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Carbonic anhydrase inhibitors. The X-ray crystal structure of human isoform II in adduct with an adamantyl analogue of acetazolamide resides in a less utilized binding pocket than most hydrophobic inhibitors ☆

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ABSTRACT

We investigated the inhibitory activity of several 1,3,4-thiadiazole-sulfonamides against all catalytically active CA (EC 4.2.1.1), CA I–XV. The tail derivatizing the 5-position in the 1,3,4-thiadiazole-2-sulfonamide scaffold was observed to be critical as an inhibitory determinant of these compounds. The high resolution X-ray crystal structure of hCA II in complex with 5-(1-adamantylcarboxamido)-1,3,4-thiadiazole-2-sulfonamide, showed the adamantyl moiety of the inhibitor residing in a less utilized binding pocket than that of most hydrophobic inhibitors, lined by the amino acid residues Ile91, Val121 and Phe131. This binding site may explain the diverse inhibition profiles of 5-carboxamide- and sufonamide-derivatized 1,3,4-thiadiazole-2-sulfonamides and offers a hot spot for designing isoform selective inhibitors, considering that residues 91 and 131 are highly variable among the 13 catalytically active isoforms.

Sulfonamides represent the main chemotype of clinically used carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs).[1,2] Members of this class include aromatic, heterocyclic or aliphatic primary sulfonamides, but most drugs belong to the heterocyclic class. Among them, 5-amino-1,3,4-thiadiazole-2-sulfonamide **1** was the lead molecule for obtaining some of the most widely used CAIs, among which acetazolamide **2** is the best known and investigated compound.[3] CAIs are clinically employed for the management of a variety of disorders connected to CA disbalances, such as glaucoma;[3,4] in the treatment of edema due to congestive heart failure,[3,5] or for drug-induced edema;[3] as mountain sickness drugs,[5] whereas other agents of this pharmacological class show applications as anticonvulsants,[6,7] antiobesity[8] or antitumor drugs/tumor diagnostic agents.[1a,9] As there are few isoform-selective inhibitors to date,[1] new sulfonamides are continuously reported to find derivatives with better inhibition profiles as compared to the promiscuous, first generation inhibitors such as **1** or **2**.[1] Furthermore, as some of the CA isoforms, such as human CA II,[1,10] are easily crystallisable in complex with various types of inhibitors, these enzyme–inhibitor complexes are widely used for structure-based drug design purposes, for understanding in detail interactions between enzymes and their ligands at molecular level, and also for detecting novel classes of CAIs. In fact, recently several interesting chemotypes have emerged as potent CAIs, mainly by combining X-ray crystallography and detailed kinetic binding assays, such as among others the thioxolones,[11] coumarins,[12] lacosamide,[13] and fullerenes.[14] Some of these compounds possess a very different mechanism of CA inhibition compared to sulfonamides and their bioisosteres such as the sulfamates and sulfamides.[1–6]

Among the many classes of heterocyclic sulfonamides investigated to date,[4b] the 1,3,4-thiadiazole-2-sulfonamides constitute a very interesting case due to the fact that on one hand acetazolamide **2** is a widely used drug, which strongly inhibits most CA isoforms of the 13 catalytically active mammalian isozymes characterized so far.[1–4] On the other hand, its 5-amino analogue **1**, is a highly versatile building block for preparing structurally different CAIs[4b] which sometimes possess physico-chemical properties and enzyme inhibitory activity strikingly different form those of acetazolamide **2** (or **1** itself).[15,16] This is in fact a highly desirable feature for new generations CAIs, as acetazolamide is a non-specific inhibitor of most mammalian isoforms, which (except CA III) are inhibited in the low nanomolar range by this compound.[1–4] The most widely used strategies for obtaining new CAIs

☆ Coordinates and structure factors have been deposited in the Protein Data Bank as entry 3MHC.

