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Saccharin: a Lead Compound for Structure-Based Drug Design of Carbonic Anhydrase IX Inhibitors

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Abstract

Carbonic anhydrase IX (CA IX) is a key modulator of aggressive tumor behavior and a prognostic marker and target for several cancers. Saccharin (SAC) based compounds may provide an avenue to overcome CA isoform specificity, as they display both nanomolar affinity and preferential binding, for CA IX compared to CA II (>50-fold for SAC and >1000-fold when SAC is conjugated to a carbohydrate moiety). The X-ray crystal structures of SAC and a SAC-carbohydrate conjugate bound to a CA IX-mimic are presented and compared to CA II. The structures provide substantial new insight into the mechanism of SAC selective CA isoform inhibition.

Keywords

saccharin; metalloenzyme; carbonic anhydrase IX; CA IX-mimic; structure-based drug design

1. Introduction

Highly aggressive cancers often result from tumors that have undergone hypoxic stress^{1,2}. This is induced by several factors including a reduction in O₂ content in the tumor microenvironment, or by factors associated with inflammation³. Hypoxic stress causes tumorigenic properties that result in an unfavourable patient prognosis, including tumor growth, invasiveness, proliferation, metastasis, and a resistance to common radiation and chemotherapies^{3,4}. As a result there has been a large effort to target factors that are

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Supporting Information: Chemical synthesis and NMR spectra for new compounds.

Accession Codes. Coordinates and structure factors for CA IX-mimic:SAC and CA IX-mimic:1 crystal structures have been deposited with the PDB, with accession codes 4RIV and 4RIU, respectively.

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important for regulating and maintaining this hypoxic phenotype. A key component that aids in tumor cell survival during hypoxic stress is pH regulation. During cases of hypoxic stress, tumor cells undergo a metabolic shift that favours the utilization of glycolytic metabolism thus producing large amounts of lactic acid and the reduction in pH in the tumor microenvironment^{5,6}. Despite this acidification, tumor cells are still able to survive due to an upregulation of pH regulator elements. This creates a differential pH environment where the intracellular pH of the tumor cell remains close to physiological levels while the extracellular pH becomes acidic^{1,7}. Disruption of this differential pH environment has been shown to be detrimental to overall tumor cell survival⁷.

A key modulator of the differential pH in the tumor microenvironment is carbonic anhydrase IX (CA IX). CA IX is a transmembrane homodimeric enzyme that has its catalytic domain positioned extracellularly^{8,9}. Similar to other isoforms, CA IX is responsible for catalysing the reversible hydration of CO₂ to HCO₃⁻ and a proton¹⁰. Inhibition of CA IX activity has shown to reduce tumor growth and proliferation, and also enhance common chemo- and radiation therapies^{4,11}. Interestingly, CA IX is highly expressed in hypoxic tumor tissues but has limited expression in normal tissue⁴. The limited levels of CA IX expression in normal cells, coupled with its extracellular location, make it a desirable drug target for the development of anti-cancer therapies^{4,11}.

Despite the effort to design compounds targeting CA IX, this task has been challenging, mainly because of the structural and sequence conservation between the active sites of CAs^{12,13}. As such, current clinically used CA inhibitors have been unsuccessful for the targeting of CA IX due to their nonspecific isoform inhibition profiles^{12,14}. However, it has been recently shown that there is some significant variability between residues within and around the CA active site surfaces¹². Furthermore, the residues of highest variability make up the distinct hydrophobic and hydrophilic sides within the CA active sites^{12,15}. Based on these amino acid differences between isoforms it has been postulated that isoform selective inhibitors using the “tail” approach may be possible^{12,16}. This is based on the utilization of a zinc binding group (ZBG) to anchor the ligand to the CA, a linker region to stabilize the ligand, and a tail moiety that interacts with key amino acid residues to facilitate CA isozyme specificity. Historically the ZBG in CA inhibitors have been sulfonamides, bisulfites, cyanates, and thiocyanates, all of which indiscriminately bind to the zinc cation (Figure 1)¹². This implied the tail of the inhibitor compound would be key to modulate the CA isoform selectivity.

