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Carbonic anhydrase inhibitors. Interaction of 2-(hydrazinocarbonyl)-3-phenyl-1*H*-indole-5-sulfonamide with 12 mammalian isoforms: Kinetic and X-ray crystallographic studies☆

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Abstract—2-(Hydrazinocarbonyl)-3-phenyl-1*H*-indole-5-sulfonamide was tested for its interaction with 12 carbonic anhydrase (CA, EC 4.2.1.1) isoforms in the search of compounds with good inhibitory activity against isozymes with medicinal chemistry applications, such as CA I, II, VA, VB, VII, IX, and XII among others. This sulfonamide is a potent inhibitor of CA I and II (K_Is of 7.2–7.5 nM), a medium potency inhibitor of CA VII, IX, XII, and XIV, and a weak inhibitor against the other ubiquitous isoforms, making it thus a very interesting clinical candidate for situations in which a strong inhibition of CA I and II is needed. The crystal structure of the hCA II adduct of this sulfonamide revealed many favorable interactions between the inhibitor and the enzyme which explain its strong low nanomolar affinity for this isoform but may also be exploited for the design of effective inhibitors incorporating bicyclic moieties.

Known for decades, the metallo-enzymes carbonic anhydrases (CAs, EC 4.2.1.1) are present in prokaryotes and eukaryotes and encoded by at least four evolutionarily un-related gene families: the α-CAs in vertebrates, bacteria, algae, and cytoplasm of green plants; the β-CAs predominantly in bacteria, algae, and chloroplasts; the γ-CAs mainly in archea and some bacteria, and the δ-CAs in diatoms and other simple marine metazoa.[1–3] In humans, 15 isozymes are presently known, 12 of which are catalytically active (CAs I–IV, CA VA, CAVB, CAVI, CA VII, CA IX, and CAs XII–XIV), whereas the CA-related proteins (isoforms CA VIII, X, and XI) are devoid of catalytic activity.[1–3] In recent years, it has emerged that in addition to their well-known role for the development of diuretics and topically acting antiglaucoma drugs,[4,5] inhibitors of these enzymes may lead to novel applications, mainly as antiobesity, anticonvulsant, and/or anticancer agents or diagnostic tools.[6–10] In fact two mitochondrial isoforms (CA VA and CA VB) are involved in lipogenesis and their inhibition leads to diminished fatty acid biosynthesis,[6] whereas at least two transmembrane, tumor associated isozymes (CA IX and CA XII) are highly overexpressed and involved in signaling/pH regulation processes within many types of hypoxic tumors.[8–10] Little is understood for the moment regarding the brain CAs involved in epileptogenesis, since a large number of isoforms are present within the CNS, among which are CA I, II, IV, VB, VII, and XIV.[7] Specific inhibitors for many such isoforms have been developed and some of them are in clinical evaluations.[5–12] In addition, many CAs isolated from other organisms than the vertebrates were shown to be targets for the drug design recently, such as among others the α-CAs from *Plasmodium falciparum* and *Helicobacter pylori*,[13,14] the β-CAs from *H. pylori*, *Mycobacterium tuberculosis*, *Candida albicans*, *Cryptococcus neoformans*, etc.[15–18] Work is in progress in several laboratories for developing stronger and more specific inhibitors targeting these enzymes, that would lead to conceptually novel therapies.[15–19] In fact a major drawback of many of the clinically used sulfonamide CA inhibitors (CAIs), such

