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Carbonic anhydrase inhibitors. Interaction of indapamide and related diuretics with 12 mammalian isozymes and X-ray crystallographic studies for the indapamide–isozyme II adduct☆

Claudia Temperini, Alessandro Cecchi, Andrea Scozzafava and Claudiu T. Supuran*

Università degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Room 188, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Firenze), Italy

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Abstract—Diuretics such as hydrochlorothiazide, hydroflumethiazide, quinethazone, metolazone, chlorthalidone, indapamide, furosemide, and bumetanide containing primary sulfamoyl moieties were reevaluated as inhibitors of 12 human carbonic anhydrases (hCAs, EC 4.2.1.1). These drugs considerably inhibit (low nanomolar range) some CA isozymes involved in critical physiologic processes, among the 16 present in vertebrates, for example, metholazone against CA VII, XII, and XIII, chlorthalidone against CA VB, VII, IX, XII, and XIII, indapamide against CA VII, IX, XII, and XIII, furosemide against CA I, II, and XIV, and bumethanide against CA IX and XII. The X-ray crystal structure of the hCA II–indapamide adduct was also resolved at high resolution.

Carbonic anhydrase (CA, EC 4.2.1.1) inhibitors, such as acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) **1**, and the structurally related sulfonamides methazolamide **2**, ethoxzolamide **3**, and dichlorophenamide **4**, inhibit non-selectively all the 16 different isoforms of this metalloenzyme characterized so far in vertebrates.[1,2] Acetazolamide played a major role in the development of renal physiology and pharmacology, as well as for the design of many of the presently widely used diuretic agents, such as among others the thiazide and high ceiling diuretics.[1–8] CAs catalyze a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion, and are thus involved in crucial physiological processes connected with the 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 many other physiologic/pathologic processes.[1–5,7,8] Many CAs isoforms have been shown to be present in various tissues of the kidney,[9,10] such as CA II, IV, VB, IX, XII, and XIV, where these play a crucial function in at least three renal physiological processes: (i) the acid–base balance homeostasis; (ii) the bicarbonate reabsorption processes, and (iii) the renal NH_4^+ output.[2,11,12] Inhibition of both cytosolic (CA II) and membrane-bound (CA IV and CA XIV) enzymes seems to be involved in the diuretic effects of these sulfonamides.[2,9–12]

Sulfonamides **1**–**4** are used for the treatment of edema due to the congestive heart failure, and for drug-induced edema, in addition to their applications as antiglaucoma agents.[1,2,11] The structurally related compound to acetazolamide, benzolamide **5**, has a renal effect on bicarbonate excretion around 10 times as potent as that of acetazolamide, but the compound remained as an orphan drug and has not been developed for clinical use. Using acetazolamide **1** as lead, a large number of other quite successful sulfonamide diuretics were developed in the 60s and 70s, such as benzothiadiazines **6** (hydrochlorothiazide **6a**, hydroflumethiazide **6b**, and the like), quinethazone **7**, metolazone **8**, chlorthalidone **9**, indapamide **10**, furosemide **11**, and bumetanide **12**.[1–3] Some of them are among the most widely clinically used diuretics,[6,11,13] and as these all possess primary SO_2NH_2 moieties, acting as excellent zinc-binding groups for the metal ion present within the CA active site,[1–3] it is to be

Keywords: Carbonic anhydrase; Isozyme; Sulfonamide; Diuretic; Benzothiadiazine; Furosemide; Indapamide; X-ray crystallography.

☆ The X-ray coordinates of the hCA II–indapamide **10** adduct are available in PDB with the ID 3BL1.