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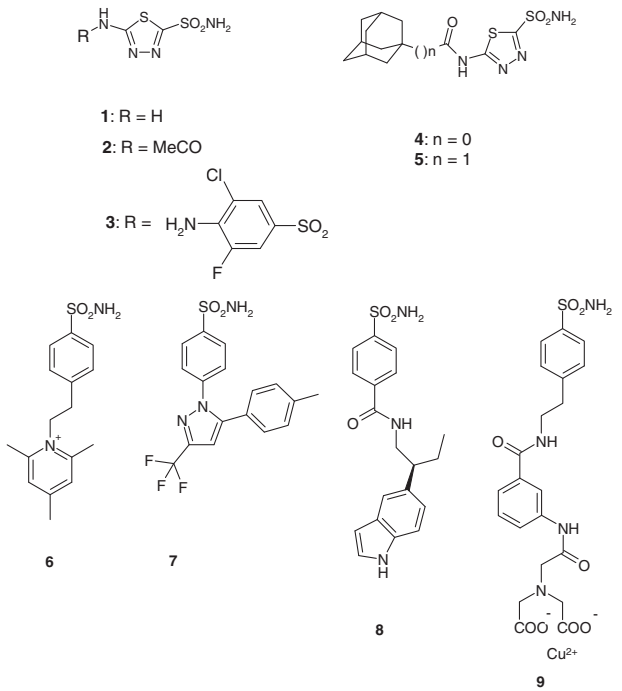
from **1** consisted in modification of its 5-amino moiety either by acylation or sulfonylation reactions.[4b,15,16] For example, compounds incorporating arylsulfonylamido moieties in the 5 position, such as among many others **3**, showed enhanced water solubility and excellent inhibition of several physiologically relevant isoforms, such as CA II and IX.[15,17,18] On the contrary, compounds with enhanced liposolubility were designed starting from **1**, such as among others the carboxamides **4** and **5**, which incorporate the bulky and highly lipophilic adamantayl moiety.[16a] Some of these derivatives also showed excellent CA II inhibitory activity and were effective anticonvulsants in experimental animals,[16a] making them interesting candidates for understanding the physiologic role of the brain CA isozymes.[19] However, no X-ray crystal structures of CAIs incorporating adamantyl moieties are known to date, which might be important for designing compounds with improved activity or selectivity profiles for the diverse CA isozymes which are drug targets, such as CA II, IV and XII (for antiglaucoma activity), CA VA and VB (for antiobesity applications), CA VII and XIV (anticonvulsants) or CA IX/XII (antitumor agents/diagnostic tools for tumors).[1] In this Letter, we report the X-ray crystal structure of the adamantyl analogue of acetazolamide, **4**, and based on these data, discuss the inhibition of this and related 1,3,4-thiadiazole-sulfonamides against the human (h) isoforms hCA I, II, IX, and XII, all of which are relevant for drug design purposes.



Data of Table 1 present the inhibition against all catalytically active human (h)/murine (m) isozymes, that is, CA I–XV of sulfonamides **1–5**, all of which have in common the 1,3,4-thiadiazole-2-sulfonamide moiety, but highly different 5-substituents. Starting from the lead **1**, in which the 5-amino group is free, acetazolamide **2** has an acetamido group in this position, whereas **3** has the 3-chloro-4-amino-5-flurophenylsulfonamido moiety. The adamantyl derivatives **4** and **5** possess either the 1-adamantyl-carboxamido moiety substituting the 5 position in the 1,3,4,-thiadiazole ring derivative **4**, or in its homolog **5**, a methylene linker is present between the 1-adamantyl and carboxamido fragments of the molecule. It may be observed that these highly different substitution patterns of compounds **1–5** influence significantly the inhibition activities against all four investigated CAs. Thus, (i) the cytosolic slow isoform hCA I was weakly inhibited by the lead **1** (K_I of 8.6 μM) whereas its acylation leads to moderately effective inhibitors, such as acetazolamide and the adamantylcarboxamides **4** and **5**, which showed K_Is in the range of 250–850 nM. The extra CH_2 moiety present in **5** with respect to **4**, led to a 2.5 times increase in the inhibitory potency for the last compound. On the other hand, the arylsulfonamide derivative **3** was a very potent hCA I inhibitor (K_I of 1.4 nM) being 6142 times more inhibitory compared to **1**, and 178 times more inhibitory compared to acetazolamide **2**; (ii) against the physiologically dominant hCA II isoform, **1** behave as a moderate inhibitor, with a K_I of 60 nM, whereas compounds **2–5** showed very good inhibitory activities, with inhibition constants in the range of 0.3–11 nM. Again the best inhibitor was the arylsulfonylated derivative **3** (K_I of 0.3 nM), whereas the three carboxamides showed rather similar activity, with K_Is of 8.9–12 nM. Thus, it may be observed that derivatization of the lead **1** by arylsulfonyla-

