Carbonic Anhydrase Inhibitors. Comparison of Chlorthalidone and Indapamide X-ray Crystal Structures in Adducts with Isozyme II: When Three Water Molecules and the Keto−Enol Tautomerism Make the Difference†

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Thiazide diuretics inhibit all mammalian isoforms of carbonic anhydrase (CA, EC 4.2.1.1) with a different profile as compared to classical inhibitors. Acting as moderate-weak inhibitors of CA II and CA I, chlorthalidone and indapamide considerably inhibit other isozymes among the 16 CAs present in vertebrates. These compounds show a different behavior against CAs I and II, with chlorthalidone being 18.3 times more potent against CA II and 150 times more potent against CA I, as compared to indapamide. In the X-ray crystal structures of the CA II−chlorthalidone adduct three active site water molecules interacting with the inhibitor scaffold were observed that lack in the corresponding indapamide adduct. Chlorthalidone bound within the active site is in an enolic tautomeric form, with the OH moiety participating in two strong hydrogen bonds with Asn67 and a water molecule. This binding mode may be exploited for designing better CA II inhibitors.

Introduction

Thiazide diuretics, such as hydrochlorothiazide, chlorthalidone, and indapamide, among others, were the first well-tolerated and efficient antihypertensive drugs that significantly reduced cardiovascular disease (CVD[a]) morbidity and mortality in placebo-controlled clinical studies.[1−6] These drugs still constitute a fundamental therapeutic option in patients with CVD but also for those suffering type II diabetes, obesity, and related metabolic complications, being highly prescribed.[1−6] Their mechanism of action is more complicated than 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),[4] 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.[7] As a consequence of the vasodilator effect of thiazide diuretics, which is primarily due to inhibition of vascular smooth muscle cell CAs, a rise in the intracellular pH (pHi) results, leading to KCa channel activation and vasorelaxation.[4] Such processes lead to improved endothelial function, reverse the abnormal arteriolar structure, and slow albumin permeation in hypertensive patients with nondiabetic or diabetic metabolic syndromes, producing an overall organ-protective, beneficial effect by preventing damage to the capillary structures and endothelium and reducing the hypertrophy of superficial glomeruli, among others.[1−6] Furthermore, among all such sulfonamide diuretics, chlorthalidone seems to be the most effective such 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 isoform CAs I and II were known, among the 16 mammalian CAs presently characterized in vertebrates.[7] Indeed, this family of enzymes comprises several cytosolic isoforms (CAs 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 (CAs VA and VB), and one secreted CA isozyme, CA VI. Three acatalytic isozymes are also known, i.e., 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]

Sulfonamide CA inhibitors (CAIs) such as acetazolamide **1**, methazolamide **2**, and ethoxzolamide **3** (Chart 1), 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, and 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** and indapamide **5** are widely used as diuretics,[8,9] as mentioned above, whereas many classes of new CAIs are constantly being reported because of at least two reasons:

(i) The large number of catalytically active isoforms (12 in primates, 13 in other mammals)[7] that are drug targets for many types of applications, as mentioned above.[7−16] Indeed, for example, the antiglaucoma sulfonamide drugs target CAs II and IV, the antiobesity CAIs target CA VA and CA VB, the anticonvulsant ones probably CAs VII and XIV, whereas the antitumor drugs/diagnostic agents target the transmembrane isoforms overexpressed in tumors, CAs IX and XII.[7−16]

(ii) The lack of isozyme selectivity of the presently available, clinically used compounds.[7]

† The X-ray coordinates of the hCA II−chlorthalidone adduct are available in PDB with the code 3F4X.

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[a] Abbreviations: CA, carbonic anhydrase; CAI, carbonic anydrase inhibitor; CVD, cardiovascular disease; hCA, human carbonic anydrase; KCa, calcium-activated potassium channels; mCA, murine carbonic anhydrase.

