Carbonic anhydrase inhibitors. Sulfonamide diuretics revisited—old leads for new applications?†

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Sulfonamide diuretics such as hydrochlorothiazide, hydroflumethiazide, quinethazone, metolazone, chlorthalidone, indapamide, furosemide and bumetanide were tested as inhibitors of the zinc enzyme carbonic anhydrases (CAs, EC 4.2.1.1). These drugs were discovered in a period when only isoform CA II was known and considered physiologically/pharmacologically relevant. We prove here that although acting as moderate to weak inhibitors of CA II, all these drugs considerably inhibit other isozymes known nowadays to be involved in critical physiologic processes, among the 16 CAs present in vertebrates. Some low nanomolar/subnanomolar inhibitors against such isoforms were detected, such as among others metolazone 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, and the binding of this sulfonamide to the enzyme was compared to that of dichlorophenamide, sulpiride and a pyridinium containing sulfonamide. Indapamide binds to CA II in a manner not seen earlier for any other CA inhibitor, which might be important for the design of compounds with a different inhibition profile.

Introduction

Diuretics are among the most widely used clinical agents and their discovery was a great success of both synthetic organic chemistry and pharmacology, with most of these agents being discovered in the late 50s and 60s.[1–3] Right from the beginning of research in this field, it was clear that many compounds incorporating SO_2NH_2 groups showed this pharmacological activity.[1–3] 5-Acetamido-1,3,4-thiadiazole-2-sulfonamide, acetazolamide **1**, originally developed as an inhibitor of the zinc enzyme carbonic anhydrases (CAs, EC 4.2.1.1) was the first non-mercurial diuretic to enter into clinical use, in 1956.[1,2] Acetazolamide played a major role in the development of fundamental 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, compounds exerting their diuretic action by promoting sodium chloride (and osmotically obligated water) excretion in different segments of the nephron.[1–6] In mammals, 16 different α-CA isozymes or CA-related proteins (CARP) were described so far, with very different catalytic activity, subcellular localization, tissue distribution and susceptibility to be inhibited by sulfonamides.[1–5,7,8] These enzymes 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 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] CAs are highly abundant in the kidneys (a total concentration of about 8–10 μM has been estimated for this organ), and many isoforms have been shown to be present in various tissues of this organ.[9,10] CA isoforms present in kidneys, such as CA II, IV, VB, IX, XII and XIV play a crucial function in at least three physiological processes: (i) the acid–base balance homeostasis (by secreting and excreting protons, due to the carbon dioxide hydration reaction catalysed by these enzymes); (ii) the bicarbonate reabsorption processes, and (iii) the renal NH_4^+ output.[2,11,12] These important functions are well localised in the different segments of the nephron: bicarbonate reabsorption occurs in the proximal tubule, whereas urinary acidification and NH_4^+ output occur in the distal tubule and collecting duct.[2,11,12] Following the administration of a CA inhibitor (CAI), such as acetazolamide **1**, the urine volume promptly increases, and its normally acidic pH (of 6) becomes alkaline (around 8.2).[2,10–12] An increased amount of bicarbonate is eliminated into the urine (120 times higher than the amount eliminated normally), together with Na^+, and K^+ as accompanying cations, whereas the amount of chloride excreted is diminished. The increased alkalinity of the urine is accompanied by a decrease in the excretion of titratable acid and ammonia, and in consequence by metabolic acidosis. This sequence of events is due to the inhibition of various CA isozymes in the proximal tubule, which leads to inhibition of H^+ secretion by this segment of the nephron. Inhibition of both cytosolic (CA II) as well as membrane-bound (CA IV and CA XIV) enzymes seems to be involved in the diuretic effects of the sulfonamides.[2,11,12] Inhibition of such CAs decreases the availability of protons for the