Table 1
Inhibition of human α-CA (hCA) isozymes I, II, IX and XII with sulfonamides **1–7**, by a stopped-flow CO_2 hydrase assay[17]

Inhibitor	K_I[a] (nM)						
	1[b]	**2**[b]	**3**[b,c]	**4**[b]	**5**[c]	**6**[b]	**7**[b]
hCA I[b]	8600	250	1.4	883	362	4000	50,000
hCA II[b]	60	12	0.3	11	8.9	21	21
hCA III	1.5×10^5	2×10^5	1.8×10^4	3×10^5	4.1×10^5	3×10^5	7×10^4
hCA IV	940	74	15	349	413	60	880
hCA VA	2300	63	21	448	401	88	794
hCA VB	2150	54	18	440	416	70	93
hCA VI	798	11	41	765	921	65	94
hCA VII	5.2	2.5	2.9	35	48	15	2170
hCA IX	41	25	38	6.4	49.5	14	16
hCA XII	34	5.7	13	2.8	4.7	7	18
mCA XIII	79	17	0.7	118	147	21	98
hCA XIV	280	41	0.9	7.5	5.1	13	689
mCA XV	48	72	30	652	719	34	45

Data for compounds **3–5** were generated in this study, although some of them (i.e., against CA I and II) were reported earlier in Refs. 15e,18.
[a] Errors in the range of ±5 % of the reported data from three different assays by a stopped-flow CO_2 hydration method.[17]
[b] From Refs. 15e,16a,18.
[c] This work.

Table 2
Data and final model statistics for the hCA II–**4** adduct.

Data-collection statistics	
Temperature (K)	100
Wavelength (Å)	1.5418
Space group	$P2_1$
Unit-cell parameters (Å,°)	a = 42.2, b = 41.3, c = 71.9, β = 104.2
Total number of reflections	25,667 (2482)[d]
Total number of unique reflections	316,281 (29,784)[d]
Resolution (Å)	24.0–1.7 (1.76–1.7)[d]
R_{sym}[a]	8.6 (54.1)[d]
$I/\sigma(I)$	11.3 (2.0)[d]
R_{cryst}[b] (%)	16.6
R_{free}[c] (%)	21.1
Amino acid residues	4–261
No. of protein atoms	2070
No. of drug atoms	22
No. of H_2O molecules	234
R.m.s.d. for bond lengths (Å), angles (°)	0.007, 1.1
Ramachandran statistics (%)	
Most favored, additionally allowed and generously allowed regions	88, 11.5, 0.5
Average B factors (Å²)	
Main-, side-chain, Zn, drug **4**, solvent	17.7, 20.8, 14.2, 30.8, 29.4

[a] $R_{sym} = \Sigma|I - \langle I\rangle|/\Sigma\langle I\rangle$.
[b] $R_{cryst} = (\Sigma||F_o| - |F_c||/\Sigma|F_{obs}|) \times 100$.
[c] R_{free} is calculated in same manner as R_{cryst}[b], except that it uses 5% of the reflection data omitted from refinement.
[d] Values in parenthesis represent highest resolution bin.