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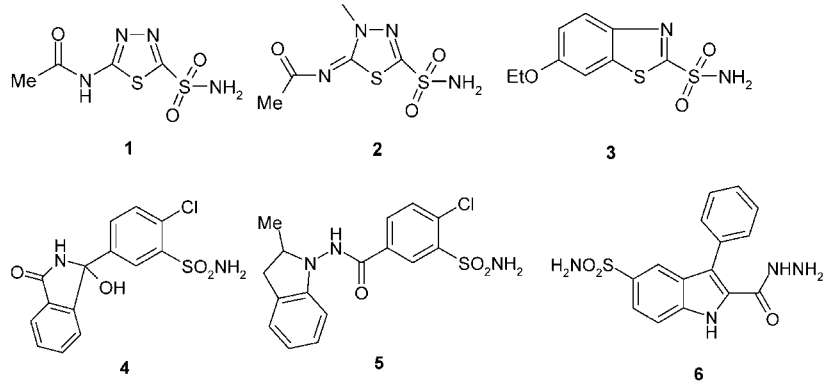
Chart 1



In a recent report we have reinvestigated the inhibition activity of sulfonamide diuretics against all catalytically active mammalian CAs, i.e., isoforms CA I−CA XIV, of human or murine origin.[8,9] Several interesting facts emerged from this study, among which is the potent inhibition observed with some of these drugs against isozymes involved in important pathologies, such as cancer and obesity. Low nanomolar (or even subnanomolar) inhibitors were detected, such as, for example, chlorthalidone **4** against CAs VB, VII, IX, XII, and XIII, indapamide **5** against CAs VII, IX, XII, and XIII, furosemide against CAs I, II, and XIV, and bumethanide against CAs IX and XII.[8,9] We have also reported the X-ray crystal structure of indapamide **5**, a modest CA II inhibitor, in complex with this ubiquitous, housekeeping isoform.[8] An unexplained issue of the preceding study[8] was, however, the important differences in the inhibition profiles of these CAs. Indeed the two structurally related drugs chlorthalidone **4** and indapamide **5** show very different inhibition profiles. Chlorthalidone **4** generally acts as a much better inhibitor of isozymes CAs I, II, IV, VB, IX, and XII, compared to indapamide **5**. Here, we report the high resolution X-ray crystal structure for the adduct of chlorthalidone **4** with hCA II, and by comparing it with that of the structurally related drug indapamide **5**, we discover a new binding mode of this diuretic never evidenced before, which exploits the interaction between some water molecules present within the enzyme active site and also favors the enol tautomric form of the bound drug. These findings may be used to design better CA II inhibitors as well as compounds with selectivity for various isoforms with medicinal chemistry applications, among the 12 such targets presently known.

Results and Discussion

Chemistry and CA Inhibition. Chlorthalidone **4** and indapamide **5** possess similar chemical structures and overall shapes of their molecules. Thus, both these compounds incorporate a 2-chlorobenzenesulfonamide moiety, which is present in many CAIs,[7] as the deprotonated sulfonamide moiety constitutes the zinc anchoring group, being coordinated to the catalytical Zn(II) ion present at the bottom of the enzyme active site.[6−13] At meta to the sulfamoyl moiety, both these drugs possess a bulky moiety, consituted by a benzoannulated five-membered ring incorporating a nitrogen heterocycle. The spacer between these two fragments is, however, different in the two drugs, as in chlorthalidone **4** there is a direct connection between them, whereas in indapamide **5** a CONH spacer connects the two fragments of the molecule. An OH moiety is also present as substituent at the connecting carbon atom between the heterocyclic and benzenesulfonamide rings of chlorthalidone, which also induces an asymmetry center, with the *S* enantiomer (used in this study) showing the highest activity as diuretic.[6b] Although there are many structural similarities between the two compounds **4** and **5**, as stressed above, their CA inhibition profiles are very different (see later in the text).