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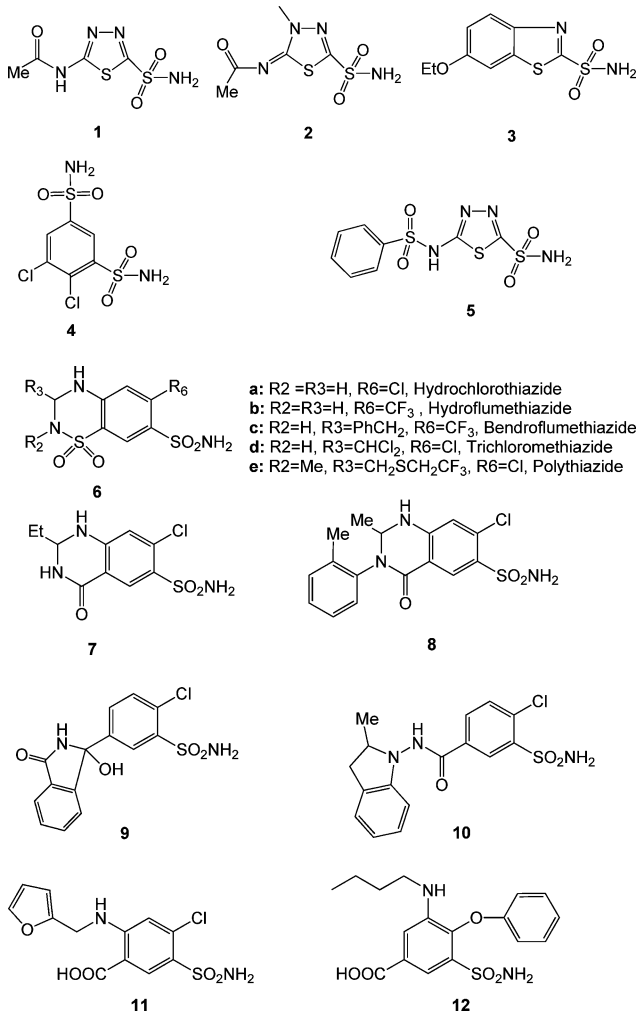
† The X-ray coordinates of the hCA II–indapamide **10** adduct are available in PDB with the ID 3BL1.

Na^+–H^+ antiporter, which maintains a low proton concentration in the cell. The net effect of these processes is the transport of sodium bicarbonate from the tubular lumen to the interstitial space, followed by movement of the isotonically obligated water, and whence augmented diuresis. CAIs also increase phosphate excretion (by an unknown mechanism) but have little or no effect on the excretion of calcium or magnesium ions.[2,11,12]



Acetazolamide **1**, and structurally related sulfonamides, such as methazolamide **2**, ethoxzolamide **3** and dichlorophenamide **4** were and are still used for the treatment of edema due to congestive heart failure, and for drug-induced edema, in addition to their applications as antiglaucoma agents.[1,2,11] However, these systemic CAIs generally produce many undesired side effects due to inhibition of CAs present in other organs than the kidneys.[1,2] The structurally related compound to acetazolamide, benzolamide **5**, with a quite acidic pK_a of 3.2 for the secondary sulfonamide group is completely ionised at physiological pH, as the sulfonamidate anion.[11] Its renal effect on bicarbonate excretion is around 10 times as potent as that of acetazolamide, the drug being maximally active at doses of 1 mg kg^{-1}, and being actively and rapidly accumulated in the kidney, but its plasma half-life is only 20 min.[11] All these facts make benzolamide a renal specific CAI, but the compound remained an orphan drug and has not been developed for wide clinical use, due to its inappropriate pharmacokinetics, although some anecdotal reports indicate that it might be beneficial for patients suffering from chronic obstructive lung disease.[1,11]

Using acetazolamide **1** as a lead, a large number of other quite successful sulfonamide diuretics were developed in the 60s and 70s, such as the 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 they 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 expected that they 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 physiological 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. Such data might be useful for the design of novel classes of more isozyme-selective CAIs and probably also to understand some new pharmacological effects of these old drugs.

