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X-ray crystallography-promoted drug design of carbonic anhydrase inhibitors†

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1-*N*-Alkylated-6-sulfamoyl saccharin derivatives were prepared and assayed as carbonic anhydrase inhibitors (CAIs). During X-ray crystallographic experiments an unexpected hydrolysis of the isothiazole ring was evidenced which allowed us to prepare highly potent enzyme inhibitors with selectivity for some isoforms with medical applications.

The artificial sweetener saccharin (**SAC**) (Fig. 1) was previously reported as an efficient inhibitor of several isoforms of the human metalloenzymes carbonic anhydrases (CAs, EC 4.2.1.1) with promising selectivity towards the cancer associated isoforms hCA IX and hCA XII,[1] both of which have been recently validated as drug targets for anti-cancer therapy or imaging of hypoxic tumors.[2] It should be noted that CAs are efficiently but indiscriminately inhibited by most sulfonamides such as acetazolamide (**AAZ**) but hCA IX selective inhibitors, such as SLC-0111, are also known, this compound being in Phase I clinical trials for the treatment of patients with advanced solid, metastatic tumors overexpressing CA IX/XII.[3]

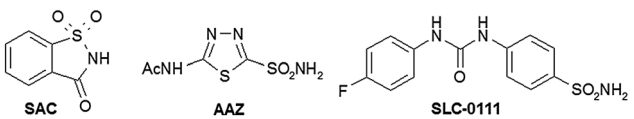

Fig. 1 Chemical structures of known CAIs.

Despite promising achievements in selective inhibition of hCA IX and hCA XII there is still a demand for more effective and selective inhibitors of various CA isoforms, such as CA II, VA, VB, IX, *etc.*[2]

The mechanism of CA inhibition by **SAC** is rather different compared to that of primary sulfonamides, the most investigated class of CA inhibitors (CAIs) including those used clinically (**AAZ**). Even though in both cases the binding to the Zn ion within the active site of CA takes place by the deprotonated nitrogen of the sulfonamide group, the **SAC** binding significantly differs from that of primary sulfonamides. The presence of the acyl group incorporated into the isothiazole ring and the absence of a proton on the nitrogen exhibit a rather different binding pattern of **SAC** to the enzyme compared to primary sulfonamides.[2]

Such different interactions directly reflect the inhibition profile of **SAC**, which efficiently inhibits only the cytosolic isoform hCA VII and the tumor associated one hCA IX compared to primary sulfonamides such as **AAZ**, which is a highly efficient inhibitor of 14 out of the 15 hCAs known to date.[1,2] For this purpose **SAC** was extensively used as a lead molecule for obtaining novel CAIs ultimately.[4–6] For example, we synthesized 6-sulfamoylsaccharin **1** and its 1-substituted derivatives **2** (Scheme 1)[4] where the opportunity to investigate competition of binding of the primary and secondary sulfonamide to the enzyme (in the case of **1**) emerged.[7]

Indeed, recently we reported the high resolution X-ray crystal structure of the adduct of hCA II with **1**, which proved that only the primary sulfonamide participates in the interaction with the metal ion.[7] Thus a series of *N*-substituted saccharin derivatives **2a–2i** appeared to be of interest for being prepared by reacting **1** with alkyl/aralkyl bromides in DMF (Scheme 1, see the ESI† for details). We investigated the inhibitory properties of these compounds and their binding to the enzyme by means of kinetic experiments and X-ray crystallography.