tion or by acylation leads to much more effective CAIs, and the nature of the groups substituting the 5-carboxamido/arylsulfonamido moiety in the obtained CAI is crucial for the biological activity; (iii) isoform CA III was weakly inhibited by all these sulfonamaides (K_Is in the millimolar range) whereas against isoforms hCA IV, VA, VB, VI, and XV (all membrane-associated or mitochondrial isozymes) acetazolamide **2** and the disulfonamide **3** were generally effective, nanomolar CAIs, whereas the adamantyl derivatives **4** and **5** showed a decrease activity (in the micromolar range); (iv) hCA VII and XIII were very potently inhibited by compounds **1–3** but around one order of magnitude less by the adamantyl derivatives **4** and **5**, which showed K_Is in the range of 35–48 nM against the first and of 118–147 nM against the second isoform (Table 1); (v) regarding the inhibition of the tumor associated isozymes CA IX and XII with these five sulfonamides it may be observed that the lead **1** was slightly less effective than its derivatives, but for example in the case of CA IX, the arylsulfonamide derivative **3** showed the same activity as **1**, whereas the longer adamantyl derivative **5** was less effective an inhibitor (K_I of 49.5 nM) compared to **1**. On the other hand, **4** was a very effective hCA IX inhibitor (K_I of 6.4 nM), being 3.9 times more effective than acetazolamide against this isoform. For hCA XII, all compounds showed effective inhibitory activity (K_Is of 2.8–33 nM), with the lead **1** being the least effective inhibitor, followed by the bis-sulfonamide 3 (K_I of 13 nM) whereas the carboxamides **2, 4** and **5** being highly potent CA XII inhibitors. Against hCA XIV, another tranmembarne isoform, as CA IX and XII; the inhibition trend among the five compounds was the same as the one discussed above. Thus, it may be observed that the structure activity relationship (SAR) for these derivatives is quite different with respect to the inhibition of the cytosolic versus the transmembrane, membrane-associated or mitochondrial isozymes. Although none of these derivatives shows selectivity for the inhibition of any isoform, the best profile is observed for **4** and **5**, which have lower affinity for hCA I (off-target isoform), are strong CA II inhibitor but shows even stronger activity against the tumor-associated isoforms hCA IX and XII. Furthermore, these compounds are less promiscuous CAIs compared to acetazolamide **2** or the disulfonamide **3**, which are potent inhibitors of all isoforms except CA III. Thus, we decided to understand the molecular basis for the strong inhibition of CAs with this compound, and report here the high resolution X-ray crystal structure of **4** in complex with hCA II, an isoform which easily crystallizes and which is a widely used model enzyme for designing CAIs.[1–4,10]

The structure of hCA II complexed with **4** has been determined to 1.7 Å resolution (Table 2).[20] Compound **4** is well ordered and refined with full occupancy, with B factors that were comparable to the solvent within the active site (Fig. 1a and Table 2). Similar to other sulfonamide inhibitors of hCA II, the sulfonamide amine nitrogen of **4** binds directly to the active site zinc atom along with the side chains of His94, His96 and His119. The overall $Zn(N)_4$ coordination can be described as a distorted tetrahedron. The thiadiazole and the adamantyl moieties protrude outwards of the active site and are stabilized by both hydrophilic and hydrophobic residues (Fig. 1b and 2). The thiadiazole ring is seated on the plane that bisects the active site into distinctive hydrophilic and hydrophobic regions, stabilized by Glu92, Leu198, Thr200, and Pro201 (Fig. 1b). The adamantyl moiety points out of the active site, primarily stabilized through hydrophobic interactions with Ile91 and Phe 131. Within the resolution limits of the structure determination **4** does not induce any conformation changes in the active site compared to the unbound form (C_αRMSD <0.15 Å). The nitrogen of the sulfonamide strongly bound to the Zn (2.0 Å), displacing the Zn bound solvent, while one of the sulfonamide oxygens occupies the position of the water molecule termed deep water (Dw). The water Dw in the unbound hCA II is located in a hydrophobic pocket and is hydrogen bonded to the Zn-solvent. It is displaced