Table 1. Inhibition Data with Sulfonamides **1**−**6** against Isozymes I−XIV[a,1,2]

	K_I[c] (nM)					
isozyme[b]	**1**	**2**	**3**	**4**	**5**	**6**
hCA I[d]	250	50	25	348	51900	7.5
hCA II[d]	12	14	8	138	2520	7.2
hCA III[d]	2.0×10^5	7.0×10^5	1.1×10^6	1.1×10^4	2.3×10^5	1.4×10^6
hCA IV[d]	74	6200	93	196	213	9000
hCA VA[d]	63	65	25	917	890	1100
hCA VB[d]	54	62	19	9	274	1100
hCA VI[d]	11	10	43	1347	1606	2650
hCA VII[d]	2.5	2.1	0.8	2.8	0.23	89
hCA IX[e]	25	27	34	23	36	102
hCA XII[e]	5.7	3.4	22	4.5	10	110
mCA XIII[d]	17	19	50	15	13	2633
hCA XIV[d]	41	43	25	4130	4950	48

[a] The isoform CAs VIII, X, and XI are devoid of catalytic activity and do not bind sulfonamides because they do not contain Zn(II) ions. [b] h = human; m = murine isozyme. [c] Mean value from at least three different measurements.[14] Errors were in the range of ±5% of the obtained value (data not shown). [d] Full length enzyme. [e] Catalytic domain.

Inhibition data of the clinically used sulfonamide CAIs acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, chlorthalidone **4**, indapamide **5**, and the newly reported indolesulfonamide derivative **6**,[17] against isoforms CAs I−XIV are shown in Table 1.

It is observed that the classical sulfonamide CAIs **1**−**3** are promiscuous inhibitors of all isozymes except CA III, which is known to possess a low affinity for this class of compounds.[18] In fact, all isozymes are generally inhibited by these sulfonamides with inhibition constants of <100 nM (except CA I inhibition with acetazolmide **1** and CA IV inhibition with methazolamide **2**) but with many low nanomolar or even subnanomolar inhibition patterns being observed 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 CA I, II, and IV inhibitor (K_I values in the range of 138−348 nM), a very effective inhibitor of CA VB, CA VII, CA IX, CA XII, and CA XIII (K_I values in the range of 2.8−23 nM), and an ineffective inhibitor of CAs III, VA, VI, and XIV (K_I values in the range of (917 to 1.1×10^4

Table 2. Crystallographic Parameters and Refinement Statistics for the hCA II−Chlorthalidone **4** Complex

parameter	value
Crystal Parameter	
space group	$P2_1$
cell parameters	
a (Å)	41.4
b (Å)	42.1
c (Å)	72.3
β (deg)	104.3
Data Collection Statistics (20.0−2.0 Å)	
no. of total reflections	42768
no. of unique reflections	21386
completeness (%)[a]	99.4 (99.0)
$F_2/\sigma(F_2)$	15.0 (1.7)
R_{sym} (%)	14.3(17.3)
Refinement Statistics (20.0−2.0 Å)	
R-factor (%)	19.9
R-free (%)[b]	20.0
rmsd of bonds from ideality (Å)	0.010
rmsd of angles from ideality (deg)	1.40

[a] Values in parentheses relate to the highest resolution shell (2.0 Å). [b] Calculated using 5% of data.

Table 3. Distances between Atoms of Chlorthalidone **4** and hCA II Active Site Residues/Metal Ion, Involved in the Binding of the Inhibitor within the Enzyme Active Site

chlorthalidone **4**	hCA II residue	distance (Å)
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

