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Discovery of 4-Hydroxy-3-(3-(phenylureido)benzenesulfonamides as SLC-0111 Analogues for the Treatment of Hypoxic Tumors Overexpressing Carbonic Anhydrase IX

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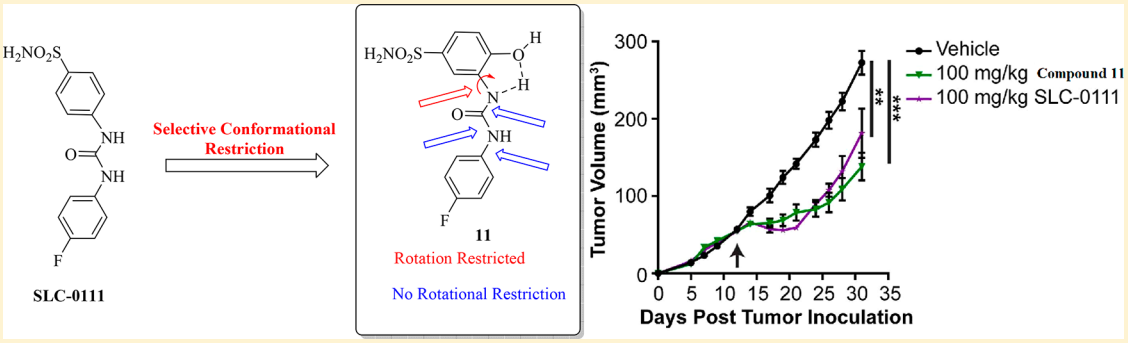
Supporting Information



ABSTRACT: Herein we report the 2-aminophenol-4-sulfonamide **1** and its ureido derivatives **2**−**23** as inhibitors of the carbonic anhydrase (CA, EC 4.2.1.1) enzymes as analogues of the hypoxic tumor phase II entering drug **SLC-0111**. This scaffold may determine preferential rotational isomers to selectively interact within the tumor-associated CAs. Most of the compounds indeed showed *in vitro* selective inhibition of the tumor associated CA isoforms IX and XII. The most potent derivative within the series was **11** (K_Is of 2.59 and 7.64 nM on hCA IX and XII, respectively), which shares the 4-fluorophenylureido tail with the clinical candidate. We investigated by means of X-ray crystallographic studies the binding modes of three selected compounds of this series to CA I. The evaluation of therapeutic efficacy of compound **11** in an orthotopic, syngeneic model of CA IX-positive breast cancer *in vivo* showed close matching antitumoral effects and tolerance with **SLC-0111**.

INTRODUCTION

Carbonic anhydrases (CAs, EC 4.2.1.1) belong to the metalloenzyme superfamily and were identified more than seven decades ago.[1−8] To date seven genetically distinct and unrelated CA families have been characterized so far (i.e., α-, β-, γ-, δ-, ζ-, η-, and θ-CAs), and they all catalyze the reversible hydration reaction of carbon dioxide to afford bicarbonate and protons.[1−8] This equilibrium is deeply involved in a variety of processes at cellular as well as at tissue levels, and among these, the pH homeostasis is the main one.[9−12] The relevance of pH regulation in normal or in hypoxic tumor cells is well documented.[9−13] A plethora of biological entities located on the cellular biomembranes are directly involved in pH modulation (i.e., V-ATPases, ion exchangers, monocarboxylate transporters, Na^+/H^+ exchangers as the main ones), and among them the CA IX isoform recently stood up as a validated drug target.[13] CA IX is highly expressed in hypoxic tumors (i.e., breast malignancies) and is associated with poor prognosis.[14−17] Conversely to other research groups, which focused on the development of human (h) CA IX antibodies,[18] we turned our attention toward the identification of selective hCA IX small molecule inhibitors.[19] Surprisingly the insertion of the ureido moiety as a linker between the sulfonamide hCA inhibitor (CAI) warhead and the molecular tail acted as the turning point in our extensive medicinal chemistry investigations within this field.[19,20] We demonstrated, by means of *in vitro* kinetic studies and X-ray crystal adducts, that the ureido group allowed higher degrees of flexibility between the two interconnected sections when compared to previous investigated linkers of the carboxyamido or of the sulfonamide type, thus allowing the entire molecule to better allocate within the enzymatic CA cavities.[20] Among the large series of compounds of this type synthesized, **SLC-0111** (also named

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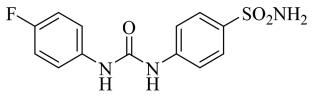
U-104, **MST-104**, and **WBI-5111** in Figure 1) showed high affinity toward the tumor associated hCA IX *in vitro* and