Results and discussion

CA inhibition studies

Table 1 shows inhibition data of 12 CA isozymes (*i.e.*, CA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII and XIV) obtained by a stopped-flow assay[14] monitoring the physiological reaction (CO_2 hydration to bicarbonate) with the clinically used sulfonamides **1–12**. Data of Table 1 show that similarly to the clinically used/orphan drug classical sulfonamide CAIs, *i.e.*, compounds **1–5**, the clinically used sulfonamide diuretics **6–12** also act as inhibitors of all twelve investigated CA isozymes, but with an inhibition profile quite different from that of inhibitors investigated earlier, and particularly different from that of sulfonamides **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 quite distinct from that of the closely structurally related diuretic **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 very weak inhibition against isozymes hCA III, VA and XIII (K_is of 10.2–870 μM). It is already apparent that even small structural changes in the benzothiadiazine scaffold, such as the substitution of the chlorine atom *ortho* to the sulfamoyl moiety by a trifluoromethyl group, such as in the pair **6a**/**6b**, has dramatic consequences for the CA inhibitory properties of the two compounds, basically against all investigated CA iozymes (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

Table 1 Inhibition data of 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 they do not contain Zn(II) ions)[12]

Isozyme[a]	K_i/nM[b]											
	1	**2**	**3**	**4**	**5**	**6a**	**6b**	**7**	**8**	**9**	**10**	**11**	**12**
hCA I[c]	250	50	25	1200	15	328	2840	35 000[d]	54 000	348	51 900	62	4930
hCA II[c]	12	14	8	38	9	290	435	1260[d]	2000	138	2520	65	6980
hCA III[c]	2×10^5	7×10^5	1×10^6	6.8×10^5	1.4×10^5	7.9×10^5	8.7×10^5	nt	6.1×10^5	1.1×10^4	2.3×10^5	3.2×10^6	3.4×10^6
hCA IV[c]	74	6200	93	15 000	nt	427	4780	nt	216	196	213	564	303
hCA VA[c]	63	65	25	630	37	4225	10 200	nt	750	917	890	499	700
hCA VB[c]	54	62	19	21	34	603	429	nt	312	9	274	322	159
hCA VI[c]	11	10	43	79	93	3655	8250	nt	1714	1347	1606	245	3890
hCA VII[c]	2.5	2.1	0.8	26	0.45	5010	433	nt	2.1	2.8	0.23	513	62
hCA IX[c]	25	27	34	50	49	367	412	nt	320	23	36	420	25.8
hCA XII[c]	5.7	3.4	22	50	3.5	355	305	nt	5.4	4.5	10	261	21.1
mCA XIII[c]	17	19	50	23	nt	3885	15 400	nt	15	15	13	550	2570
hCA XIV[c]	41	43	25	345	33	4105	360	nt	5432	4130	4950	52	250

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

USA),[6,11] and this derivative was not available to be investigated here. Literature data[3b] 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), and is a low micromolar inhibitor (thus, not a very efficient one) 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 know 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). These results showing **9** to be such a strong CAI against many isoforms are quite unexpected, and considering the wide clinical use of the compound for the treatment of hypertension,[16] at least two issues can be raised: is the inhibition of these CA isozymes responsible for some of the therapeutic effects of the drug (or for some side effects observed with it), and, secondly, can these observations be useful for the design of CAIs with increased selectivity for some CA isoforms or for envisaging novel therapeutic applications of the drug (*e.g.*, an adjuvant to antitumor therapies, considering its strong inhibitory effects against the tumor-associated CA isozymes IX and XII)?[17] We shall reply to some of these questions after considering in detail the inhibition profile of the remaining three drugs. (vi) Indapamide **10** acts as an inefficient CA I and III inhibitor (K_is in the range of 51.9–230 μM), is a 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 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] (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, namely 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 **12** 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 and XIII (K_is in the range of 2570–6980 nM), probably due to the quite bulky phenoxy moiety *ortho* to the sulfamoyl zinc-binding group. The compound shows better inhibitory activity against isoforms CA IV, VA and XIV (K_is in the range of 250–700 nM) but excellent inhibition of the tumor-associated isoforms CA IX and XII (K_is in the range of 21.1–25.8 nM, *i.e.*, the same order of

magnitude as acetazolamide **1**, methazolamide **2** or ethoxzolamide **3**). Thus, it may be observed that bumethanide is an effective inhibitor of only the tumor-associated isoforms CA IX and XII, discriminating thus between these drug targets[17] and other CA isoforms that should not be inhibited by a cancer-specific CAI.