nM). The structurally related indapamide **5** is a quite ineffective inhibitor of CAs I, II, III, VA, VI, and XIV (K_I values in the range of 890 to 2.3 × 10^5 nM), a medium potency CA IV and CA VB inhibitor (K_I values in the range of 213−274 nM), and a very effective inhibitor of CAs VII, IX, XII, and XIII (K_I values in the range of 0.23−36 nM). The recently reported bicyclic sulfonamide **6**[17] (possessing some structural resemblance to **4** and **5**) shows an even better, more isozyme selective inhibition profile as compared to **4** and **5**. As it is an efficient inhibitor of only CAs I and II (K_I values in the range of 7.2−7.5 nM), it behaves as a medium potency inhibitor of CAs VII, IX, XII, and XIV (K_I values in the range of 48−110 nM), being a much weaker inhibitor of all other isozymes (K_I values in the range of 1100 to 1.4 × 10^6 nM, Table 1). Thus, the crucial question for the drug design of isoform-selective CAIs is the following: What are the structural elements present in the molecules of these sulfonamides that are responsible for the varying degrees of isozyme-selectivity evidenced so far? As the X-ray crystal structures of the adducts of hCA II with **5** and **6** were previously reported by this group,[8,17] we shall analyze here the corresponding X-ray crystal structure of the hCA II−chlorthalidone **4** adduct, which may shed some light on this question, critically important for the design of compounds with a better pharmacologic profile.

X-ray Crystallography. Crystallographic parameters and refinement statistics for the hCA II−**4** complex are shown in Table 2, whereas Table 3 presents hydrogen bonds and other contacts of the hCA II−**4** adduct. The overall hCA II− **4** complex is shown in Figure 1, whereas Figures 2 shows the

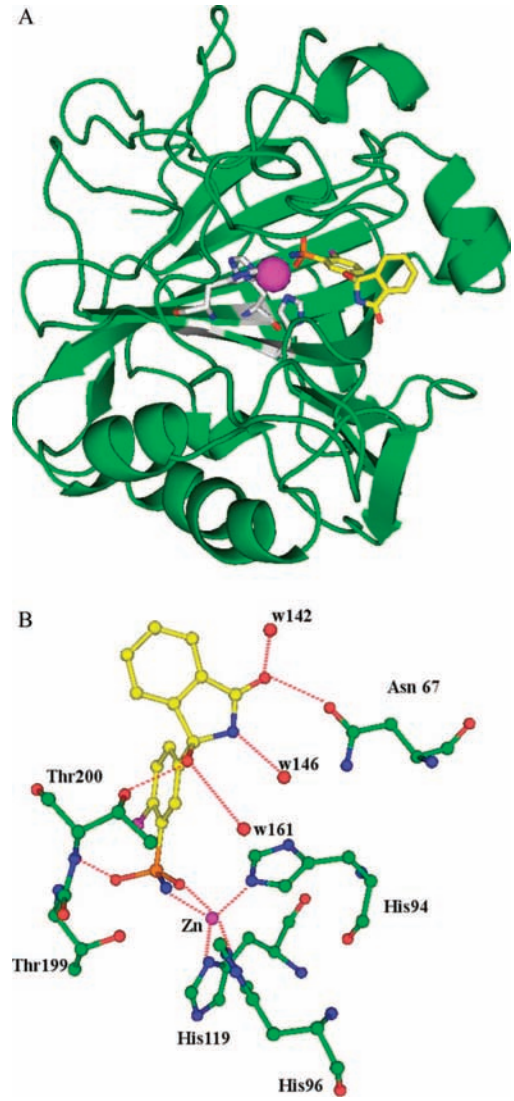


Figure 1. (A) hCA II−chlorthalidone **4** complex. The protein backbone is shown in green and the catalytic Zn(II) ion as violet sphere. Its three histidine ligands (His94, His96, and His119) are shown as CPK color, and the inhibitor **4** is shown in yellow. (B) Detailed interactions in which chlorthalidone **4** (in yellow) participates when bound within the hCA II active site. Active site residues coordinating the metal ion (His94, His96, His119) as well as those involved in the binding of the inhibitor (Asn67, Thr199, and Thr200) are also shown. Three ordered water molecules critical for the binding of this inhibitor to the CA II active site are indicated as red spheres. Distances are shown in Table 3.

omit map of the inhibitor bound within the enzyme active site. Figure 3A shows the detailed interactions of the inhibitor **4** bound within the enzyme active site, whereas Figure 3B and Figure 3C show the interactions with amino acid residues present in the hCA II active site in which the structurally related inhibitors **5** and **6** participate. Superpositions of the active site of hCA II complexed with chlorthalidone **4**, indapamide **5**, and the bicyclic sulfonamide **6** are shown in Figure 4.

