crystal structures of their CA II adducts, we observed several (2–4) active site water molecules interacting with the chlorthalidone, trichloromethiazide, and furosemide scaffolds which may be responsible for this important difference of activity. Indeed, indapamide bound to CA II has no interactions with active site water molecules. Chlorthalidone bound within the CA II active site is in an enolic (lactimic) tautomeric form, with the enolic OH also participating in two strong hydrogen bonds with Asn67 and a water molecule. The newly evidenced binding modes of these diuretics may be exploited for designing better CA II inhibitors as well as compounds with selectivity/affinity for various isoforms with medicinal chemistry applications.

4. Experimental

4.1. Materials

Sulfonamides **1–7** are commercially available compounds (from Sigma–Aldrich, Milan, Italy). The twelve CA isozymes used in the experiments were recombinant ones obtained and purified as reported earlier by this group.[18–22]

4.2. CA inhibition assay

An Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining the ionic strength constant), following the CA-catalyzed CO_2 hydration reaction.[23] The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled–deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations), and dilutions up to 0.01 μM were done thereafter with distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, and represent the mean from at least three different determinations.[23]

4.3. X-Ray crystallography

The hCA II–**4** adduct was crystallized as previously described.[8,17] Diffraction data were collected under cryogenic conditions (100 K) on a CCD Detector KM4 CCD/Sapphire using Cu Kα radiation (1.5418 Å). The unit cell dimensions were determined to be a = 41.4 Å, b = 42.1 Å, c = 72.3 Å and $\alpha = \gamma = 90°$, $\beta = 104.3°$ in the space group P21. Data were processed with CrysAlis RED (Oxford Diffraction 2006).[24] The structure was analyzed by difference Fourier technique, using the PDB file 1CA2 as starting model. The refinement was carried out with the program REFMAC5;[25] model building, and map inspections were performed using the COOT program.[26] The final model of the complex CAII/Indapamide had an R-factor of 22.8% and R-free 29.0% in the resolution range 20.0–2.1 Å, with an rms deviation from standard geometry of 0.015 Å in bond lengths and 1.8° in angles. The correctness of stereochemistry was finally checked using PROCHECK.[27] Coordinates and structure factors have been deposited with the Protein Data Bank (accession code 3F4X).

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