In order to visualize the binding mode of saccharin sulfonamide derivatives **2** to hCA II, we solved the high resolution

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† Electronic supplementary information (ESI) available: A detailed description of the synthesis and characterization of compounds **1–4**, as well as the enzyme inhibitory assays. See DOI: 10.1039/c5cc01854d

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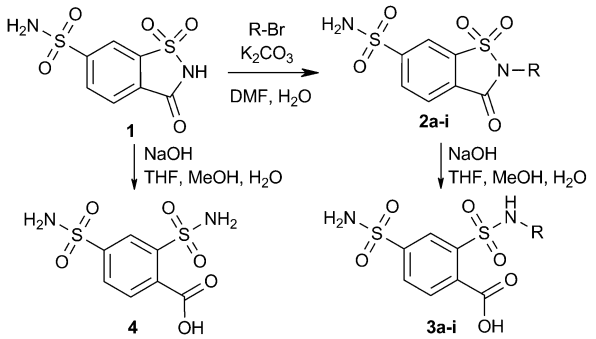

Scheme 1 Synthesis of 1-*N*-substituted 6-sulfamoylsaccharins **2** and their hydrolysis products **3** and **4**.

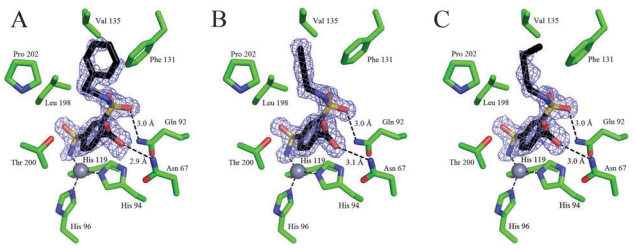

Fig. 2 Comparison of binding modes of compounds **2i**, **2e** and **2d** within the hCA II active site. Compound **2i**/**3i** is shown in panel A, **2e**/**3e** is shown in panel B and **2d**/**3d** is shown in panel C. The zinc ion is the gray sphere and its coordinating residues (His94, 96 and 119) are shown in green. Residues 67, 92, 131, 135, 198, 200 and 202 participating in hydrogen bonding, hydrophobic and van der Waals contacts with inhibitors are also indicated. For the sake of clarity, $F_o - F_c$ OMIT electron density is shown only for ligands and contoured at 3σ. The figure was prepared by using Pymol (DeLano ThePyMOL Molecular Graphics System San Carlos, CA, USA, DeLano Scientific).

crystal structures of hCA II in complex with compounds **2i**, **2e** and **2d** reported here. The electron density was interpretable for all inhibitors (Fig. 2) and surprisingly revealed that the isothiazole ring was open in all of them. Even though the isothiazole ring opening occurs most probably by alkaline hydrolysis due to the relatively high pH (of 9.0) in the crystallization buffer it unexpectedly and clearly revealed a new possibility of designing CAIs. One should mention that initially we explored the possibility that the enzyme itself hydrolyzed the amide bond from derivatives **2**, but this did not occur (data not provided). Indeed, although the CAs have esterase and thioesterase activity,[8,9] they do not possess peptidase activity. Notably all three compounds were bound in a very similar fashion, coordinating to the zinc ion with their primary sulfonamide, whereas oxygens of carboxyl and sulfone groups made H-bonds with the Asn67 and Gln92 side chains. The R moieties occupied a hydrophobic pocket, formed by the side chains of residues Phe131, Val135, Leu198, Leu204 and Pro202 (Fig. 2).

The observed binding mode of all three compounds is substantially different from that previously reported for unsubstituted saccharin (PDB code 2Q38),[1] or derivative **1**,[7] when the two inhibitors were not hydrolysed. Thus, the binding observed for compounds **3**, obtained by hydrolysis of derivatives **2** reported here, is indeed very different compared to other saccharin based CAIs reported so far[4–7] (Fig. 3). Inspired by these crystallographic results

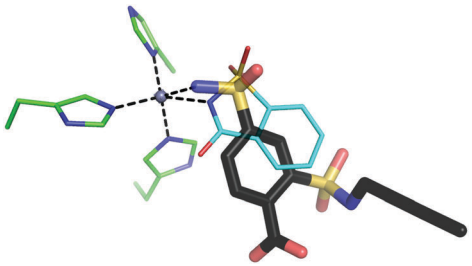

Fig. 3 Different binding modes of **SAC** (grey carbons, thin sticks) and *N*-substituted 'open' saccharin **3e** (black carbons, thick sticks). Both compounds are coordinating active site Zn ions (grey sphere), but **3e** is bound to the metal ion by its primary sulfonamide whereas **SAC** by the secondary, acylated sulfonamide.

we thereafter prepared all the corresponding open forms of the 6-sulfamoyl saccharins **1** and **2**, obtaining the bissulfamoyl carboxylic acids **3** and **4** (Scheme 1), under alkaline hydrolytic conditions. Even though we expected that these carboxylic acids **3** and **4** might undergo a ring closure under neutral or acidic conditions, we did not observe the isothiazole ring closure even by storing compounds **3** and **4** for a prolonged period at ambient temperatures.