However, more recently it has been shown that saccharin (**SAC**), the main additive in the artificial sweetener, “Sweet N’ Low”, selectively binds to CA IX with nanomolar affinity¹⁷, and SAC-based compounds were highly selective for CA IX (with >1000-fold selectivity over other CA isoforms)^{18,19} (Figure 1). However until this study, structural information on specific interactions of either **SAC** or SAC-based compounds to the active site of CA IX were not known. This is mainly due to difficulties with the expression and crystallization CA IX^{8,9}. To circumvent this issue and gain insights into the specific binding interactions of both **SAC** and a SAC-based compound to CA IX, we use a CA IX-mimic. The CA IX-mimic is a CA II template with selective amino acid replacements that reflect a CA IX active site, which is easily expressed and crystallized like CA II but structurally analogous to

the catalytic domain of CA IX (~80% sequence identity between active site residues)²⁰. The CA II to CA IX-mimic substitutions are A65S, N67Q, E69T, I91L, F131V, K170E, and L204A²¹. Here we compare the specific interactions of **SAC** between CA II and CA IX, and provide a rationale for the >1000-fold preference of a SAC-based compound for CA IX over other CAs. This study provides insight into both the use of acyl-secondary sulfonamide moieties as a ZBG and the use of **SAC** as a lead compound for the design of selective CA isoform inhibitors towards the development for anti-cancer targeting of CA IX.

2. Results and Discussion

2.2 Inhibitor Design

The synthesis of compound **1** is described in Moeker, et al.¹⁹ and an improved synthesis is reported here (Supplementary Data), the purchase of **SAC** is as in Köhler, et al.¹⁷ **SAC** is a benzoic sulfimide that utilizes a cyclic secondary sulfonamide as the zinc binding group (ZBG) (Figure 2A). Compound **1** was designed utilizing the tail approach with a polar carbohydrate as the tail moiety of the CA inhibitor^{12,14}. Specifically, compound **1** consists of the SAC pharmacophore as the ZBG, a 1,2,3-triazole linker group and a glucose tail moiety (Figure 2B). The use of this arrangement has been previously shown to selectively target CA IX via preferential inhibition and physiochemical attributes^{12,19,22}.

2.3 Selective CA IX Inhibition

Several approaches have been employed to modify the tail region of compounds to produce CA IX selective inhibition^{12,14,16,23}. This includes the use of different ZBG, bulky chemical moieties to take advantage of the extracellular location of the CA IX catalytic domain, and prodrug-like properties^{24,25}. Ideally a CA IX inhibitor would be highly selective over other CA isoforms and have low membrane permeability^{4,11}. The measured inhibition constants (K_i s) of **SAC** and compound **1** for CA IX are 100 nM and 50 nM, respectively, and their selective inhibition of CA IX in comparison to CA II were 60 and >1000-fold, respectively. In addition, they both demonstrate favorable selectivity of CA IX over CA XII, another tumor-associated but more widespread CA isoform^{24,26}. CA I and II are ubiquitously expressed cytosolic enzymes therefore their inhibition profiles were used to distinguish CA IX selectively^{4,12,18}. The data for **SAC** and **1** were also compared to the inhibition of acetazolamide (**AZM**), a clinically used CA inhibitor that does not display isoform selective inhibition (Table 1).

SAC binds to both CA IX and CA XII in the nanomolar range. This comes as a surprise as cyclic sulfimide compounds are typically poor CA inhibitors²⁷. In addition **SAC** showed a 60-fold higher affinity for CA IX over CA II and an even greater preference for CA IX over CA I. **SAC** also shows preferential binding for CA XII over CA I and II. The nanomolar potency and preferential binding of **SAC** to CA IX and CA XII over CA I and CA II, make it a promising lead compound for anti-cancer CA targeting. Compound **1**, with its extended glucose tail moiety compared to **SAC**, displays a >1000-fold selectivity for CA IX over both CA I and CA II, the carbohydrate tail moiety also contributing both reduced membrane permeability and increased solubility^{27,28}. This result highlights the possible utilization of **SAC** modifications to increase CA selectivity to produce more potent CA IX inhibitors.