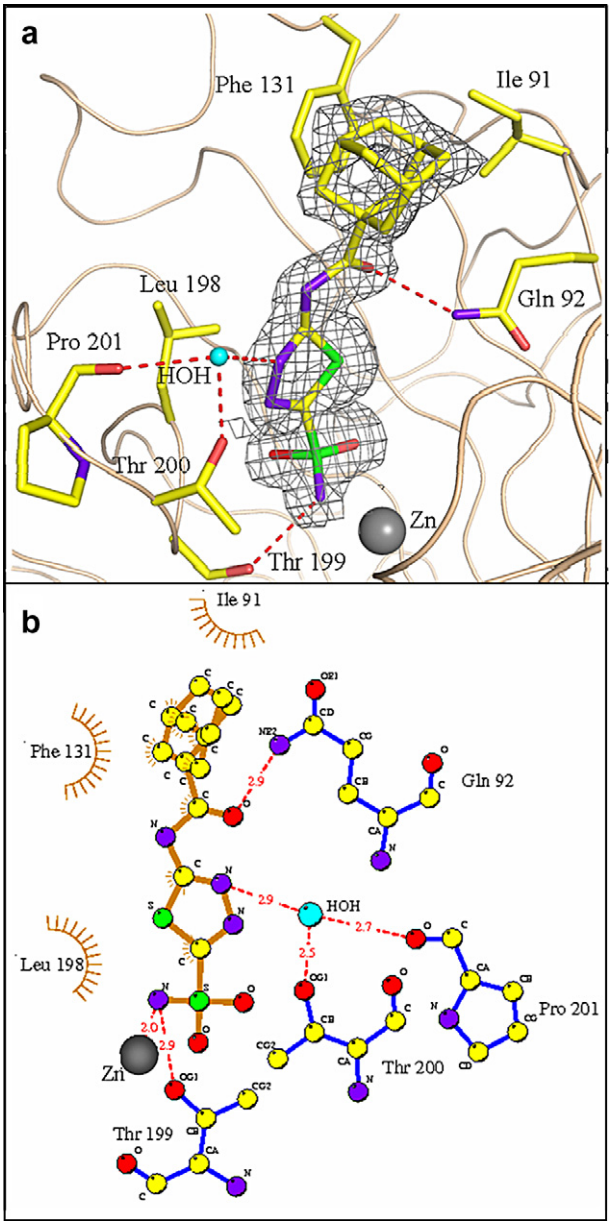


Figure 1. (a) Stick representation of 5-(1-adamantylcarboxamido)-1,3,4-thiadiazole-2-sulfonamide (**4**). The electron density is a σ-weighted $2F_o - F_c$ Fourier map (grey mesh). Figure made using PyMOL (DeLano Scientific). (b) Schematic 2D-representation of hCA II–**4** interactions. Hydrophobic contacts are indicated by orange hash marks, and H-bond lengths (Å) are indicated by red dashed lines. Figure made using Ligplot. Amino acids are as labeled. Atoms are colored as: zinc, grey; carbon, yellow; oxygen, red; nitrogen, purple; and sulfur, green spheres.

upon CO_2 substrate binding.[1d] Compound **4** also disrupts the well-ordered network of waters, on the hydrophilic side of the active site and pushes the proton shuttle residue His64, due to steric clashes, into an out conformation. These waters and His64 have been shown to play a critical role in the proton transfer reaction which is the rate-determining step for the hCA II catalytic turnover.[21,28]

In order to understand whether the tails of inhibitors **3** and **4** bind in the same active site region of hCA II, we have superposed the hCA II—**4** adduct reported here with the hCA II—**3** adduct (PDB file 2HOC) reported earlier by one of our groups.[29] As seen from data of Figure 2, the 1,3,4-thiadiazole-sulfonamide moieties of the two inhibitors **3** and **4** are superposable in the two hCA II adducts, whereas the side chains are orientated in two very diverse