* Corresponding author. Tel.: +39 055 457 3005; fax: +39 055 4573385; e-mail: claudiu.supuran@unifi.it

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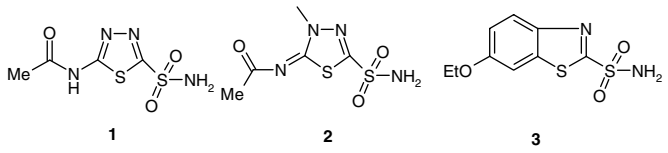
expected that these should also have CA inhibitory properties. However, this issue has been investigated only in the 60s or 70s when these drugs were launched, and when only one CA isozyme (i.e., CA II) was presumed to exist and be responsible for all the physiologic effects of the sulfonamide drugs.[11] Here, we reinvestigate the interaction of some of these clinically used diuretics with all 12 catalytically active mammalian CA isoforms and also report the X-ray crystal structure of one of them (indapamide **10**) with CA II.

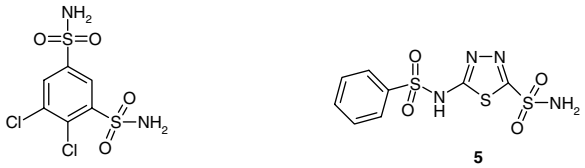
The data listed in Table 1 show that similar to the classical CAIs **1**–**5**, the clinically used sulfonamide diuretics **6**–**12** also act as inhibitors of all 12 investigated CA isozymes, with an inhibition profile different from that of inhibitors investigated earlier, such as **1**–**5**. The following should be noted from these inhibition data. (i) Hydrochlorothiazide **6a** acts as a medium potency inhibitor of isoforms hCA I, II, VB, IX, and XII, with inhibition constants in the range of 290–603 nM, the compound being a weaker inhibitor of isoforms hCA VA, VI, VII, XIII, and XIV (K_Is in the range of 3.655–5.010 μM) and an exceedingly weak one against hCA III (K_I of 0.79 mM). (ii) Hydroflumethiazide **6b** shows an inhibition profile distinct from that of the closely structurally related **6a**, being a rather efficient inhibitor of the following isoforms hCA II, VB, VII, IX, XII, and XIV, with inhibition constants in the range of 305–435 nM. This sulfonamide is a weaker inhibitor of hCA I, IV, and VI (K_Is in the range of 2.84–8.25 μM) and shows a very weak inhibition against isozymes hCA III, VA, and XIII (K_Is of 10.2–870 μM). Thus, even small structural changes in the benzothiadiazine scaffold, such as the substitution of the chlorine atom in *ortho* to the sulfamoyl moiety by a trifluoromethyl group, such as in the pair **6a/6b**, have dramatic consequences for the CA inhibitory properties of the two compounds (Table 1). (iii) Quinethazone **7** is the only diuretic among compounds **1**–**12** investigated here which is not approved for clinical use in Europe (but it is approved in USA),[6,11b] and this derivative was not available to be investigated here. The literature data[6] show it to be a very weak hCA I and a modest hCA II inhibitor, with inhibition constants in the range of 1.26–35 μM (Table 1). (iv) Metolazone **8** shows very weak hCA I and III inhibitory properties (K_Is in the range of 54–610 μM), being a low micromolar inhibitor of hCA II, VI, and XIV, with inhibition constants in the range of 1.714–5.432 μM. However, the drug is a medium potency inhibitor of isozymes hCA IV, VA, VB, and IX (K_Is in the range of 216–750 nM) and a very efficient one against hCA VII, hCA XII, and mCA XIII (K_Is in the range of 2.1–15 nM). (v) Chlorthalidone **9** also