2.4. X-Ray Crystallography

The mode of **SAC** and compound **1** binding was studied using X-ray crystallography (Table 2). The **SAC** was seen to bind directly to the catalytic zinc of the CA IX-mimic displacing the zinc-bound OH⁻/H₂O, via its deprotonated imide (pK_A of **SAC** is 1.3)¹⁷. In addition the benzene ring, part of the benzoic sulfimide structure, interacts weakly with Leu198 (~3.5 Å) (Figure 3A). **SAC** binding does not displace the active site solvent network, as typically seen by other CA inhibitors, for example **AZM**^{14,21,29}. Instead, solvent W2, W3a, and W3b are clearly ordered, with potential H-bonds between the carbonyl of **SAC**, W2, and His64 creating further stabilizing interactions (Figure 3A). This result suggests that entry of **SAC** into the active site may be similar to that of the substrate CO₂, interacting with Leu198 on the hydrophobic face of the active site³⁰. This rationale might also explain the preference in binding of the **SAC** to CA IX and CA XII over CA I and II (Table 1). The higher affinity **SAC** has for CA IX compared to CA II might be due to the substitution of Phe131 for Val in CA IX. With the Val131 allowing passage of **SAC** into the active site, whereas the phenylalanine of CA II would cause significant steric hindrance and deter **SAC** entering the active site of CA II since **SAC** is a structural rigid molecule (Figure 3B). The same can be postulated in regards to the observed weak CA I binding of **SAC** where phenylalanine resides at positions 66 and 91 which is situated toward the active site entrance, can cause similar steric hindrance¹². This would not be an issue for CO₂ because of its smaller size compare **SAC**. This however does not explain why there is a 6-fold difference in **SAC** binding to CA IX over CA XII (Table 1) as both isoforms do not contain a Phe in their active sites. Instead the overall hydrophobic nature of the catalytic site may contribute to **SAC** entry where the hydrophobicity of the active site residues in CA IX is much greater than those in the same positions of CA XII¹². This rationale can also justify the reduced binding of **SAC** to CA I versus CA II (~3-fold), where CA II has a higher hydrophobicity in its active site residues¹².

The binding mode of **SAC** to the CA IX-mimic is very similar to that previously reported for CA II as shown by the superimposition between the two structures **SAC**:CA IX-mimic and **SAC**:CA II (PDB ID: 2Q1B¹⁷, with a rmsd = 0.129 Å; Figure 3C). Similarly, the crystal structure of compound **1** with CA IX-mimic showed that the compound readily bound to the catalytic zinc via the **SAC** moiety with no displacement of W2, W3a, and W3b, and a H-bond between the carbonyl of the **SAC**, W2, and His64 (Figure 4A). Also similar to **SAC**, compound **1** interacts weakly with Leu198 (~3.5 Å). Additionally there is a weak hydrophobic interaction between the *O*-methylene of the tail and the side chain of Leu91. Leu91 has previously been described as being a key residue that determines an inhibitors selectivity between CA IX and other CAs^{12,13}. This interaction, although weak, may contribute to the additional selective inhibition of compound **1** over **SAC** for CA IX. The oxygen of the 1'-OH of the glucose fragment also forms weak H-bonds with the amides of Gln67 and Gln92. However, the electron density around this group is weak, indicating that there is most likely a significant amount of flexibility in this region of the compound (Figure 2B). Also, similar to the **SAC** binding, Phe131 would greatly reduce the binding of compound **1** to CA II. The overlay of Phe131 in CA II onto the CA IX-mimic highlights the steric clash that would occur between the benzyl of Phe131 of CA II and compound **1** (Figure 4B). This clearly indicates that compound **1** is simply too bulky to bind to CA II. It

should be noted that attempts to obtain crystals of compound **1** with CA II were made, but we were unsuccessful, and this result correlates directly with inhibition data presented in Table 1.

To further show that **SAC** can function as a useful ZBG for the design of CA IX specific CAIs, we superimposed the structure of compound **1**:CA IX-mimic onto **SAC**:CA IX-mimic and this clearly shows the mode of binding of **SAC** and the **SAC** moiety in compound **1** are conserved (Figure 4C; rmsd = 0.068 Å). This suggests that **SAC** can be classified as a useful ZBG moiety for the design of CA inhibitors (Figure 1C); specifically, the design of CA IX selective inhibitors (Figure 4C).

The overall analysis of specific interactions of both **SAC** and compound **1** suggests the preferential binding is due to clear interactions in the variable hydrophobic pocket of the CA active site (Figure 5A and B)¹². The differences in residues of the hydrophobic pocket of CA IX versus CA II provide a rationale to the selective inhibition profiles of both **SAC**, and **1**.