SLC-0111

Figure 1. Structure of **SLC-0111**.

promising antiproliferative and antimetastatic properties in *in vivo* breast cancer models.[13] Such data granted **SLC-0111** entering phase II clinical trials for the treatment of advanced solid tumors.[21]

Many contributions in the literature reported CAIs analogues of the **SLC-0111** type, and among others, it is worth mentioning the thioureido,[22] the sulfamate,[23] and, very recently, the selenoureido analogues.[24] Derivatives of **SLC-0111** bearing the ^{19}F or the $^{11}C{=}O$ isotopes for imaging purposes were also synthesized.[25,26]

In the search of more effective hCA IX selective inhibitors of the **SLC-0111** type, we report for the first time a series of ureido containing compounds based on the 2-aminophenol-4-sulfonamide **1**. In this study, we explored their *in vitro* inhibition data on the physiologically relevant hCAs (i.e., I, II, IX, and XII), we determined the binding modes of selected ones by means of X-ray experiments on the hCA I-ligand adducts, and, finally, we assessed their antitumor properties *in vivo* using an orthotopic, syngeneic CA IX-positive breast tumor model in comparison to **SLC-0111**.

RESULTS AND DISCUSSION

Drug Design and Synthesis. We considered developing our ureido-containing compound synthetic strategy on the 2-aminophenol-4-sulfonamide **1** scaffold, whose phenolic OH was primarily intended to form a stable intramolecular five-membered ring with the ureido N*H* moiety placed at the position 2. The introduction of such a moiety was thought to induce a rotational restriction between C2 and N1′, which may determine preferential rotational isomers to interact within the hCAs enzymatic sites, thus resulting in enhancement of CAs enzymatic selectivity (Figure 2).

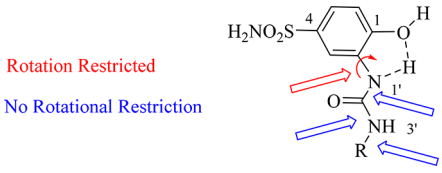
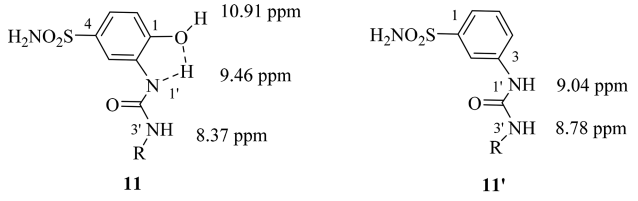


Figure 2. Formation of the intramolecular hydrogen bond leading to a five-membered ring in the target compounds may lead to C2−N1′ rotational restriction.

Additionally, the formation of an intramolecular ring, as discussed above, represents a means to selectively downtune only the C2−N1′ bond frequency rotation without affecting the rotational freedom of the remaining scaffold. Our theoretical considerations on the formation of the intramolecular five-membered ring were supported by a comparative ^1H NMR structural characterization on compound **11** (as the structural analogue of **SLC-0111**) and its 4-unsubstituted analogue **11′** previously reported by our group[27,28] (Figure 3).



R= 4-Fluorophenyl

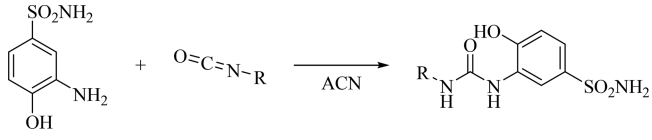
Figure 3. ^1H NMR 400 MHz signals at 23 °C in DMSO-d_6 of compounds **11** and **11′**.

According to the 400 MHz ^1H NMR experiment at 23 °C in DMSO-d_6, the 1′-*H*, 3′-*H* and O*H* in **11** were assigned to the broad singlet peaks, all exchangeable with D_2O, centered at δ 9.46, 8.37, and 10.91 respectively. The ^1H NMR experiment of **11′** in the same conditions revealed that the 1′-*H* and 3′-*H* were at δ 9.04 and 8.78 respectively. The δ 0.42 downfield difference between **11** and **11′** 1′-*H* was ascribed to the formation of the intramolecular hydrogen bond interaction.