Crystals of the hCA II - **4** adduct were isomorphous with those of the native protein,[8,13,17] allowing for the determination of the crystallographic structure by difference Fourier techniques. The refined structure presented a good geometry with rmsd from ideal bond lengths and angles of 0.010 Å and 1.4°, respectively (Table 2). The overall quality of the model was good with all residues in the allowed regions of the Ramachandran plot. Inspection of the electron density maps at various

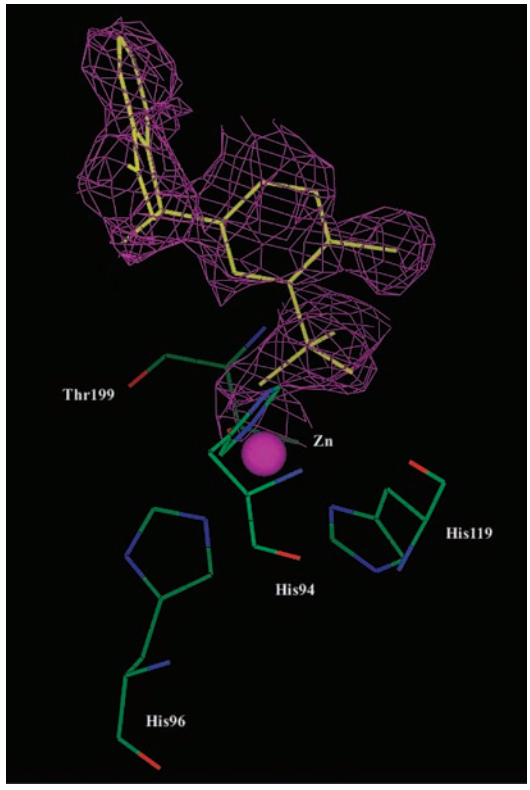


Figure 2. Composite omit $2F_o - F_c$ electron density map corresponding to the chlorthalidone molecule bound within the hCA II active site and of some relevant enzyme residues.

stages of the refinement showed features compatible with the presence of one molecule of inhibitor **4** bound within the active site (Figure 1). Interactions between the protein and Zn^{2+} ion were entirely preserved in the adduct (data not shown), as in all other hCA II−sulfonamide/sulfamate/sulfamide complexes investigated so far.[8,13,17] 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 inhibitor and enzyme active site all retained (Figures 1, 2, and 3A and Table 3).[8,13,17] In particular, the ionized nitrogen atom of the sulfonamide group of **4** is coordinated to the zinc ion at a distance of 2.02 Å, (Table 3). This nitrogen is also hydrogen-bonded to the hydroxyl group of Thr199 (N···Thr199OG = 2.77 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 Å away from the catalytic Zn^{2+} ion, whereas the second one participates in a hydrogen bond (of 2.92 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 (Figures 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 (Figure 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 3 and Figure 3 are 0.10−0.17 Å shorter as compared to the corresponding ones in other such adducts, for example, with the structurally related, bicyclic sulfonamides **5** and **6**.[8,17] However, the most salient 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 3) 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 (Figures 2 and 3A). It is obvious that in order to participate in both these hydrogen bonds, chlorthalidone must adopt the enolic tautomeric form shown in Figure 3A, when bound to the CA II active site. This is as far as we know, the first evidence showing that probably the biologically active form of this drug is the enolic one evidenced in this study. Indeed, this conformation of the inhibitor is clearly the one corresponding to the electron density map shown in Figure 2 and to the pattern of hydrogen bonds mentioned above.