3. Conclusion

Targeting CA IX in the hypoxic tumor microenvironment may provide novel treatment for several cancers, however designing isoform selective drug-like inhibitors to target the enzyme is challenging. As a result we present the structural rationale as to why **SAC** and **SAC**-based compounds selectively target CA IX versus other CA isoforms. **SAC** binds in a conserved manner between both CA II and CA IX. Most likely the significant difference in inhibitory profiles between CA IX and the other isoforms is a result of **SAC** entry into the active site, which is predicted to be via hydrophobic interactions similar to CO₂ binding. However unlike CO₂, **SAC** entry to the CA active site can be perturbed by the bulky side-chains of Phe at the active site surface¹². Furthermore, it is predicted that overall hydrophobicity of active site residues can also play a role in **SAC** active site entry hence the observed difference in binding between CA IX and CA XII¹². It has previously been shown that **SAC** can be used as a lead group for CA inhibitor design^{19,31}. However until now, there was no structural information available as to how the tail regions of these compounds interact with the CA active site. Differential interactions between compound **1** and residues Leu198 and Leu91, and the amino acid change of a Phe for Val at position 131 in CA IX compared to CA II, suggests that the >1000-fold isoform selectivity is attributed to interactions in the hydrophobic pocket (Figure 5A and B). Sequence alignment of CA IX and CA II suggests that residues in the hydrophobic pocket of the CA active site have the most variability between isoforms, and thus may be exploited further for design of selective CA inhibitors^{4,12}. In addition, there are predicted weak interactions occurring between residues of the hydrophilic pocket and the glucose moiety of compound **1** that may also contribute to this effect. We have also shown that **SAC** binds to the zinc in a conserved manner through superimposition with **SAC** and compound **1**. This suggests that **SAC** can be used as a ZBG for design of other CA IX inhibitors (Figure 1C). However what sets apart **SAC** from the other ZBGs is that it naturally shows preferential inhibition for CA IX over other CAs and inhibits CA IX in the nanomolar range (Table 1).

The use of **SAC** as a ZBG in combination with carbohydrate tail moieties will further provide a novel class of promising drug candidates that target CA IX as an anti-cancer therapy, and progress the development of cancer treatment.

4. Experimental Section

4.1. Protein Expression, Purification and CA IX-mimic design

CA II and CA IX-mimic were expressed and purified using BL21DE3 competent cells as described by Pinard et al.²¹. The CA IX-mimic used for this study was designed based on previous studies by Genis, et al.²⁰ (containing two active site amino acid substitutions) and reconstructed by Pinard et al.²¹. The CA IX-mimic utilized the easily crystallizable CA II as structural scaffold, with 7 point mutations in the active site to create a CA IX catalytic domain mimic as a model for structural analysis of ligand binding. Active site mutations in the CA IX-mimic include; A65S, N67Q, E69T, I91L, F131V, K170E, and L204A. Purity of each enzyme was confirmed by SDS-PAGE. Concentrations were determined by UV/Vis spectroscopy, and measured at 22 mg/mL and 55 mg/mL for CA IX-mimic and CA II, respectively.

4.3. CA Inhibition Assay

CA Inhibition assays were performed previously by Moeker, et al.¹⁹ and Köhler, et al.¹⁷. Methods for assays include the use of an Applied Photophysics stopped-flow instrument for assaying the CA-catalyzed CO₂ hydration activity²⁵. IC₅₀ values were obtained from dose response curves working at seven different concentrations of test compound, by fitting the curves using PRISM (www.graphpad.com) and non-linear least squares methods, values represent the mean of at least three different determinations as described previously³². The inhibition constants (K_i) were then derived by using the Cheng-Prusoff equation³³ as follows: $K_i = IC_{50}/(1 + [S]/K_m)$ where [S] represents the CO₂ concentration at which the measurement was carried out, and K_m the concentration of substrate at which the enzyme activity is at half maximal. All enzymes used were recombinant, produced in *E.coli* as reported earlier²⁵. The concentrations of enzymes used in the assay were: hCA I, 10.4 nM; hCA II, 8.3 nM; hCA IX, 8.0 nM and hCA XII, 12.4 nM.

4.2. X-Ray Crystallography

Purified CA II and CA IX-mimic were crystallized in 1.6 M Na-Citrate, 50 mM Tris, pH 7.8 using hanging drop vapor diffusion²¹. Crystals for both were observed after 5 days. Stock solutions of **SAC** were made by dissolving 1 packet (~1 g) of "Sweet N' Low" in deionized water. The final stock concentration of **SAC** was estimated at 6mM. 1 µl of this solution was used to soak the CA IX-mimic crystals 4 hours prior to data collection. For compound **1** a stock solution of ~50 mM was made by dissolving the compound in deionized water. Both CA IX-mimic and CA II crystals were then soaked with the respective compound solutions 24 h prior to data collection. Diffraction data was collected "in-house" using an RU-H3R rotating Cu anode ($\lambda=1.5418 \text{ \AA}$) operating at 50 kV and 22 mA utilizing an R-Axis IV⁺⁺ image plate detector (Rigaku, USA). Each data set was processed using HKL2000³⁴. All data sets were scaled to the $P2_1$ space group, X-ray diffraction data statistics are summarized in Table 2. Initial phases for each data set were determined using molecular replacement

methods with PDB: 3KS3³⁵ as a search model. Model refinement, and generation of ligand restraint files were performed using Phenix³⁶ suite of programs. Models for ligand-protein complexes, and PDB files for ligands were generated using Coot^{37,38}. Coot was also used to determine bond lengths used for analysis. Figures were generated using PyMol³⁹.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard abbreviations