shows a very interesting inhibition profile, acting as a weak hCA III inhibitor (with a K_I of 11 μM, this compound is one of the most effective hCA III inhibitors ever detected among all known sulfonamides except trifluoromethanesulfonamide which has a K_I of 0.9 μM),[3b,15] and a rather weak hCA VI and hCA XIV inhibitor (K_Is in the range of 1.347–4.95 μM). Chlorthalidone is a moderate hCA VA inhibitor (K_I of 917 nM) and an effective, or very effective inhibitor of the other mammalian CA isozymes. Thus, the ubiquitous hCA I and II, as well as hCA IV, show inhibition constants in the range of 138–348 nM, but isoforms VB, VII, IX, XII, and XIII are inhibited in the low nanomolar range (K_Is in the range of 2.8–23 nM). (vi) Indapamide (the clinically used R-enantiomer) **10** acts as an inefficient CA I and III inhibitor (K_Is in the range of 51.9 to >200 μM), is a rather weak inhibitor of isoforms CA II, VA, VI, and XIV (K_Is in the range of 890–4950 nM) but shows significant inhibitory activity against CA IV and VB (K_Is in the range of 213–274 nM) and excellent inhibition of CA VII, IX, XII, and XIII, with inhibition constants in the low nanomolar range (K_Is in of 0.23–36 nM). These data are indeed remarkable, also considering the wide use of the drug as diuretic and its beneficial effects in patients with type 2 diabetes mellitus, as recently reported in an important clinical trial.[13] A special mention should be done regarding CA VII (K_I of 0.23 nM) since this isoform is present only within the brain, unlike other cytosolic CAs.[1,15b] In this organ, CA VII is involved in epileptogenesis among others, being one of the targets of the anticonvulsant sulfonamides and sulfamates.[1,15b] There are no literature data regarding a possible anticonvulsant effect of this compound, but the present data strongly suggest one, and experiments are warranted to test this activity which may lead to novel applications for the drug or to the design of novel classes of CA VII-selective inhibitors.[1,15b] (vii) Furosemide **11** acts as a very weak hCA III inhibitor (K_I of 3200 μM), but it shows moderate inhibitory activity against many isoforms, such as CA IV, VA, VB, VI, VII, IX, XII, and XIII, with K_Is in the range of 261–564 nM. The compound is, on the other hand, a much better inhibitor of CA I, II, and XIV, with K_Is in the range of 52–65 nM. (viii) Bumethanide is again an extremely weak hCA III inhibitor (K_I of 3400 μM), similarly to furosemide with which it is structurally related. However, bumethanide is also a weak inhibitor of hCA I, II, VI, and XIII (K_Is in the range of 2570–6980 nM), probably due to the quite bulky phenoxy moiety in *ortho* to the sulfamoyl zinc-binding group. The compound shows better inhibitory activity against isoforms CA IV, VA, VB, and XIV (K_Is in the range of 159–700 nM) but very good inhibition of the tumor-associated isoforms CA IX and XII (K_Is in the range of 21.1–25.8 nM, that is, the same order of magnitude as acetazolamide **1**, methazolamide **2**, or ethoxzolamide **3**) and CA VII (K_I of 62 nM).