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☆ The coordinates of the hCA II–indolesulfonamide adduct have been deposited in PDB, ID code 3B4F.
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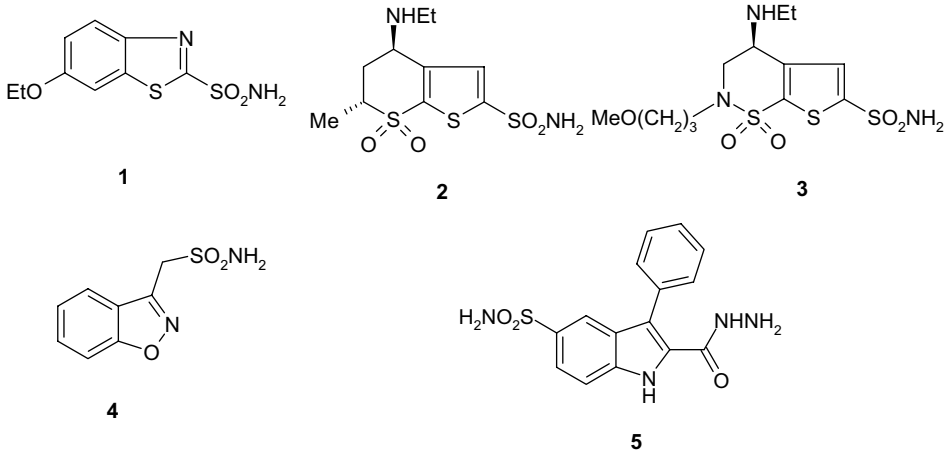
as among others ethoxzolamide **1**, dorzolamide **2**, brinzolamide **3** (used as antiglaucoma drugs)[5], and zonisamide (an antiepileptic drug),[20,21] is their promiscuous inhibition of many of the 12 catalytically active mammalian isoforms. Thus, screening of large libraries of derivatives or the rational drug design of inhibitors targeting a precise isoform proved ultimately to be potent tools for obtaining derivatives with some degree of selectivity for inhibiting some CA isoforms.[22,23]

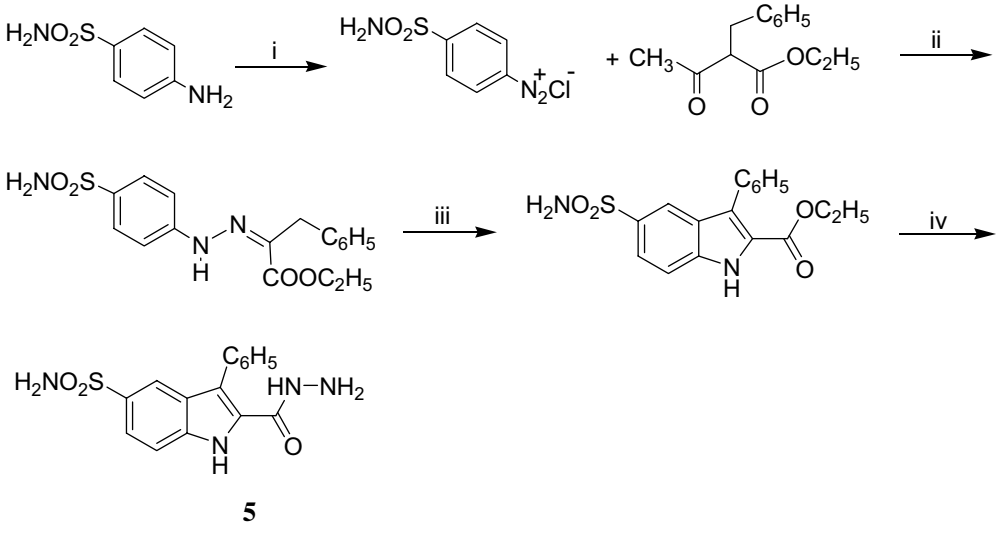
2-(Hydrazinocarbonyl)-3-phenyl-1*H*-indole-5-sulfonamide **5**,[26] a bicyclic compound structurally related with ethoxzolamide **1**, but also possessing two side chains similarly to **2** and **3**, with a different orientation as compared to all these clinically used compounds, attracted our attention as a possible candidate for obtaining CAIs with different inhibition profiles as compared to the presently available compounds. Sulfonamide **5** was previously reported by Salman's group,[26] being easily prepared from



Ethoxzolamide **1**, a first generation, very potent CAI against most isoforms,[19] has been used as lead molecule for the design of second generation such compounds, among which dorzolamide **2** and brinzolamide **3** are widely used as topically acting antiglaucoma agents.[5] A more recent such bicyclic sulfonamide CAI, zonisamide **4**, has been discovered serendipitously to possess significant anticonvulsant activity and is presently used as an antiepileptic.[20,21] No X-ray crystal structures of **1** in complex with CAs are available for the moment, however, the other three bicyclic derivatives **2**–**4** were crystallized in adducts with various isoforms, such as CA II and IV among others, by our and Christianson's groups.[21,24,25]

sulfanilamide as shown in Scheme 1. Diazotization of sulfanilamide followed by condensation of the diazonium salt with ethyl 2-benzylacetoacetate leads to an intermediate which was cyclized in acidic medium with formation of the ethyl ester derivative of **5**, which was converted to **5** by treatment with hydrazine (Scheme 1).[26]

Sulfonamide **5** has been investigated for the inhibition of the 12 catalytically active mammalian CA isoforms CA I–XIV (h, human; m, mouse isozyme) (Table 1). Data for the structurally related, clinically used derivatives **1**–**4** are also included for comparison, as they were published



Scheme 1. Preparation of the indole-5-sulfonamide derivative **5**. Reagents and conditions: (i) $NaNO_2$, 37% HCl, 0 °C; (ii) KOH, 0 °C; (iii) 37% HCl, reflux, 4 h; (iv) $H_2NNH_2{\cdot}H_2O$, reflux, 6 h.

