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X-Ray Crystallography-Promoted Drug Design of Carbonic Anhydrase Inhibitors†

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Cite this: DOI: 10.1039/k0xx00000x

Received ooth January 2012, Accepted ooth January 2012

DOI: 10.1039/k0xx00000x

www.rsc.org/

Abstract. 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) (Figure 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 being recently validated as a drug target for anti-cancer therapy or imaging of hypoxic tumors.2 It should be noted that CA 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 Despite promising achievements on selective inhibition of hCA IX and hCA XII there is still a demand on more effective and selective inhibitors of various CA isoforms, such as CA II, VA, VB, IX, etc.4 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 in the isothiazole ring and the absence of a proton on the nitrogen raises a rather different binding pattern of SAC to the enzyme compared to primary sulfonamides.5

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)7 where the opportunity to investigate competition of binding between the primary and secondary sulfonamide to the enzyme (in the case of 1) emerged.7

![Chemical structures of known CAIs.](Fig. 1)

![Scheme 1 Synthesis of 1-N-substituted 6-sulfamoylsaccharines 2 and their hydrolysis products 3 and 4.](Scheme 1)
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 of interest to be prepared by reacting 1 with alkyl/arylalkyl bromides in DMF (Scheme 1, see Supporting information 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 hCAII, we solved the high resolution crystal structures of hCAII 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 to design 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 shown). Indeed, although the CAs have esterase and thioesterase activity,8,9 they do not possess peptidase activity. Notably all these 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 of previously reported for unsubstituted saccharin (PDB code 2Q38)4, 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 (Figure 3). Inspired by these crystallographic results we thereafter prepared all the corresponding open forms of the 6-sulfamoyl saccharins 1 and 2, obtaining the bis(sulfamoyl) 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 in 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.

Four hCA isoforms were included in this study: two cytosolic ones hCA I and hCA II, and the two tumor-associated transmembrane isoforms hCA IX and hCA XII, all of which are drug targets for various applications of their inhibitors.10,11 Data of Table 1 shows the following interesting findings. Against the slow cytosolic isoform hCA I the activity range of compounds 1-4 was between 2.6-451 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, a 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.

![Fig. 2 Comparison of binding modes of compound 2i, 2e and 2d within the hCA II active site. Compound 2f/3 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$_2$F$_7$ DMT electron density is shown only for ligands and contoured at 3o. The figure was prepared by using Pymol (Delano ThePyMOL Molecular Graphics System San Carlos, CA, USA, Delano Scientific).

![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 ion (grey sphere), but 3e is bound to the metal ion by its primary sulfonamide whereas SAC by the secondary, acylated sulfonamide.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>$K_i$(nM)</th>
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<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>251.8</td>
</tr>
<tr>
<td>2a</td>
<td>Et</td>
<td>257.1</td>
</tr>
<tr>
<td>2b</td>
<td>nPr</td>
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<tr>
<td>2c</td>
<td>nBu</td>
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</tr>
<tr>
<td>2d</td>
<td>nC$<em>6$H$</em>{11}$</td>
<td>4.3</td>
</tr>
<tr>
<td>2e</td>
<td>CH$_2$CH$_2$Me</td>
<td>4.6</td>
</tr>
<tr>
<td>2f</td>
<td>Bn</td>
<td>2.6</td>
</tr>
<tr>
<td>2g</td>
<td>CH$_2$(C$_6$H$_4$(4-NO$_2$))</td>
<td>4.9</td>
</tr>
<tr>
<td>2h</td>
<td>CH$_2$CH$_2$(4-Br)</td>
<td>57.8</td>
</tr>
<tr>
<td>2i</td>
<td>CH$_2$CH$_2$Ph</td>
<td>9.1</td>
</tr>
<tr>
<td>3a</td>
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<tr>
<td>3d</td>
<td>nC$<em>6$H$</em>{11}$</td>
<td>29.9</td>
</tr>
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<td>3e</td>
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<td>3g</td>
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<td>38.7</td>
</tr>
<tr>
<td>3i</td>
<td>CH$_2$CH$_2$Ph</td>
<td>59.0</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>451.3</td>
</tr>
</tbody>
</table>

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

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This table shows the data for the inhibition of the hCA isoforms by the compounds mentioned. The values are given in nanomolar (nM).

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Four hCA isoforms were included in this study: two cytosolic ones hCA I and hCA II, and the two tumor-associated transmembrane isoforms hCA IX and hCA XII, all of which are drug targets for various applications of their inhibitors.10,11 Data of Table 1 shows the following interesting findings.

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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 were 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 that of AAZ, with Ks in 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 among many of the closed ones were low nanomolar hCA II inhibitors and as thus, this reduction seems to be for this isoform less relevant 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 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 from compounds 2e-2i, i.e., benzyl, 4-substituted benzoyl moieties or phenethyl.

An opposite inhibition pattern was observed in 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 Ks in 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 paper 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 the addition to the primary and secondary sulfamoyl moieties. This type of sulfonamides were in fact not available so far by 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 (Figs 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 bissulfamyl benzic 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 paper 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.

Acknowledgments. This research was in part financed by two FP7 EU projects (InnovaBalt and Dynano).

Notes and references
†Electronic Supplementary Information (ESI) available: Detailed description of the synthesis and characterization of compounds 1-4, as well as the enzyme inhibitory assays. See DOI: 10.1039/c000000x