But what is the relevance of this study for the drug design of CAIs with diverse pharmacological applications? Up to now, these widely used drugs were considered to be inactive as CAIs, due to the fact that these were launched in a period when only CA II was well known (and considered as responsible of all physiologic effects of CAIs). It may indeed be observed that in contrast to the classical CAIs of type **1**–**5** (generally low nanomolar CA II inhibitors), all compounds **6**–**12** (except furosemide **11**) are much weaker inhibitors of this isozyme, usually in the micromolar range. Indeed, only furosemide **11** is a good CA II inhibitor among these diuretics, with a K_I of 65 nM, whereas all others show K_Is in the range of 138–6980 nM (Table 1). Again with the exception of furosemide **11**, diuretics **6**–**12** have low affinity

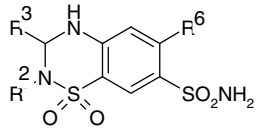
Table 1. Inhibition data with some of the clinically used sulfonamides **1**–**12** against isozymes I–XIV (the isoforms CA VIII, X, and XI are devoid of catalytic activity and probably do not bind sulfonamides as these do not contain Zn(II) ions)[1,2]



1 **2** **3**

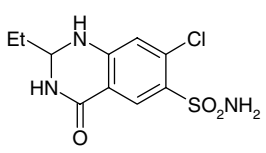


4 **5**



6

a: R^2 =R^3=H, R^6=Cl, Hydrochlorothiazide
b: R^2=R^3=H, R^6=CF_3 , Hydroflumethiazide
c: R^2=H, R^3=$PhCH_2$, R^6=CF_3, Bendroflumethiazide
d: R^2=H, R^3=$CHCl_2$, R^6=Cl, Trichloromethiazide
e: R^2=Me, R^3=$CH_2SCH_2CF_3$, R^6=Cl, Polythiazide



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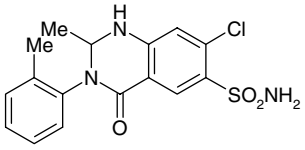

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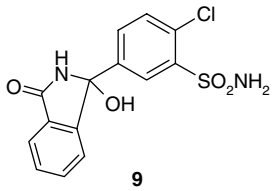

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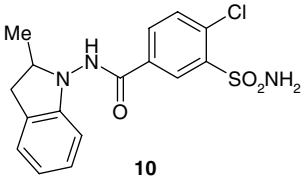

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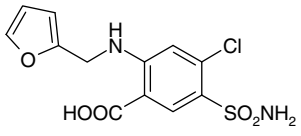

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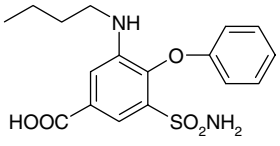

12

Isozyme[d]	K_I[d] (nM)												
	1	**2**	**3**	**4**	**5**	**6a**	**6b**	**7**	**8**	**9**	**10**	**11**	**12**
hCA I[a]	250	50	25	1200	15	328	2840	35,000[c]	54,000	348	51,900	62	4930
hCA II[a]	12	14	8	38	9	290	435	1260[c]	2000	138	2520	65	6980
hCA III[a]	2×10^5	$>2 \times 10^5$	$>2 \times 10^5$	$>2 \times 10^5$	1.4×10^5	$>2 \times 10^5$	$>2 \times 10^5$	nt	$>2 \times 10^5$	1.1×10^4	$>2 \times 10^5$	$>2 \times 10^5$	$>2 \times 10^5$
hCA IV[a]	74	6200	93	15,000	nt	427	4780	nt	216	196	213	564	303
hCA VA[a]	63	65	25	630	37	4225	10,200	nt	750	917	890	499	700
hCA VB[a]	54	62	19	21	34	603	429	nt	312	9	274	322	159
hCA VI[a]	11	10	43	79	93	3655	8250	nt	1714	1347	1606	245	3890
hCA VII[a]	2.5	2.1	0.8	26	0.45	5010	433	nt	2.1	2.8	0.23	513	62
hCA IX[b]	25	27	34	50	49	367	412	nt	320	23	36	420	25.8
hCA XII[b]	5.7	3.4	22	50	3.5	355	305	nt	5.4	4.5	10	261	21.1
mCA XIII[a]	17	19	50	23	nt	3885	15,400	nt	15	15	13	550	2570
hCA XIV[a]	41	43	25	345	33	4105	360	nt	5432	4130	4950	52	250

h, human; m, murine isozyme; nt, not tested, data not available.
[a] Full length enzyme.
[b] Catalytic domain.
[c] From Ref. 3b.
[d] Mean value from at least three different measurements.[14] Errors were in the range of ±5% of the obtained value (data not shown).

for CA I, the other isoform known when these drugs have been discovered.[16] However, the data listed in Table 1 show that many of the drugs **6**–**12** appreciably inhibit CAs discovered after their introduction in clini-