Comparing the X-ray crystal data of the chlorthalidone−hCA II adduct reported here with those of the adducts of the same isoform with the structurally related compounds **5** and **6**,[8,17] we have some hints regarding the diverse inhibition profiles of these three sulfonamides against various CAs but also some insights for the design of CA II inhibitors with improved efficacy. Thus, data of Figures 3 and 4 show that the three structurally related, bicyclic sulfonamides **4**−**6** make a lot of very different interactions with amino acid residues and water molecules when bound within the hCA II active site. The main similarities for the binding of the three CAIs to the enzyme active site regard the benzenesulfonamide moiety present in all of them. Basically the sulfonamidate zinc binding group is anchored in the same manner to the metal ion and Thr199 in all three compounds (Figures 3), whereas the chlorophenyl fragment of **4** and **5** as well as the corresponding phenylsulfonamido one present in **6** are almost (but not entirely) superposable (Figure 4), occupying 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 ortho to the sulfamoyl moiety of **4** and **5** has no influence on the binding of the benzenesulfonamide moiety to the enzyme (Figures 3 and 4). However, the conformation in which the remaining scaffold of inhibitors **4**−**6** bind within the CA II active site and the interactions they make with amino acid residues and water molecules are very different for the three inhibitors examined here. Thus, the superposition of the hCA II−**4** and hCA II−**5** adducts shown in Figure 4 demonstrates that the protein scaffold is unchanged in the two complexes, whereas the moieties substituting in the 3-position the benzenesulfonamide scaffolds of the two inhibitors lie in very different active site regions extending toward the exit of the cavity. These two 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. Indeed, only an edge-to-edge stacking interaction 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 for the indapamide adduct (Figure 3B), whereas, as mentioned above, the corresponding moieties of chlorthalidone participate in several strong hydrogen bonds (of 2.54−3.16 Å) with Thr200, Asn67, and three water molecules (W142, W146, and W161), which were not present