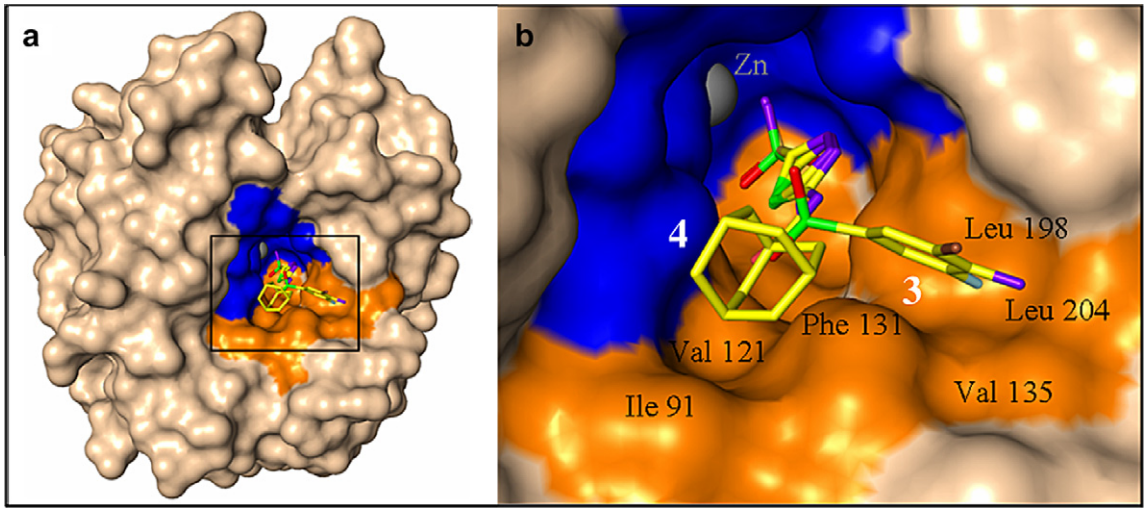


Figure 2. Overall (a) and zoom-in (b) view of superposed hCA II–**3** (PDB file 2HOC)[29] and –**4** adduct (this study), showing the two separate hydrophobic pockets in which the tails of the inhibitors bind, separated by Phe131. hCA II is depicted as a surface representation (bulk solvent accessible area, light pink; hydrophilic and hydrophobic regions of the active site, blue and orange, respectively). Inhibitors **3** and **4** are represented as sticks. Atoms of inhibitor molecules are colored as in Figure 1. Figure made using PyMOL (DeLano Scientific).