cal use, with some low nanomolar (or even subnanomolar) inhibitors against many of them. Examples of such situations, are among others: metholazone **8** against CA VII, XII, and XIII, chlorthalidone **9** against CA VB, VII, IX, XII, and XIII, indapamide **10** against CA VII, IX, XII, and XIII, furosemide **11** against CA I, II, and XIV, and bumethanide **12** against CA IX and XII among others (Table 1). As already mentioned above, bumethanide **12** is a tumor-specific (targeting CA IX and XII) CAI, of equal potency to acetazolamide **1**, but without the promiscuity of acetazolamide which is a potent CAI against most mammalina isozymes. Indeed, bumethanide is a weak inhibitor of all other isoforms except CA IX and XII, which are overexpressed in tumors.[17] Indapamide **10** and chlorthalidone **9** are also strong inhibitors of the tumor-associated CAs, but these are also effective in inhibiting CA VII and XIII too (Table 1). It is thus clear, that these old drugs may indeed have newer applications in therapy or as experimental agents, in situations in which the selective inhibition of some CA isozymes is needed, and which cannot be obtained with the presently used compounds of types **1**–**5**.

Recently, it has been observed that indapamide **10** in combination with an ACE inhibitor (as diuretics) are highly beneficial for the treatment of patients with hypertension and type 2 diabetes.[13] On the other hand, classical sulfonamide CAIs such as acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, and other compounds possessing such properties, are known to induce vasodilation in a variety of tissues and organs, including the kidneys, eye vasculature, and brain vessels.[18,19] However, the exact mechanisms by which these produce this beneficial effect for many pathologies (e.g., hypertension, glaucoma, and diabetic retinopathy), or the isoforms involved in it, are unknown for the moment.[18,19] A very recent report shows that indapamide **10** has a protective role against ischemia-induced injury and dysfunction of the blood–brain barrier, probably due to its vasodilating effects.[20] An organ-protective effect of indapamide in the animal models of renal failure has also been reported, showing the drug to be beneficial in preventing damage to the capillary structures, the endothelium, and in reducing the hypertrophy of superficial glomeruli among others.[21] All these effects are probably mediated by the inhibition of CAs present in blood vessels or in the kidneys, but no specific pharmacologic or biochemical studies are available so far, except for these clinical observations mentioned here.[13,19–21] The lesson we learn from all these data is that probably many of the recently reported beneficial clinical properties of indapamide **10** are due indeed to its diuretic effects, but in conjuction with its strong inhibition of some CA isozymes (such as CA IV, VB, VII, IX, XII, and/or XIII) reported here for the first time, isoforms present in kidneys and blood vessels.

In order to understand at molecular level the interactions between indapamide **10** and the active site of a CA isozyme, we report here the X-ray crystal structure of its adduct (the R-enantiomer) with the ubiquitous, highly investigated isoform hCA II, since other isoforms for which the compound has good affinity (such as CA VII and IX) were not crystallized yet.[1–4,22–26] Inspection of the electron density maps at various stages of the refinement, showed features compatible with the presence of one molecule of inhibitor **10** bound within the active site (Fig. 1).[27–31] Interactions between the protein and Zn^{2+} ion were entirely preserved in the adduct, as in all other hCA II–sulfonamide/sulfamate/sulfamide complexes investigated so far.[23–26] Analysis of the three-dimensional structure of the complex revealed a compact binding between the inhibitor and the enzyme active site, 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 (Figs. 2 and 3, and Table 2).[22–26] In particular, the ionized nitrogen atom of the sulfonamide group of **10** is coordinated to the zinc ion at a distance of 2.15 Å. This nitrogen is also hydrogen bonded to the hydroxyl group of Thr199 (N–Thr199OG = 2.86 Å), which in turn interacts with the Glu106OE1 atom (2.5 Å, data not shown). One oxygen atom of the sulfonamide moi-