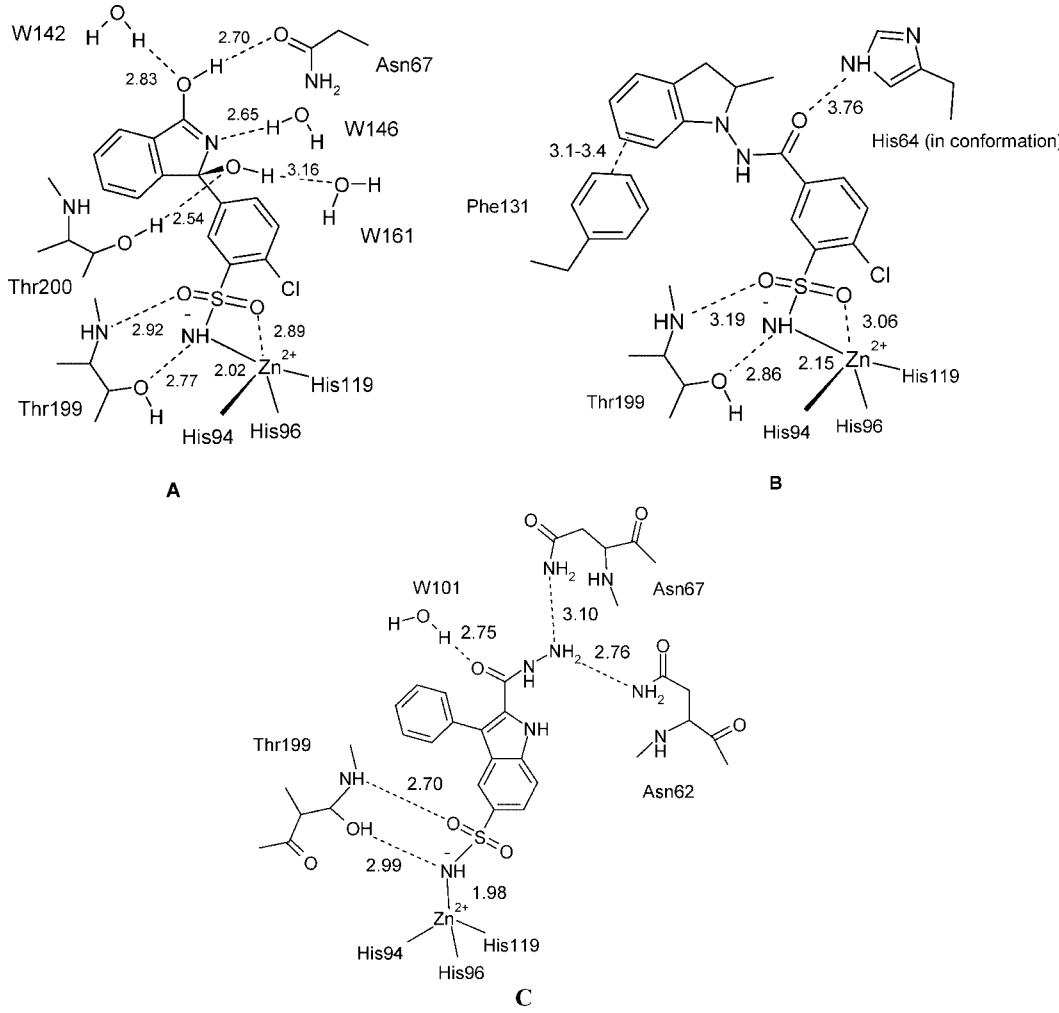


Figure 3. Comparison of interactions in which chlorthalidone **4** (A), indapamide **5** (B), and sulfonamide **6** (C) participate when bound to the CA II active site.

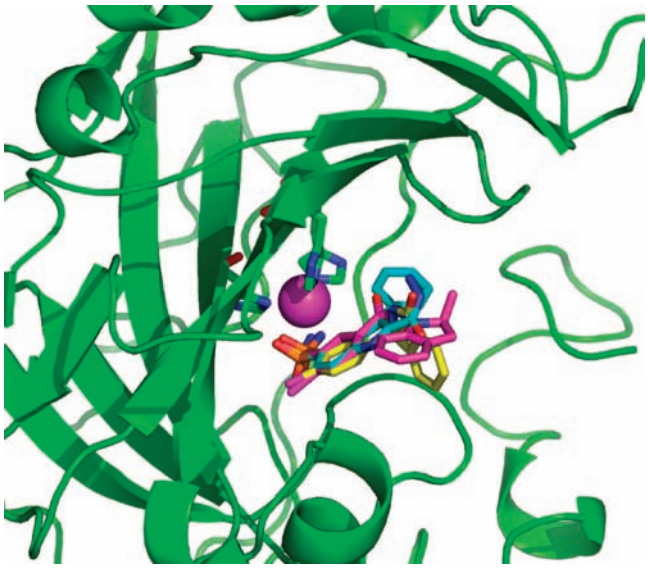


Figure 4. Superposition of the hCA II−chlorthalidone **4** (yellow), hCA II−indapamide **5** (magenta), and hCA II−**6** adduct (sky blue) complexes. The active site zinc ions (violet sphere), their His ligands, and protein backbones (green) are entirely superposable.

in the hCA II−indapamide adduct (Figure 3A). The enolization of the keto moiety of **4** allows for supplementary hydrogen bonding (as compared to the keto tautomer). Furthermore, the various heteroatoms present in the molecule of **4**, i.e., the enol group mentioned above, the nitrogen of the isoindole moiety, and the central OH functionality, are all exposed to the solvent and prone to hydrogen-bond to the three water molecules (W142, W146, and W161) and amino acid residues, which stabilize the enzyme−inhibitor adduct. In contrast, there are fewer heteroatoms present in the structurally related indapamide **5**, and except for the CONH ones, they are buried and unexposed to the solvent. As a consequence, no water molecules participate in hydrogen bonds with them, and only the oxygen atom of the carboxamide functionality makes a long and weak hydrogen bond with His64. Thus, the particular enantiomeric form in which chlorthalidone 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 compared to indapamide. As far as we know, this is the first documented case in which ordered water molecules in the enzyme active site (coupled with a normally unstable enol tautomeric form) can lead to such a strong discrimination between two structurally related inhibitors. In order to obtain better CAIs based on the indapamide/chlorthalidone scaffold, the second compound is obviously the most interesting one. Such a compound should preserve the different distribution of heteroatoms present in **4** in such a way to ensure their exposure to the solvent and formation of hydrogen bonds with water molecules, which stabilize the enzyme−inhibitor complex.