Table 1. Inhibition data with the clinically used derivatives **1**–**4** as well as sulfonamide **5** against 12 mammalian α-CA isoforms

Isozyme[c]	K_I[d] (nM)				
	1	**2**	**3**	**4**	**5**
hCA I[a]	25	50,000	45,000	56	7.5
hCA II[a]	8	9	3	35	7.2
hCA III[a]	1.1×10^6	7.7×10^5	1.1×10^5	2.2×10^6	1.4×10^6
hCA IV[a]	93	8500	3950[a]	8590[a]	9000
hCA VA[a]	25	42	50	20	1100
hCA VB[a]	19	33	30	6033[a]	1100
hCA VI[a]	43	10	0.9	89[a]	2650
hCA VII[a]	0.8	3.5	2.8	117[a]	89
hCA IX[b]	34	52	37	5.1	102
hCA XII[b]	22	3.5	3.0	11,000	110
mCA XIII[a]	50[a]	18	10	430[a]	2633
hCA XIV[b]	25	27	24	5250[a]	48

Data for the inhibition of these CAs with compounds **1**–**4** are from Ref. 28.
[a] Recombinant enzyme. Data reported for the first time here.
[b] Catalytic domain.
[c] h, human; m, murine isozyme.
[d] Errors in the range of ±5% of the reported data from three different assays, by a stopped flow CO_2 hydration method.[27]

earlier by our group.[28] It may be observed that except CA III, which has a low affinity for all sulfonamide inhibitors,[28b] the other 11 investigated isoforms are generally all well inhibited by compounds **1**–**5**. Ethoxzolamide is a promiscuous, potent inhibitor of all of them, with K_Is < 50 nM for inhibition of all these CAs (Table 1). However, dorzolamide **2** and brinzolamide **3**, which have been obtained through rational drug design and comparison of the X-ray crystal structures of their various congeners with isoforms CA I and II,[19,24,25] already show a better inhibition profile as compared to dorzolamide, since they are also weak inhibitors of other two isoforms, hCA I and IV (in addition to hCA III) (Table 1). However, both compounds generally act as very potent, non-selective inhibitors of isozymes CA II, VA, VB, VI–XIV, with inhibition constants in the range of 0.9–50 nM. Zonisamide **4** is a rather special case among sulfonamide CAIs, being one of the very few aliphatic sulfonamides possessing a heterocyclic tail investigated in detail as an inhibitor.[21] Data of Table 1 show that zonisamide is a good hCA I, II, VA, and IX inhibitor (K_Is in the range of 5.1–56 nM), a medium potency CA VI and VII inhibitor (K_I of 89–117 nM), and a quite weak inhibitor of isozymes CA III, IV, VB, XII, XIII, and XIV, with K_Is > 430 nM (Table 1). These data clearly show that it is possible to design inhibitors with a certain degree of selectivity for target isozymes, zonisamide for example being a rather CA IX-selective compound, the next best inhibited isozymes being CA VA and CA II, but the selectivity ratio for the inhibition of the tumor-associated isoform IX over the mitochondrial one CA VA is around 4, and versus the cytosolic isozyme CA II is around 7. Thus, this sulfonamide is four times a better CA IX than CA VA inhibitor, and a seven times better CA IX than CA II inhibitor. The newly investigated bicyclic sulfonamide **5** also shows a completely different inhibition profile as compared to the other structurally related compounds **1**–**4** discussed here (Table 1). Thus, **5** is a very strong, nanomolar CA I and II inhibitor (K_Is of 7.2–7.5 nM), an ineffective CA III, IV, VA, VB, VI, and XIII inhibitor (K_Is > 1100 nM), and a medium potency CA VII, CA IX, CA XII, and CA XIV inhibitor (K_Is in the range of 48–110 nM). Since CA VII is present only in the brain,[7] CA IX and XII are generally restricted to tumors[8] and CA XIV to the liver and kidneys (with a rather low level of expression),[10] compounds such as **5**, which have lower affinity for ubiquitous isozymes such as CA IV, VA, VB, VI, and XIII but a very high one for hCA I and II, may have better profiles when used for the management of diseases due to the imbalance of CA activity (e.g., glaucoma and other eye diseases). Indeed, recently, in a seminal paper, Feener's group[29] reported elevated levels of CA I in vitreous from individuals with diabetic retinopathy, the enzyme being involved in retinal hemorrhage and erythrocyte lysis characteristics of this disease. It was proved that CA I induced alkalinization of vitreous which increased kallikrein activity and generation of factor XIIa. Such results prove that inhibition of CA I and/or kallikrein-mediated innate inflammation could provide new therapeutic opportuni-