All compounds obtained were subjected to CA inhibition studies summarized in Table 1.

Four hCA isoforms were included in this study: two cytosolic ones hCA I and hCA II, and two tumor-associated transmembrane isoforms hCA IX and hCA XII, all of which are drug targets for various applications of their inhibitors.[2,3] Data of Table 1 show the following interesting findings.

Against the slow cytosolic isoform hCA I the activity range of compounds **1**–**4** was between 2.6 and 451 nM. Almost all compounds showed a better inhibition compared to the non-selective compound **AAZ**. Only compound **4** showed low inhibition against this isoform, whereas derivatives **1**, **2a** and **3g** had a comparable inhibition profile to that of **AAZ**. However the most interesting observation was that in pairs of closed/open ring derivatives **1**/**4** and **2**/**3**, the net reduction of the inhibitory activity for the open forms **4** and **3** (1.7 to up to 43 times) compared to the corresponding closed form ones **1** and **2** occurred. The most significant reduction of activity, by two orders of magnitude, was observed for compounds **3e** and **3g**, which were 27 and 43 times, respectively, less inhibitory compared to the corresponding benzisothiazoles **2e** and **2g**. The only exceptions to this rule were the pairs **2a**/**3a** and **2h**/**3h**, for which the closed form was less inhibitory than the open ones (Table 1).

For the rapid isoform hCA II a similar inhibition pattern was observed as for hCA I discussed above. All compounds except **4** showed an excellent, better inhibitory activity than **AAZ**, with K_is in the range of 0.2–8.4 nM. A similar reduction of the inhibitory activity of the open *versus* the closed forms was also observed with most compounds, but already many of the closed ones were low nanomolar hCA II inhibitors and thus, this reduction seems to be less relevant for this isoform than for hCA I. The nature of the R group also influenced the inhibition pattern of these derivatives significantly. Thus, an increase of the aliphatic chain from C2 to C4 led to an increase of the hCA II

Table 1 CA inhibition data of isoforms hCA I, II, IX and XII with saccharin derivatives **1–2** and the corresponding open forms **3–4** reported in this communication, by a CO_2 hydrase stopped-flow assay[12]

		K_i (nM)			
Compound	R	hCA I	hCA II	hCA IX	hCA XII
1	—	251	8.4	337	52.9
2a	Et	257	1.0	452	7.2
2b	*n*Pr	49	0.6	278	5.9
2c	*n*Bu	4.8	0.6	51.2	5.7
2d	nC_5H_{11}	4.3	3.5	380	5.2
2e	$CH_2CHCHMe$	4.6	0.4	271	9.5
2f	Bn	2.6	0.4	51.8	5.8
2g	$CH_2C_6H_4(4\text{-}NO_2)$	4.9	0.2	52.9	7.9
2h	$CH_2C_6H_4(4\text{-}Br)$	57.8	0.8	321	14.3
2i	CH_2CH_2Ph	9.1	0.4	378	6.7
3a	Et	66.2	1.7	92.8	77.7
3b	*n*Pr	81.4	0.2	89.5	63.6
3c	*n*Bu	41.4	1.1	130	58.7
3d	nC_5H_{11}	29.9	6.0	333	68.3
3e	$CH_2CHCHMe$	125	0.7	78.1	67.8
3f	Bn	64.0	1.5	67.4	50.5
3g	$CH_2C_6H_4(4\text{-}NO_2)$	213	2.2	73.6	195
3h	$CH_2C_6H_4(4\text{-}Br)$	38.7	0.3	70.5	27.0
3i	CH_2CH_2Ph	59.0	2.8	71.6	48.6
4	—	451	31.7	42.1	63.3
AAZ[a]	—	250	12	25	5.7