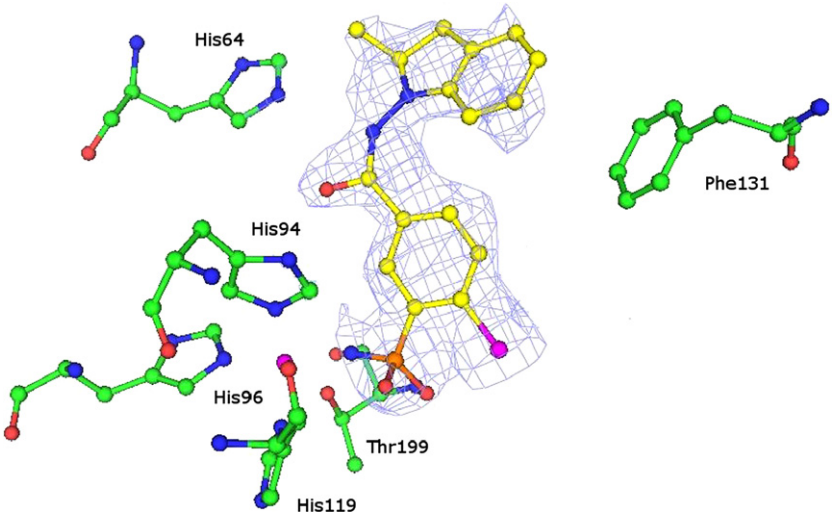

Figure 1. Simulated annealing omit $|2F_o - F_c|$ electron density map of indapamide **10** bound within the hCA II active site.

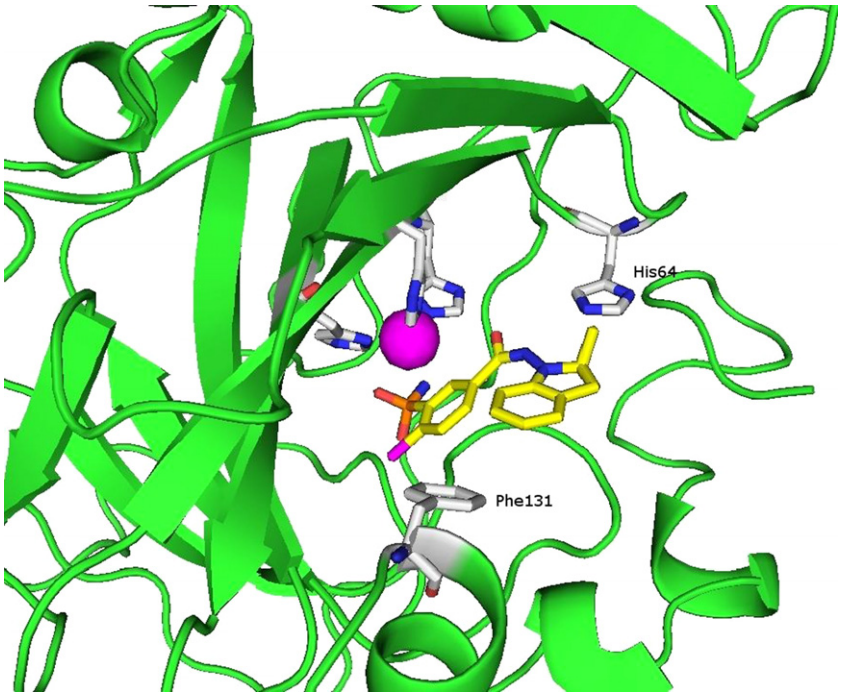

Figure 2. The hCA II–indapamide **10** complex. View of the zinc coordination sphere and neighboring amino acid residues involved in the binding of inhibitor **10** (in yellow).

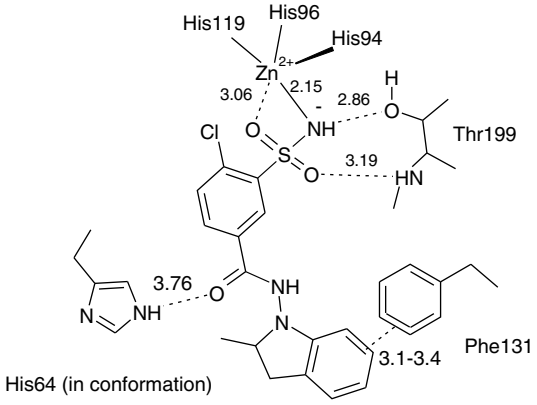

Figure 3. Detailed interactions in which indapamide **10** participates when bound within the hCA II active site. Active site residues coordinating the metal ion (His94, 96, and 119) as well as those involved in the binding of the inhibitor (His64, Phe131, and Thr199) are shown. Figures represent distances (in Å).