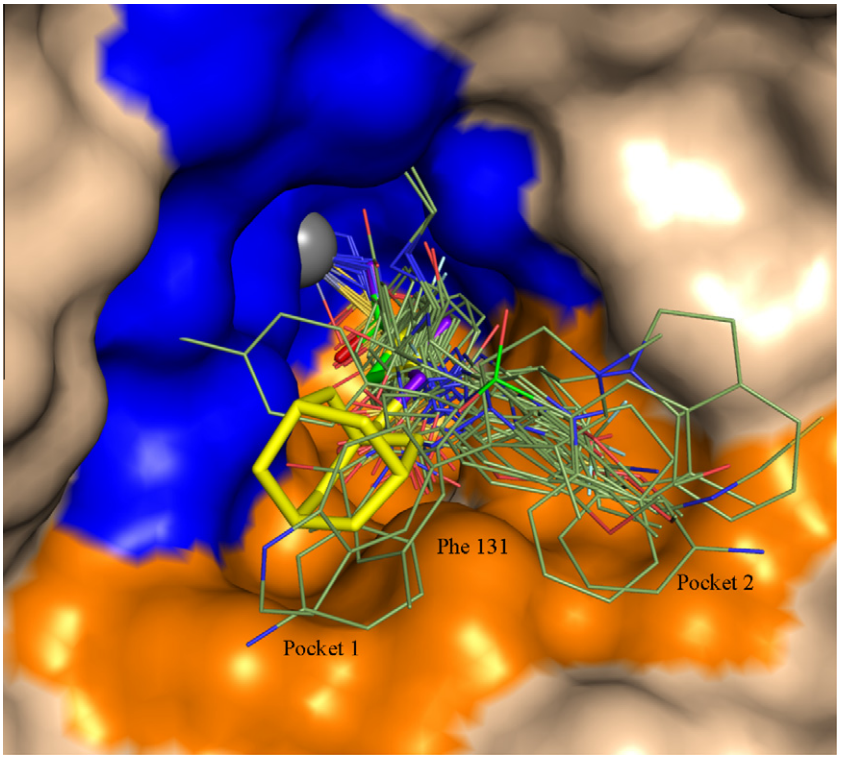


Figure 3. Superposition of hCA II–**4** adduct with selected hCA II–inhibitors complexes. All the inhibitors have the same sulfonamide-binding mode to the Zn. The hydrophobic tails of these inhibitors, however segregate into two distinct hydrophobic pockets that are separated by Phe 131. The tails of hCA II–**4** adduct and a select few other hCA II–inhibitors , compounds **6**–**9**, (PDB ID: 1IF9, 1OQ5, 1ZE8 and 2FOU) have a preferential binding to the less utilized 'pocket 1' whilst the tails of the majority of the hCA II–inhibitors (PDB IDS: 1A42, 1BN1,1BN3, 1BN4, 1BNM, 1BNQ, 1BNT, 1BNU, 1BNW, 1IF4, 1IF5, 1IF6, 1IF7,1IF8, 1KWQ, 1OKL, 1OKM, 10KN, 1TTM, 1Z9Y, 1ZFK, 1ZH9, 2FOQ and 2FOS) bind in the more commonly observed 'pocket 2' binding-site. hCA II is represented as a surface-model (hydrophobic, orange; hydrophilic, blue). The hCAII-**4** adduct is represented as a stick-model (C, yellow). All the superposition hCA II–inhibitors from PDB are represented as stick-model (C, green). Atoms N, O and S of inhibitor molecules are colored as in Figure 1.

orientations. In the case of the arylsulfonamide moiety present in **3**, the amino acid residues with which this moiety interacts are Leu198, Phe131, Val 135 and Leu204. On the contrary, in the case of the adamantyl derivative **4**, the bulky hydrophobic chain of the inhibitor interacts with amino acid residues Ile91, Val121 and Phe131, almost on the opposite side of the active site compared to the previous inhibitor (Fig. 2). These two hydrophobic pockets, designated as **1** and **2**, respectively, are thus separated by Phe131, a critically important residue for the binding of many classes of inhibitors.[7,10]

To check whether these binding patterns are more general, 28 hCA II sulfonamide inhibitors that possessed hydrophobic groups