Table 2. Crystallographic parameters and refinement statistics for the hCA II–**5** adduct

Parameter	Value
Crystal parameter	
Space group	$P2_1$
Cell parameters	a = 42.00 Å
	b = 41.35 Å
	c = 72.34 Å
	β = 104.35°
Data collection statistics (20.0–1.89 Å)	
No. of total reflections	88,138
No. of unique reflections	19,119
Completeness[a] (%)	99.14 (99.78)
F_2/sig(F_2)	10.53 (1.88)
R-sym (%)	11.0 (46.0)
Refinement statistics (20.0–1.89 Å)	
R-factor (%)	19.6
R-free[b] (%)	23.9
Rmsd of bonds from ideality (Å)	0.009
Rmsd of angles from ideality (°)	1.54

[a] Values in parentheses relate to the highest resolution shell (2.00–1.89).
[b] Calculated using 5% of data.

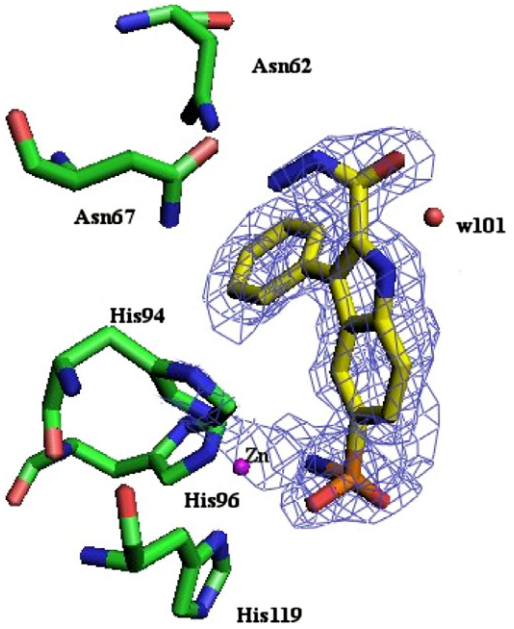
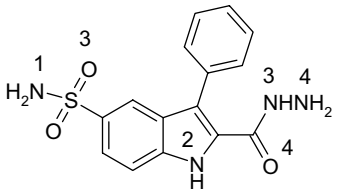


Figure 1. Electron density map of **5** (in yellow) bound within the hCA II active site. The Zn(II) ion of the enzyme, its three histidine ligands (His94, 96, and 119), residues involved in the binding of the inhibitor (Asn62 and Asn67) as well as one water molecule participating in hydrogen bonds with the CO moiety of the inhibitor are also shown.

ties for the treatment of hemorrhage-induced retinal and cerebral edema.[29] It should be stressed that untill this study was published, CA I was considered a typical anti-target among the many CA isoforms,[1,2,28a] since this enzyme is ubiquitous in the gastrointestinal tract and blood, being present at very high concentrations in many such tissues, but its precise physiological function being far less understood.[1,2,28a] In fact, CA I has a rather low catalytic activity as compared to CA II (also a ubiquitous isoforms present in the same tissue in which CA I is found)[1,2,28a] and is also inhibited to a large extent by physiological levels of chloride and bicarbonate (CA II is much more resistant to inhibition by these anions).[1,2,28a] As a consequence, many of the CAIs developed as anti-glaucoma drugs in the 90s, among which dorzolamide **2**

Table 3. Strong interactions in which compound **5** participates with amino acid residues/metal ion from the hCA II active site, in the hCA II–**5** adduct