ety is 3.06 Å away from the catalytic Zn^{2+} ion, being considered as weakly coordinated to the metal ion, whereas the second one participates in a hydrogen bond (of 3.19 Å) with the backbone amide group of Thr199.[23–26] His64 (in its *in* conformation) makes strong van der Waals contacts (<4 Å) with the CONH moiety of the inhibitor, but these interactions cannot actually be considered as hydrogen bonds. A very strong interaction is on the other hand the strong offset face-to-face stacking between the annulated *ortho*-phenylene moiety of inhibitor **10** and the phenyl group of Phe131 (Figs. 2 and 3), which has been observed previously for several other adducts of hCA II with sulfonamides such as a pyridinium derivative[8] and sulpiride.[26] Such a stacking

Table 2. Crystallographic parameters and refinement statistics for the hCA II–**10** adduct

Parameter	Value
Crystal parameter	
Space group	$P2_1$
Cell parameters	
a	41.32 Å
b	42.05 Å
c	72.25 Å
β	104.29°
Data collection statistics (20.0–2.1 Å)	
No. of total reflections	24,686
No. of unique reflections	24,373
Completeness (%)[a]	85.0 (82.0)
$F_2/\mathrm{sig}(F_2)$	7.8 (1.7)
R-sym (%)	14.0 (30.0)
Refinement statistics (20.0–2.1 Å)	
R-factor (%)	22.8
R-free (%)[b]	29.9
Rmsd of bonds from ideality (Å)	0.015
Rmsd of angles from ideality (°)	1.80

[a] Values in parentheses relate to the highest resolution shell (2.1–2.0 Å).

[b] Calculated using 5% of data.

interaction was in fact demonstrated to be highly important for the orientation of the inhibitor within the active site and for the potency of a sulfonamide as CAI against this isoform.[8] The second feature which is salient for the adduct of **10** with hCA II regards the stacking interaction in which Phe131 participate with the phenylene moiety of the bicyclic ring present in indapamide. As seen from Figures 2 and 3, the two rings, that is, the *ortho*-phenylene moiety of inhibitor **10** and the phenyl

group of Phe131 are strictly parallel to each other, being at a distance of 3.41–3.46 Å.

In conclusion, we investigated whether the widely used benzothiadiazines and high ceiling diuretics, such as hydrochlorothiazide, hydroflumethiazide, quinethazone, metolazone, chlorthalidone, indapamide, furosemide, and bumetanide, which contain primary sulfamoyl moieties acting as potential zinc-binding functions, may act as inhibitors of 12 catalytically active mammalian CAs. These drugs are widely used clinically and were launched in a period when only isoform CA II was known and considered physiologically/pharmacologically relevant, and thus no inhibition data against other CA isoforms are available in the literature. Although acting as moderate–weak inhibitors of CA II, and CA I, all these drugs considerably inhibit other CA isozymes known nowadays to be involved in critical physiologic processes, among the 16 CAs present in vertebrates. Some low nanomolar (or even subnanomolar) inhibitors against such isoforms were detected, such as metholazone against CA VII, XII, and XIII, chlorthalidone against CA VB, VII, IX, XII, and XIII, indapamide against CA VII, IX, XII, and XIII, furosemide against CA I, II, and XIV, and bumethanide against CA IX and XII. The X-ray crystal structure of the CA II–indapamide adduct was also resolved at high resolution, showing features that may be useful for the drug design of novel classes of CAIs. We also propose that the recently observed beneficial effect of indapamide for the treatment of patients with hypertension and type 2 diabetes is due to its potent inhibition of CA isoforms present in kidneys and blood vessels, which explain both the blood pressure lowering effects and organ-protective activity of the drug.

Acknowledgments

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