But what is the relevance of this study for the drug design of CAIs with diverse pharmacological applications? We think that there are at least several aspects that need to be considered here for answering this question. First, these widely used drugs were considered to be inactive as CAIs, due to the fact that they were launched in a period when only CA II was well known (and considered to be responsible for all physiological 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**, the diuretics **6–12** have low affinity for CA I, the other isoform known when these drugs were discovered. However, data in Table 1 show that many of the drugs **6–12** appreciably inhibit CAs discovered after their introduction in clinical use, with some low nanomolar (or even subnanomolar) inhibitors against many of them. Examples of such situations, are among others: metolazone **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 (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 mammalian 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 they are also effective in inhibiting CA VII and XIII (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**.

A second important aspect of these findings is whether we can explain some recent observations of clinical trials in which such diuretic agents have been employed, and how this is reflected in the drug design process. Thus, a relevant question arising is how these inhibitory activities of compounds of type **6–12** against CA isoforms known nowadays to play important physiological roles,[1–8] is reflected by the pharmacological effects (or side effects profile) of these drugs? It is in fact known that many interesting classes of novel drugs have been discovered just by observing side effects of some clinically used agents.[1–3] 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] Treatment with indapamide was also shown to lead to a significant decrease of plasma adiponectin concentration in patients with essential hypertension.[13] Adiponectin is considered to participate in the pathogenesis of carbohydrate metabolism disturbances often found in patients treated with other thiazide-type diuretics.[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, brain vessels, *etc.*[18,19] However, the exact mechanisms by which they produce this beneficial effect for many pathologies (*e.g.*, hypertension, glaucoma, diabetic retinopathy, *etc.*), or the isoforms involved in it, are unknown for the moment.[18,19] In line with these studies,[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 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.[13,21] All these effects are probably mediated by inhibition of CAs present in blood vessels or in the kidneys, but no specific pharmacological 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 conjunction 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. This hypothesis may explain both the blood pressure lowering effects as well as organ-protective activity of the drug. For medicinal chemists this means that probably it is possible to design sulfonamide CAIs possessing an inhibition profile similar to **10**, but with a stronger activity against the target isoform(s) involved in these pathologies, provided that it will be possible to understand which these isozymes are.

X-Ray crystallography

Crystallographic parameters and refinement statistics for the hCA II–**10** complex are shown in Table 2, whereas Table 3 presents hydrogen bonds and other contacts of the hCA II–**10** adduct.

The electron density map of **10** bound to the active site of hCA II is shown in Fig 1, whereas Fig. 2 and 3 show the detailed interactions of the inhibitor **10** bound within the enzyme active site. A superposition of the active site of hCA II complexed with indapamide **10**, with that of the hCA II complexes of other sulfonamide CAIs such as dichlorophenamide **4**, the positively-charged sulfonamide **13** and sulpiride **14**, is presented in Fig. 4. 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 with the ubiquitous, highly investigated isoform hCA II.[1–4] Crystallographic refinement of the hCA II–**10** adduct was performed at a final resolution of 2.1 Å. Crystals of the adduct were isomorphous with those of the native protein,[22–26] 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 0.015 Å and 1.8°, respectively (Table 2). The overall quality of the model was good with all residues in the allowed regions of the Ramachandran plot. Refinement statistics are summarized in Table 2. Inspection of the electron density maps at various stages of the refinement, showed features compatible

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
	$\beta = 104.29°$
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)
$F2/\mathrm{sig}(F2)$	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.

Table 3 Hydrogen bonds and contacts of indapamide **10** in complex with hCA II

Indapamide atom	hCA II residue	Distance/Å
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

with the presence of one molecule of inhibitor **10** bound within the active site (Fig. 1).