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Atom of 5	hCA II residue	Distance (Å)
N1	Zn	1.98
O3	Zn	3.16
N1	Oγ1Thr199	2.99
O2	N Thr199	2.70
O3	Zn	3.16
N2	w101	2.75
N3	Oδ1 Asn67	2.91
O4	w101	3.05
N4	Oδ1 Asn67	2.41
N4	Nδ2 Asn67	3.10
N4	Nδ2 Asn62	2.76

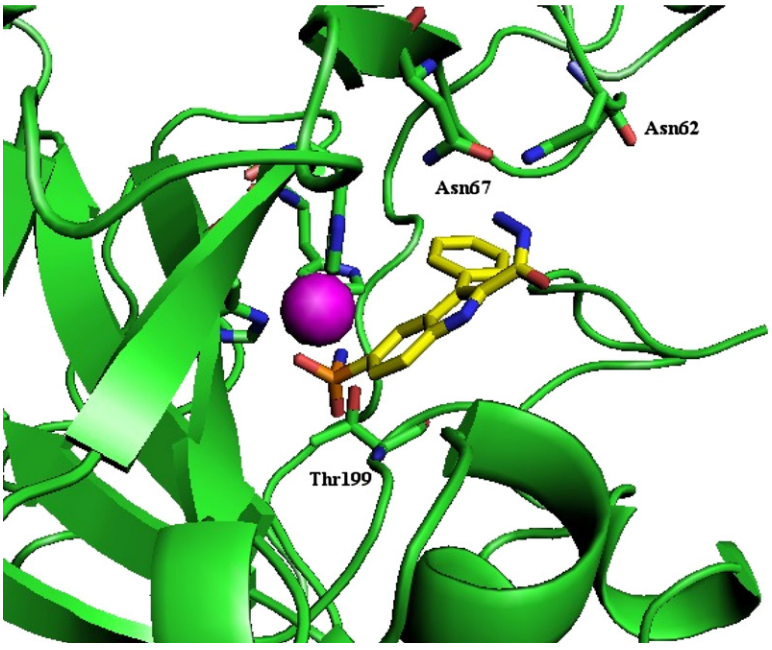

Figure 2. Detailed schematic representation for interactions in which sulfonamide **5** (in yellow) participates when bound to the hCA II active site. It should be noted that the bound inhibitor is oriented toward the hydrophilic half of the enzyme active site cavity. The Zn(II) ion is the central violet sphere.

and brinzolamide **3**, were designed in such a way as to act as very weak CA I inhibitors (see Table 1 and discussion above). However, these last data obtained recently[29] show that potent CA I inhibitors might be quite important for the management of such terrible diseases as diabetic retinopathy and cerebral edema.[29] Few potent CA I inhibitors are described in the literature,[1,2,28a] and compound **5** investigated here is one of the most potent such CAIs.

In order to better understand the effective hCA II inhibitory activity of **5** and also to learn some lessons for the drug design of new CAIs based on such bicyclic ring systems, we report the X-ray crystal structure of the hCA II–**5** adduct (Table 2).[30] The three-dimensional structure of the enzyme was very similar to that of hCA II without any ligand bound,[19,21,23–25] as judged by an r.m.s. deviation for Cα atoms of 0.31 Å only. Examination of the initially calculated electron density maps in the active site region showed the clear evidence for the binding of one inhibitor molecule within the active site cavity. The electron density of all moieties of the inhibitor is in fact very well defined (Fig. 1).

The tetrahedral geometry of the Zn^{2+} binding site and the key hydrogen bonds between the SO_2NH_2 moiety of **5** and enzyme active site are all retained with respect to other hCA II-sulfonamide/sulfamate/sulfamide complexes structurally characterized so far (Figs. 1 and 2).[5,19,21,23] In particular, the ionized nitrogen atom of the sulfonamide group of **5** is coordinated to the zinc ion at a distance of 1.98 Å. This nitrogen atom is also hydrogen bonded to the hydroxyl group of Thr199 (N–Thr199OG = 2.70 Å), which