The insertion of structural modifications within small molecules greatly affect their solubility properties in aqueous media, which represents one of the major concerns in early stages of drug development, as it seriously affects their bioavailability. Usually the enhancement of water solubility properties of a drug parallels the increase of the dissolution rate in the same medium. This kinetic parameter has important implications mainly on first-pass metabolism and susceptibility of the drug to efflux mechanisms, which in turn affects pharmacological effectiveness and patient compliance. Therefore, we compared the solubility of **SLC-0111** and **11** at room temperature (r.t.) using a spectrophotometric method at the maximum absorbance of 217 nm, which revealed that the latter was only slightly less soluble (1.66 fold) in aqueous pH 7.4 buffer solution. This data suggested that the structural modification introduced in our series (i.e., 2-aminophenol-4-sulfonamide **1** scaffold) did not significantly affect one of the main parameters of druggability, at least for these closely related **SLC-0111** derivative.

The synthetic plan adopted for the synthesis of compounds **2−23** consisted of coupling **1** with commercially available isocyanates using acetonitrile as solvent and according to our previously reported procedures (Scheme 1).[19,20,27,28]

Scheme 1. General Synthesis of 2-Ureidosubstituted Benzenesulfonamides 2−23[19,20,27,28]



1 **2-23**

All compounds obtained were properly characterized by means of ^1H-, ^{13}C-, and ^{19}F-NMR spectroscopy and HRMS and were ≥95% HPLC pure (see Experimental Section for details).

CA Inhibition. We investigated here the CA inhibitory properties of our reported compounds **1−23**, and the obtained results were compared to the clinically used drug acetazola-

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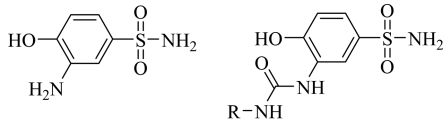
Table 1. Inhibition Data of Human CA Isoforms hCA I, II, IX, and XII with Sulfonamides 1−23 and the Acetazolamide (AAZ) by a Stopped Flow CO_2 Hydrase Assay[29]



1 **2-23**

		K_I (nM)*			
compd	R	hCA I	hCA II	hCA IX	hCA XII
1		5354.1	3333.8	26.1	6.0
2	cyclopentyl	354.0	>10000	254.1	5.5
3	phenethyl	409.3	1647.7	231.4	6.9
4	$C_6H_5CH_2$	378.6	2343.9	216.1	5.5
5	4-$MeC_6H_4CH_2$	273.3	667.8	262.5	5.7
6	C_6H_5	825.3	1394.7	169.0	44.1
7	2-MeC_6H_4	309.3	136.9	19.9	43.5
8	4-ClC_6H_4	1434.0	997.6	205.6	6.6
9	3-ClC_6H_4	377.1	1253.5	2.9	7.1
10	2-ClC_6H_4	323.1	368.7	14.2	3.0
11	4-FC_6H_4	441.0	1107.5	2.6	7.6
12	4-$NO_2C_6H_4$	2493.0	2103.1	13.9	8.0
13	2-NO_2−C_6H_4	2079.8	>10000	2.6	7.8
14	3,5-$Me_2C_6H_3$	2476.5	7290.5	25.8	5.8
15	2,5-$Me_2C_6H_3$	306.1	94.3	329.5	48.0
16	2-*i*-PrC_6H_4	345.8	762.3	179.7	44.7
17	3-$MeSC_6H_4$	739.0	728.5	23.6	35.9
18	4-EtC_6H_4	665.6	771.7	26.3	57.8
19	4-*n*-BuC_6H_4	551.8	3406.2	157.0	5.2
20	2-$MeOC_6H_4$	255.1	951.3	23.2	7.3
21	2-$EtOC_6H_4$	654.3	373.2	134.1	9.6
22	4-$PhOC_6H_4$	2262.9	4416.5	31.0	69.0
23	1-naphthyl	344.5	842.3	12.3	8.7
SLC-0111[b]		5080	960	45.1	4.5
AAZ		250.0	12.0	25.0	5.7

[a]Mean from three different assays, by a stopped flow technique (errors were in the range of ±5−10% of the reported values). [b]From ref 19.

mide (**AAZ**), used as standard CAI, against four physiological relevant isoforms (i.e., hCAs I, II, IX, and XII) by means of the stopped flow CO_2 hydrase assay.[29]

The following structure−activity relationship (SAR) can be drawn from the data reported in Table 1:

(i) The cytosolic and kinetically slowest hCA I isoform was poorly inhibited by all compounds in the series (K_Is 255.1−5354.1 nM). The most effective inhibitor among all was the 2-methoxyphenylureido derivative **20**, which showed comparable potency with the reference CAI **AAZ** (K_Is of 255.1 and 250.0 nM, respectively). Weaker inhibition potencies were observed for derivatives **2**−**5**, **7**, **9**−**11**, **15**, **16**, and **23** whose K_Is were comprised between 273.3 to 441.0 nM, whereas the remaining compounds resulted ineffective against the hCA I (K_Is between 551.8 to 5354.1 nM). It should be noted that the weakest inhibitor among the series was the amino unsubstituted compound **1** (K_I of 5354.1 nM).