with sufficient size to extend to Phe131 were selected from the Protein Data Base[30] (1IF9, 1OQ5, 1ZE8, 2FOU, 1A42, 1BN1,1BN3, 1BN4, 1BNM, 1BNQ, 1BNT, 1BNU, 1BNW, 1IF4, 1IF5, 1IF6, 1IF7, 1IF8, 1KWQ, 1OKL, 1OKM, 10KN, 1TTM, 1Z9Y, 1ZFK, 1ZH9, 2FOQ and 2FOS) and least-square superposed onto the hCA II coordinates of hCAII-**4** with RMSDs of <0.3 Å and compared (Fig. 3). It may be observed that in addition to **4**, only four other sulfonamides, compounds **6–9**,[7c,d,31,32] used the hydrophobic pocket evidenced in this work, which we designate as 'pocket 1'. The tails of the majority of the other hCA II–inhibitors (PDB IDS: 1A42, 1BN1, 1BN3, 1BN4, 1BNM, 1BNQ, 1BNT, 1BNU, 1BNW, 1IF4, 1IF5, 1IF6, 1IF7, 1IF8, 1KWQ, 1OKL, 1OKM, 10KN, 1TTM, 1Z9Y, 1ZFK, 1ZH9, 2FOQ and 2FOS) were observed in the more commonly used 'pocket 2' binding-site, on the right of Phe131 (Fig. 3). Whereas compounds **6**[7d] and **7**[7c] are potent CAIs (K_I of 21 nM against CA II, Table 1), comparable to **4** (K_I of 11 nM), derivative **8**[31] was reported to be a very potent CA II inhibitor (K_I of 0.3 nM) and **9**[32] a much weaker one (K_I of 80 nM against CA II and of 76 nM against CA I). Thus, the binding within the hydrophobic pocket 1 or 2 does not correlate with the potency of the sulfonamide inhibitor, but it may be important for delineating features which lead to some isoform selectivity. Indeed, whereas residue 121 is conserved in most CAs (being a Val in all isoforms except CA I, in which is Ala), the remaining two residues defining the hydrophobic pocket 1, that is, 91 and 131, are among the most variable ones in the active site of α-CAs. For example, position 91 is Phe in CA I, Ile in CA II, Arg in CA III and XIII, Lys in CA IV, VA, VB and VII, Gln in CA VI, Leu in CA IX and XV, Thr in CA XII, and Ala in CA XIV.[33] It is thus clear that this hydrophobic pocket 1 represents a hot spot for designing inhibitors which interact with it, in order to target specifically different CA isozymes. In fact, as seen form data of Table 1, compounds **4, 6** and **7** which bind to hydrophobic pocket 1 do show a better inhibition profile (being less promiscuous CAIs) compared to **3** which binds with the tail in the hydrophobic pocket 2 and which is a very potent CAI for most isoforms except CA III (acetazolamide **2** has too short a tail to be discussed here).

In conclusion, we investigated the inhibitory activity of several 1,3,4-thiadiazole-sulfonamides against the physiologically relevant isoforms hCA I, II, IX and XII. The tail derivatizing the 5-position in the 1,3,4-thiadiazole-2-sulfonamide scaffold is crucial for the inhibitory activity of these compounds. We have resolved the high resolution X-ray crystal structure of hCA II in complex with 5-(1-adamantylcarboxamido)-1,3,4-thiadiazole-2-sulfonamide, evidencing a less utilized hydrophobic pocket in which the adamantyl moiety of the inhibitor lies, which is lined by the amino acid residues Ile91, Val121 and Phe131.

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20. Co-crystals of hCA II–**4** complex were obtained using the hanging drop vapor diffusion method as reported earlier.[1d,11,21] 10 μl drops (0.2 mM hCA II; 0.4 mM drug **4**; 0.8 M sodium citrate; 50 mM Tris-Cl; pH 8.0 were equilibrated against precipitant solution (1.6 M sodium citrate; 50 mM Tris-Cl; pH 8.0) at room temperature (~20 °C).[21] Useful crystals were observed 4 days after the crystallization setup. A crystal was cryoprotected by quick immersion into 25% glycerol precipitant solution and flash-cooled by exposure to a gaseous stream of nitrogen at 100 K. X-ray diffraction data were obtained using an R-AXIS IV^{++} image plate system with Osmic Varimax HR optics and a Rigaku RU-H3R Cu rotating anode operating at 50 kV and 22 mA. The detector-crystal distance was set to 80 mm. The oscillation steps were 1° with a 6 min exposure per image. Indexing, integration, and scaling were performed using HKL2000.[22] Starting phases were calculated from Protein Data Bank entry 2CBA[23] with waters removed. Refinement using Phenix package,[24] with 5% of the unique reflections selected randomly and excluded from the refinement data set for the purpose of R_{free} calculations,[25] was alternated with manual refitting of the model in Coot.[26] The final model refined to an R_{cryst} of 16.6% and R_{free} of 21.1%. The validity of the final model was assessed by PROCHECK.[27] Complete refinement statistics and model quality are included in Table 2.
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