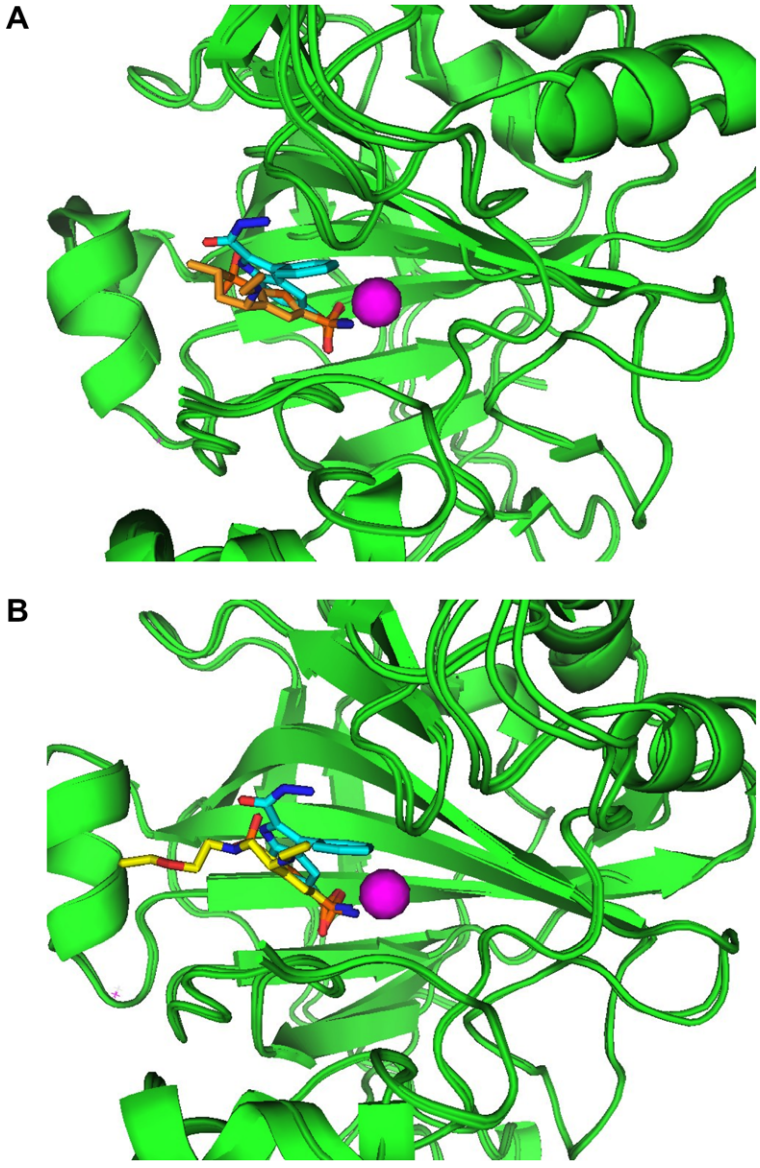

Figure 3. Superposition of the: (A) hCA II–dorzolamide **2** (gold), PDB entry 1cil,[24] with hCA II–**5** (blue) adducts, and (B) hCA II–brinzolamide **3** (yellow), PDB entry 1a42,[25] with hCA II–**5** (blue) adducts. Compound **5** binds in distinct active site regions of the enzyme cavity as compared to dorzolamide/brinzolamide.

in turn interacts with the Glu106OE1 atom (2.50 Å, data not shown). One oxygen atom of the coordinated sulfamoyl moiety is hydrogen bonded to the backbone amide of Thr199 (ThrN–O2 = 2.99 Å), whereas the second oxygen atom of this moiety is 3.16 Å away from the catalytic Zn^{2+} ion, being considered as weakly coordinated to the metal ion.[1,1,19,21,23] All these interactions have also been observed in the adducts of hCA II with other sulfonamide inhibitors, such as dorzolamide **2**, brinzolamide **3** or zonisamide **4**, but the corresponding distances are of course different.[19,21–25] The bicyclic heterocyclic scaffold of **5** is accommodated perfectly within the active site channel, being oriented towards the hydrophilic half of it, and participating to several favorable interactions with various amino acid residues and water molecules (Figs. 1 and 2). It should be noted that only the terminal amino moiety, the oxygen atom of the $CONHNH_2$, and the endocyclic NH indole group participate in such strong interactions, more precisely, the oxygen (CO) atom makes a hydrogen bond with water 101 (of 3.05 Å) which also makes a second hydrogen bond with the endocyclic NH moiety of **5** (of 2.75 Å). The terminal NH_2 moiety on the other hand participates in three hydrogen bonds with the oxygen atom of Asn67 (of 2.41 Å), with the NH_2 moiety of the same amino acid residue (of 3.10 Å), and with the NH_2 moiety of the neighboring Asn62 (of 2.76 Å) (Table 3). The 3-phenyl-indole scaffold of the inhibitor makes a host of favorable van der Waals interactions (< 4 Å) with amino acids lining the hCA II cavity (data not shown) which also contribute to the tight binding of **5** within the active site.