Interactions between the protein and the 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.[23–26] 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 (Fig. 2 and 3, and Table 3).[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 (Table 3). 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 moiety 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 (Table 3). 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 (Table 3 and Fig. 2 and 3), which has been observed previously for several other adducts of hCA II with sulfonamides such as the pyridinium derivative **13**[8b] and sulpiride

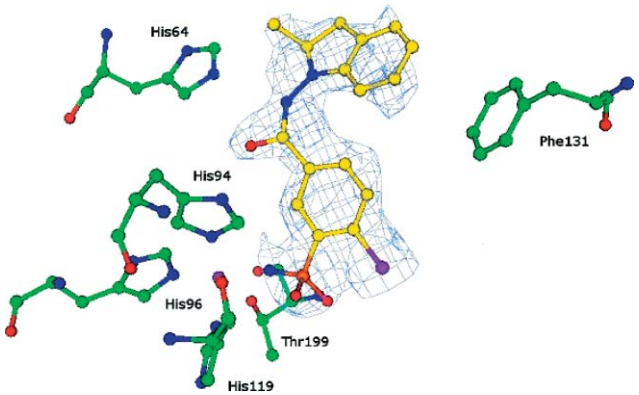


Fig. 1 Simulated annealing omit |2Fo–Fc| electron density map of indapamide **10** bound within the hCA II active site.

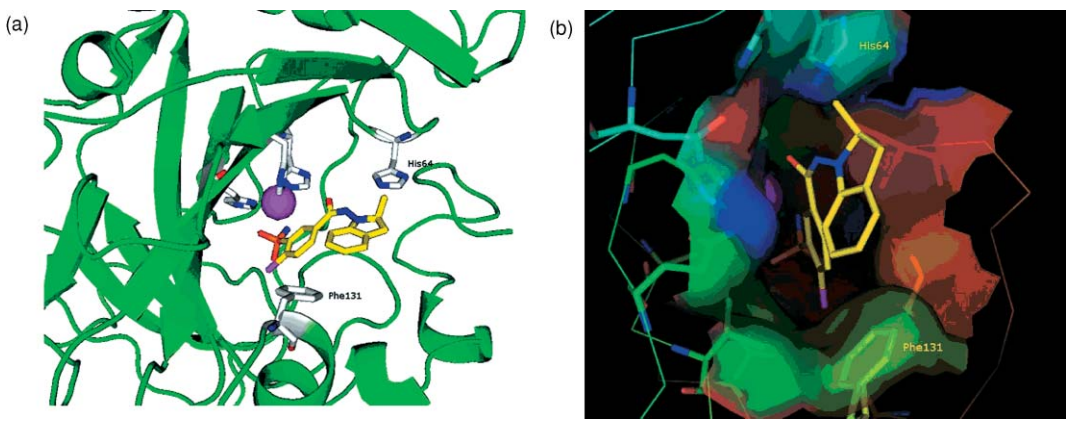


Fig. 2 The hCA II–indapamide **10** complex. (a) View of the zinc coordination sphere and neighboring amino acid residues involved in the binding of the inhibitor **10** (in yellow). (b) Detailed representation of the hCA II–indapamide active site.

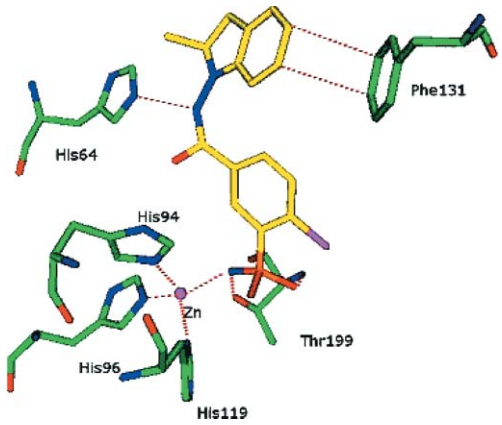
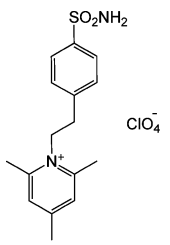