(ii) In analogy to the previous one, the second cytosolic isoform (i.e., hCA II) was weakly inhibited by most of the compounds reported. Among the series, the 2,5-dimethylphenylureido derivative **15** was the most potent inhibitor (K_I of 94.3 nM). A modest decrease of potency (1.5-fold) was obtained with the 2-methylphenylureido derivative **7** (K_I of 137.0 nM). All remaining compounds were ineffective inhibitors against the hCA II (see Table 1).

(iii) Better inhibition results were obtained for the transmembrane and tumor associated isoform hCA IX (Table 1). As reported above, derivatives **2**−**6**, **8**, **15**, **16**, **19**, and **21** showed the lowest inhibitory potencies among the compounds tested with K_I values in the range of 134.1−329.5 nM, thus far less potent when compared to the reference CAI **AAZ** (K_I of 25.0 nM). Interestingly the unsubstituted **1** as well as its derivatives **7**, **14**, **17**, **18**, **20**, and **22** showed remarkable potencies and comparable to the standard **AAZ** (K_I values comprised between 19.9 and 26.1 nM). The introduction of the 2-chloro, 4-nitro, and 1-naphthylureido substituents into the 3-amino-4-hydroxybenzenesulfonamide **1** scaffold (compounds **10**, **12**, and **23**) determined a clear enhancement of the inhibitory potencies against the hCA IX (K_Is of 14.2, 13.9, and 12.3 nM, respectively). Finally, compounds **9**, **11**, and **13** were the most potent inhibitors within the series tested against the IX isoform (K_Is of 2.9, 2.6, and 2.6 nM, respectively), and they deserve deeper SAR considerations. Among the chlorophenylureido substituted derivatives **8**−**10**, compound **9** (which is the most active) possess the halogen in *meta* position, whereas the introduction of the chloro

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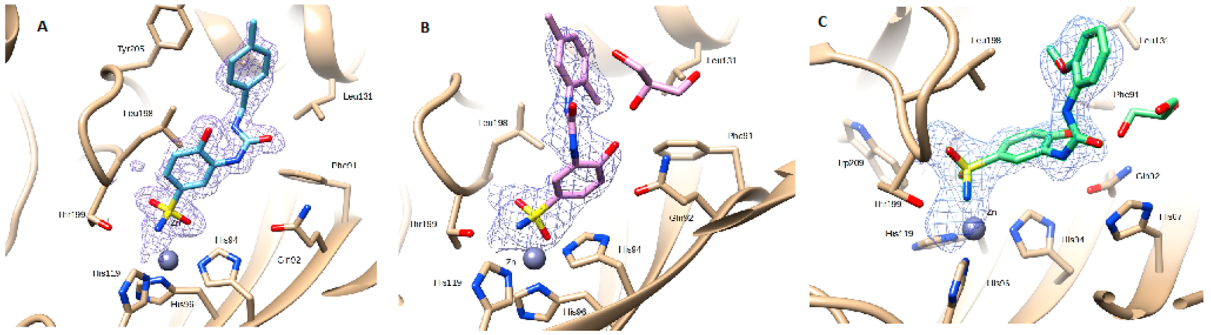

Figure 4. Active site region in the hCA I–**5** (A), **15** (B), and **20** (C) complexes. The omit Fo − Fc electron density maps (contoured at 2.0 σ) for the three inhibitors are also shown. PDB accession codes 6F3B, 6FAF and 6FAG, respectively.

in *ortho* or in *para* clearly reduced the inhibition potencies against the hCA IX of 4.9- and 70.9-fold, respectively. Fluctuations of the kinetic properties in a regioisomer-dependent manned were also observed for compounds **12** and **13**. Shift of the nitro moiety from *para* (compound **12**) to *ortho* to afford the derivative **13** resulted in a 5.3-fold enhancement of the inhibitory potencies and thus obtaining the second most potent inhibitor against the hCA IX within the entire series (see Table 1).

As reported above in Table 1, compounds **11** and **13** match each other in terms of inhibition potency against the hCA IX (K_Is of 2.6 nM) but presented quite different substitution patterns. Specifically, compound **11** contains the 4-fluorophenylureido moiety in analogy to **SLC-0111**. Interestingly, the latter was 17.3-fold less potent against the hCA IX when compared to **11**.