Compound **6** is, on the other hand, a much more powerful hCA II inhibitor as compared to chlorthalidone **4**, but its scaffold is also more complex, with the additional phenyl moiety substituting the indole ring participating in a lot of favorable hydrophobic interactions.[17] Furthermore, also for this compound, the polar hydrazido moiety participates in at least three strong hydrogen bonds (of 2.76−3.10 Å) with two amino acid residues (Asn62 and Asn67) as well as an ordered water molecule, W101 (Figure 3C).[17] Figure 4 also shows that chlorthalidone **4** and compound **6** are not at all superposable (except for the benzenesulfonamide moiety mentioned above) when bound to the enzyme active site, which explains the very different CA II inhibitory activities of these derivatives.

As the entrance of the active site cavity in the various CA isoforms is the region with the least conserved amino acid residues among the investigated isozymes,[7−13] we may also explain the quite diverse inhibition profiles observed with sulfonamides **1**−**6** discussed here (Table 1). Indeed, compounds without a bulky tail orientated toward the exit of the active site cavity, such as **1**−**3**, indistinctively inhibit all CA isoforms with high efficacy (usually with inhibition constants of <100 nM), leading to promiscuous, unselective CAIs. On the contrary, compunds possessing such bulkier moieties that bind toward the exit/edge of the active site cavity (such as **4**−**6**) and may thus interact differently with the various amino acid residues present in those regions do show some levels of selectivity for inhibition of certain CA isozymes, as exemplified here by the three sulfonamides **4**−**6**. However, as the X-ray crystal structure is not available yet for all CA isoforms (i.e., CAs VB, VI, VII, IX, and XV),[7] the nature and number of these amino acid residues responsible for the selective inhibition of CA isozymes are still unknown.

Conclusions

We reinvestigated the CA inhibitory activity of two thiazide, widely clinically used sulfonamide diuretics. These two compounds, chlorthalidone and indapamide, inhibit all mammalian CA isoforms but with a very different profile as compared to classical inhibitors, such as acetazolamide, methazolamide, and ethxzolamide. Acting as moderate-weak inhibitors of CA II and CA I, chlorthalidone and indapamide considerably inhibit other isozymes involved in critical physiologic processes, among the 16 CAs present in vertebrates. These two structurally related compounds showed a very different behavior against the widespread isozyme CAs I and II, with chlorthalidone being 18.3 times more potent an inhibitor against CA II and 150 times more potent against CA I, as compared to indapamide. Examining the two X-ray crystal structures of their CA II adducts, we observed three active site water molecules interacting with the chlorthalidone scaffold that are responsible for this important difference of activity. Chlorthalidone bound within the CA II active site is in an enolic tautomeric form, with the enolic OH 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 for various isoforms with medicinal chemistry applications.

Experimental Section

Materials. Sulfonamides **1**−**5** are commercially available compounds (from Sigma-Aldrich, Milan, Italy). The 12 CA isozymes used in the experiments were recombinant ones obtained and purified as reported earlier by this group.[18−22]

CA Inhibition Assay. An Applied Photophysics (Oxford, U.K.) 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 constant the ionic strength), 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]

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 the following: $a = 41.4$ Å, $b = 42.1$ Å, $c = 72.3$ Å and $\alpha = \gamma = 90°$, $\beta = 104.3°$ in the space group *P*21. 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 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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