Fig. 3 Detailed interactions in which indapamide **10** (in yellow) participates when bound within the hCA II active site. Active site residues coordinating the metal ion (His94, 96, 119) as well as those involved in the binding of the inhibitor (His64, Phe131 and Thr199) are shown. Distances are presented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

14.[26a] Such a stacking 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.[8b] Thus, in order to better understand the binding of **10** to hCA II, the superpositions of its hCA II adduct with those of dichlorophenamide **4**[26b] as well as those of sulfonamides **13**[8b] and **14**[26a] are shown in Fig. 4. Two facts can be observed. First, a quite unexpected orientation for the phenyl ring of inhibitors **4** and **10** bound within the enzyme active site may be observed with respect to that of other benzene-sulfonamides whose structures in complex with hCA II have been reported (Fig. 4a).[22–26] It may be seen that the plane of the phenyl moiety of the benzene-1,3-disulfonamide **4** and of indapamide **10** appears clearly rotated by almost 30° and tilted by approximately 10° with respect to its most recurrent orientation, as the one of derivatives **13**[8b] and **14**[26a] (Fig. 4b and 4b).



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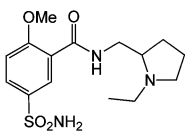

14

This peculiar conformation may be ascribed to the presence of the secondary sulfonamide group in the *meta* position of **4**, and the corresponding bulky carbohydrazide substituent in the *meta* position to the sulfamoyl moiety of **10**, which probably orientates the entire molecule in this unusual conformation, when bound within the enzyme cavity. In addition, both these CAIs (*i.e.*, **4** and **10**) possess a chlorine atom *ortho* to the sulfamoyl moiety coordinated to the Zn(II) present in the enzyme cavity, which could also influence the orientation of the organic scaffold due to steric impairment in the rather restricted environment near the metal ion. In fact, Fig. 4a shows that the sulfamoyl-chloro-phenyl fragment present in the scaffold of inhibitors **4** and **10** is practically completely superposable in these two hCA II–sulfonamide complexes, whereas the second sulfamoyl group of dichlorophenamide **4** binds in the same active site region as the much bulkier carbohydrazide moiety of indapamide **10** (Fig. 4a). The second feature which is salient for the adduct of **10** with hCA II regards the stacking interaction in which Phe131 participates with the phenylene moiety of the bicyclic ring present in indapamide. As seen from Fig. 2–4, the two rings, *i.e.*, the *ortho*-phenylene moiety of the inhibitor **10** and the phenyl group of Phe131 are strictly parallel to each other, being at a distance of 3.41–3.46 Å. The same stacking has been previously evidenced between the phenyl moiety of Phe131 and the 2,4,6-trimethylpyridinium ring of the positively-charged sulfonamide **13**,[8b] or the same phenyl moiety and the *N*-ethyl-pyrrolidine group of sulpiride **14**.[26a] In both these complexes this stacking interaction has been one of the main factors assuring a strong affinity of the inhibitor for the hCA II active site. As seen from Fig. 4b and 4c, although the phenylene moiety of **10** binds in the same active site region as the trimethylpyridinium ring of the positively-charged sulfonamide **13**,[8b] and the *N*-ethyl-pyrrolidine group of sulpiride **14**,[26a] these three cyclic moieties are not very well superposable with each other, which in part probably explains the net difference of activities as hCA II inhibitors between the three compounds. Thus, whereas **13** is a very strong hCA II inhibitor (K_i of 21 nM),[8b] sulpiride **14** is a slightly weaker one (K_i of 40 nM)[26a] whereas **10** is a moderate-weak inhibitor (K_i of 2.52 μM, Table 1). However, we consider this as a quite positive and interesting feature of indapamide as a CAI (as discussed above), which might be used to design compounds with reduced affinity for the ubiquitous, house-keeping enzyme hCA II, but strong inhibitory properties against other isoforms, such as hCA IV, VB, VII, IX, XII or XIII, against which indapamide acts as a much more efficient CAI (Table 1).