CA	carbonic anhydrase
K_i	inhibition constant
ZBG	zinc binding group

Figure 1. Schematic depiction of ZBGs used for CAIs. Shown (A) sulphonamides, (B) thiocyanates, and (c) cyclic secondary sulphonamides bound to the zinc. Figure was generated using ChemDraw⁴⁰.

Figure 2.
A) SAC (green) and B) compound **1** (grey), with their respective electron density as observed, bound to CA IX-mimic.

Figure 3.

SAC (green) bound in the active site of (A) CA IX-mimic (cyan) and (B) an overlay of F131 of CA II (yellow). C) Superposition of **SAC** bound in CA II (magenta, PDB ID: 2Q1B¹⁷) and CA IX-mimic (green). Specific interactions and hydrogen-bond distances (Å) are as shown. Figure was made using PyMol⁴¹. Residues and waters are as labelled (CA II numbering).

Figure 4.

Compound **1** (grey) bound in the active site of (A) CA IX-mimic (cyan) and (B) an overlay of F131 of CA II (yellow). C) Superposition of compound **1** and **SAC** in CA IX-mimic (green). Specific interactions and hydrogen-bond distances (Å) are as shown. Figure was made using PyMol⁴¹. Residues and waters are as labelled (CA II numbering).

Figure 5. Surface rendition of CA IX-mimic (cyan) with (A) **SAC** (green) and (B) compound **1** (grey) bound. Hydrophobic (orange) and hydrophilic (purple) residues are highlighted. Figure was made using PyMol⁴².

Table 1
Inhibition and selectivity ratio for CA I, II, IX, and XII with **SAC**, compound **1**, and **AZM**.

	K_i (nm) ^a					Selectivity Ratio ^b		
	CA I	CA II	CA IX	CA XII	I/IX	II/IX	I/XII	II/XII
SAC ¹⁷	20000	6000	100	650	200	60	30	1
1 ¹⁹	>50000	>50000	50	600	>1000	>1000	>100	>100
AZM ¹⁷	250	12	25	6	10	0.5	40	2

^aThe K_i values are averaged values with experimental errors of 5%.

^b Selectivity is determined by the ratio of K_i s for the cytosolic CAs I and II relative to the extracellular CAs IX and XII. Data for **1**, and **SAC** and **AZM** were taken from Moeker, et al.¹⁹, Köhler, et al.¹⁷, respectively.

Table 2X-ray crystallography statistics for **SAC** and **1** in complex with CA IX-mimic.

Compound PDB accession #	SAC 4RIV	1 4RIU
Space Group Cell Dimensions (Å;°)	<i>P2₁</i> <i>a</i> = 42 ± 0.1, <i>b</i> = 42 ± 0.2, <i>c</i> = 72 ± 0.3; β = 104 ± 0.2	
Resolution (Å)	20.0-1.64	19.9-1.66
Total Reflections	29217	27169
R _{sym} ^a (%)	4.1 (6.7)	6.4 (38.0)
I/Iσ	48.1 (22.0)	15.7 (3.7)
Completeness (%)	97.1 (92.1)	94.0 (90.4)
R _{cryst} ^b (%)	16.3 (18.2)	15.0 (19.3)
R _{free} ^c (%)	18.7 (21.6)	17.7 (24.1)
# of Protein Atoms	2191	2093
# of Water Molecules	241	205
# of Ligand Molecules	24	30
Ramachandran stats (%): Favored, allowed, outliers	96.3, 3.7, 0.8	97.3, 2.7, 0.0
Avg. B factors (Å ²): Main-chain, Side-chain, Solvent, Ligand	15.5, 20.0, 36.6, 28.2	18.7, 23.8, 29.5, 38.3

$$^a R_{\text{sym}} = (\sum |I - \langle I \rangle| / \sum \langle I \rangle) \times 100$$

$$^b R_{\text{cryst}} = (\sum |F_o - F_c| / \sum |F_o|) \times 100$$

^c R_{free} is calculated in the same way as R_{cryst} except it is for data omitted from refinement (5% of reflections for all data sets).

^e Values in parenthesis correspond to the highest resolution shell.