[a] Acetazolamide (**AAZ**) was used as a standard inhibitor for all CAs investigated in this communication.

inhibitory properties but a further increase to C5 was detrimental for the inhibitory activity (compare **2d** to **2a–c**, Table 1). However, unsaturated or aralkyl chains (as in **2e–2i**) led again to highly effective, subnanomolar CAIs, for all the substitution patterns of compounds **2e–2i**, *i.e.*, benzyl, 4-substituted benzyl moieties or phenethyl.

An opposite inhibition pattern was observed in the case of the tumor-associated transmembrane isoform hCA IX with compounds **2–4** reported here. Even though none of the compounds was superior to **AAZ**, the inhibitory activity increased going from the closed to the open forms for the compound pair **1**/**4** and most of the pairs **2**/**3**. As shown in Table 1, the highest increase, more than 4 times, was observed for compounds **3a**, **3h**, **3i** and **4** with K_is in the range of 42.1–92.9 nM, which are effective inhibitors of this tumor-associated isoform.

For the second transmembrane isoform, hCA XII, the inhibition pattern was similar to those of the cytosolic isoforms hCA I and hCA II discussed above. All closed forms except **1** and **2h** exhibited comparable inhibitory activity with **AAZ**, whereas the open forms **3a–3f**, **3h–4** were around one order of magnitude less inhibitory compared to **AAZ**. Overall, many low nanomolar hCA XII inhibitors were detected such as for instance **2a–2i**, which had inhibition constants ranging between 5.2 and 14.3 nM, in the same range as the classical sulfonamide inhibitor **AAZ**.

The most interesting finding of this communication is however the fact that our drug design has been guided by the crystallographic work, which evidenced a hydrolytic process taking place during the crystallization experiments. Unexpectedly, the hydrolysis afforded compounds possessing a free COOH moiety in addition to the primary and secondary sulfamoyl moieties. This type of sulfonamide was in fact not synthesized so far using other synthetic procedures, and as shown above, they possess notable inhibitory properties, with a profile quite different from that of the structurally related, closed form (or the primary sulfonamide **AAZ**). In fact all sulfonamides **3a–3i** were highly effective, CA II-selective inhibitors, and this type of profile is very rare or even absent among the many sulfonamide CAIs reported so far.[10] Furthermore, the crystallographic experiments (Fig. 2 and 3) also showed that the R moiety present in these compounds may adopt a variety of orientations within the CA II active site, which may explain their very high affinity for this isoform and the relatively lower ones for other isoforms such as hCA I, IX and XII (Table 1). As hCA II is the main target for designing anti-glaucoma CAIs (in clinical use for decades but with many side effects due to inhibition of other isoforms),[11] these findings may lead to the design of water-soluble (due to the presence of the COOH moiety, which may form sodium salts), highly effective and selective hCA II inhibitors belonging to a novel chemical space.

In conclusion we report here new CAIs obtained by a 'side reaction' which occurred during an X-ray crystallographic study of sulfonamide–CA adducts. We have demonstrated the high potential of the newly obtained compounds (open/closed forms of 1-*N*-substituted saccharins or the unsymmetrically substituted bissulfamoyl benzoic acids), possessing an improved selectivity towards some CA isoforms with medical applications. Considering the chemical simplicity and good water solubility of the newly obtained CAIs, their scaffold may find applications in the development of new types of CAIs, probably by modulating the nature of the moieties substituting in position 1 the saccharin derivatives (the R moiety). Indeed, in this communication we explored few substitution patterns which are aliphatic, alkenyl and aralkyl groups. By extending the type and nature of these moieties, which as shown in the crystal structures, interact with amino acid residues critical for the binding of inhibitors, compounds with improved potency and selectivity may presumably be obtained.

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