In order to try to understand the different inhibition profiles of compound **5** and dorzolamide **2** or brinzolamide **3**, we have also superposed the 3 D structures of the three sulfonamides complexed within the hCA II active site (Fig. 3). These figures show that although all three compounds have an excellent affinity for hCA II, in the low nanomolar range, their binding to the enzyme is rather different, since only the SO_2NH_2 moieties of the three inhibitors are superposable. We estimate that the degree of isozyme selectivity observed with sulfonamide **5** as compared to other CAIs investigated up to now (e.g., **2**–**4**) is due to the particular orientation which **5** adopts when bound to the CA II active site, which is characteristic only to this sulfonamide (see also Fig. 3).

In conclusion, we investigated the interaction of the sulfonamide **5** with all the catalytically active mammalian CA isoforms. This sulfonamide is a potent inhibitor of CA I and II, a medium potency inhibitor of CA VII, IX, XII, and XIV, and a weak inhibitor against the other ubiquitous isoforms, making it thus a very interesting clinical candidate for situations in which a strong inhibition of CA I and II is needed. The high resolution crystal structure of the hCA II–**5** adduct revealed many favorable interactions between the sulfonamide **5** and the enzyme which explain its strong low nanomolar affinity for this isoform but may also be exploited for the design of effective inhibitors incorporating bicyclic moieties.

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26. Compound **5** has been prepared as reported in: Ergenç, N.; Salman, A.; Bankaoğlu, G. *Pharmazie* **1990**, *45*, 346. *2-Benzyl-2-[N-(4-sulfonamidophenyl)hydrazono]ethanoate*. To a solution of 0.01 mol sulfanilamide in 4 ml of 37% HCl, 10 ml of 7% $NaNO_2$ aqueous solution was added dropwise at 0 °C. The solution containing the diazonium salt was poured into an ice-cold mixture of 2.3 g (a little excess of 0.01 mol) ethyl 2-benzylacetoacetate, 10 ml of C_2H_5OH, 20 ml of H_2O, and 2.7 g of KOH. The mixture was kept cold overnight. The hydrazone produced as an oil was separated, dissolved in $(C_2H_5)_2O$, washed with H_2O, and dried over anhydrous Na_2SO_4. $(C_2H_5)_2O$ was distilled, the oily residue was treated with 5 ml of 37% HCl, and set aside for 5 h at room temperature. The resulting solid substance was recrystallized from C_2H_5OH. *Ethyl 5-(aminosulfonyl)-3-phenyl-1H-indole-2-carboxylate*. A mixture of 0.01 mol ethyl 2-benzyl-2-[*N*-(4-sulfonamidophenyl)hydrazono]ethanoate and about 10 ml of 37% HCl was heated on a water bath for 4 h, cooled, and poured into 100 ml of H_2O, the crude product was filtered, washed with H_2O, and recrystallized from C_2H_5OH. *2-(Hydrazinocarbonyl)-3-phenyl-1H-indole-5-sulfonamide*. 3.45 g (0.01 mol) ethyl 5-(aminosulfonyl)-3-phenyl-1*H*-indole-2-carboxylate was dissolved in 20 ml of C_2H_5OH, 4 ml of $H_2NNH_2{\cdot}H_2O$ was added and refluxed for 6 h, cooled, and kept cold overnight. The resulting crystals were filtered off, washed with $(C_2H_5)_2O$, and recrystallized from C_2H_5OH/DMF.
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30. The hCA II–**5** complex was crystallized as previously described. 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: $a = 42.00$ Å, $b = 41.35$ Å, $c = 72.34$ Å, and $\alpha = \gamma = 90°$, $\beta = 104.35°$ 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 hCA II–**5** had an *R*-factor of 19.6% and *R*-free 23.9% in the resolution range 20.0–1.89 Å, with a rms deviation from standard geometry of 0.009 Å in bond lengths and 1.54° in angles. The correctness of stereochemistry was finally checked using PROCHECK.[34] Coordinates and structure factors have been deposited with the Protein Data Bank (PDB ID 3B4F). Crystallographic parameters and refinement statistics are summarized in Table 2.
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