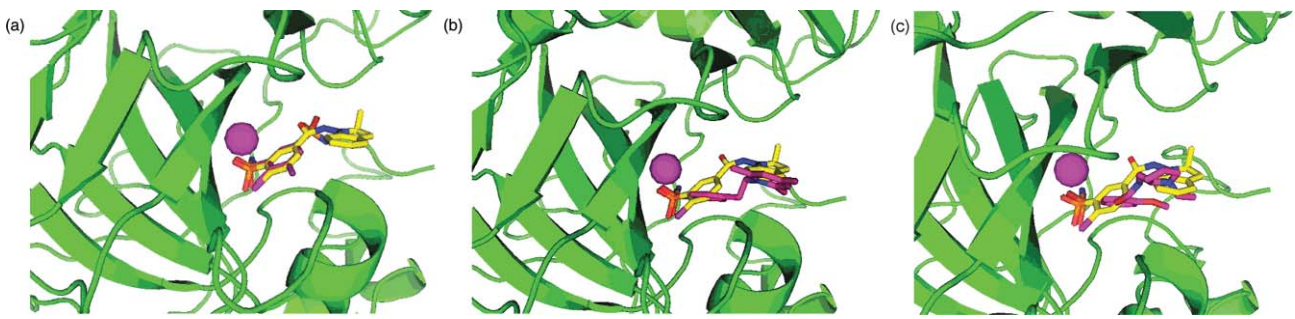

Fig. 4 Superposition of: (a) hCA II–indapamide **10** (yellow) with hCA II–dichlorophenamide **4** (magenta); (b) hCA II–indapamide **10** (yellow) with hCA II–pyridinium sulfonamide **13** (magenta);[8b] (c) hCA II–indapamide **10** (yellow) with hCA II–sulpiride **14** (magenta) adducts.[26a]

Experimental

Materials

Sulfonamides **1–12** 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.[27–30]

CA inhibition assay

An Applied Photophysics (Oxford, UK) stopped-flow instrument was used for assaying the CA catalysed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, and 0.1 M Na_2SO_4 (for maintaining constant ionic strength), following the CA-catalyzed CO_2 hydration reaction.[14] 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 were 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 non-linear least-squares methods using PRISM 3, and represent the mean from at least three different determinations.[14]

X-Ray crystallography

The hCA II–**10** adduct was crystallized as previously described.[25,26] Diffraction data were collected under cryogenic conditions (100 K) on a CCD Detector KM4 CCD/Sapphire using CuKα radiation (1.5418 Å). The unit cell dimensions were determined to be: $a = 41.32$ Å, $b = 42.02$ Å, $c = 72.25$ Å and $\alpha = \gamma = 90°$, $\beta = 104.25°$ in the space group $P2_1$. Data were processed with CrysAlis RED (Oxford Diffraction 2006).[31] 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;[32] model building and map inspections were performed using the COOT program.[33] 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 a 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.[34] Coordinates and structure factors have been deposited with the Protein Data Bank (accession code 3BL1). Crystallographic parameters and refinement statistics are summarized in Table 2.

Conclusions

We investigated whether such widely used drugs as the benzothiadiazines and high ceiling diuretics, among which hydrochlorothiazide, hydroflumethiazide, quinethazone, metolazone, chlorthalidone, indapamide, furosemide and bumetanide, which contain primary sulfamoyl moieties acting as potential zinc-binding functions, may show significant inhibitory effects against 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 to weak inhibitors of CA II and CA I, all these drugs considerably inhibit other CA isozymes known nowadays to be involved in critical physiological processes, among the 16 CAs present in vertebrates. Some low nanomolar (or even subnanomolar) inhibitors against such isoforms were detected, such as metolazone 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 explains both the blood pressure lowering effects as well as organ-protective activity of the drug. Lessons for the drug design of novel sulfonamide CA inhibitors based on these findings are also discussed.

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