(iv) The second tumor associated isoform (i.e., hCA XII) was weakly inhibited by derivatives **6**, **7**, **15**–**18**, and **22**, which showed K_Is spanning between 35.9 and 69.0 nM. Conversely, the remaining derivatives in the series were far more potent, with K_Is in the low nanomolar range comparable to the standard **AAZ**. As reported in Table 1, the inhibition data of these compounds were similar to each other, thus not allowing to draw detailed SAR data.

In summary, we reported for the first time the *in vitro* inhibitory activity of 2-aminophenol-4-sulfonamide **1** and its phenylureido derivatives **2**–**23**. Overall, the entire series showed preferential inhibition of the tumor associated isoforms hCA IX and XII over the cytosolic, and herein considered, off-target hCA isoforms I and II. Only compound **15** resulted to be an inhibitor of hCA II even if the K_I value was rather high (94.3 nM). Of particular interest in this study is a comparative analysis of the inhibition values relative to compound **11** over **SLC-0111**, which share the same 4-fluorophenylureido tail. As reported in Table 1, **11** was 11.5- and 17.4-fold more effective when compared to **SLC-0111** in inhibiting hCA I and IX, respectively, whereas it showed K_I values 11.5- and 1.7-fold lower for the cytosolic hCA II and for the second tumor associated XII. The calculated selectivity indexes (K_{IhCAII}/K_{IhCAIX} and $K_{IhCAII}/K_{IhCAXII}$) for **SLC-0111** were 21.3 and 213.3, respectively, whereas for its structural analogue **11**, 426 and 145.7. Thus, our new compound was more effective in inhibiting the tumor associated isoform IX when compared to **SLC-0111**. Therefore, in consideration of the inhibition data reported above we consider compound **11** worth to be further investigated, as an **SLC-0111** analogue.

X-ray Crystallography. We assessed the binding modes of compounds **5**, **15**, and **20** within the hCA I active site by means of X-ray crystallographic experiments. Among the series reported, these compounds were the most active *in vitro* in inhibiting the hCA I isoform. In this study, we particularly focused on the hCA isoform I since the largest majority of X-ray crystal adducts reported in this field is related to the ubiquitous hCA II or to the tumor associated IX mimic,[30] but hCA I is very abundant in red blood cells (at 1 μM concentrations level) and may influence the pharmacokinetics of drugs interacting with it or other CA isoforms of pharmacological interest.

As reported below in Figure 4, all compounds were coordinated to the Zn(II) ion by means of the deprotonated sulfonamide moiety, which in turn is also engaged in a strong H-bond with the OH of Thr199 residue. One of the sulfonamide oxygen is also involved in a hydrogen interaction with the amide nitrogen of Thr199. This coordination pattern, which is typical for all primary sulfonamides, is respected also in these cases.[30]

Slight differences of the allocated inhibitors within the enzymatic cavity were observed when a superimposition of the three structures was performed (Figure 5).

As reported above, **15** and **20** superimpose almost perfectly, whereas **5** (the only one with an additional CH_2 moiety in the tail) showed a different orientation. Surprisingly, the insertion

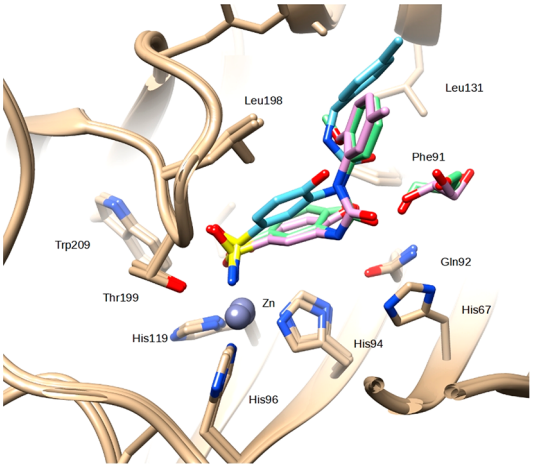

Figure 5. Superimposition of hCA I–**5** (light blue), **15** (pink), and **20** (green) adducts.

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of the small CH_2 spacer forces the inhibitor **5** to interact mostly with the hydrophobic part of the CA I active site cavity.

Deeper structural considerations of the hCA I-**15** and **20** adducts revealed the aromatic rings of the sulfamoylphenyl heads making hydrophobic contacts with residues Phe91, Ala121, and Leu198 and a T-shaped stacking with His94. Additional hydrogen bonding interactions occurred between the phenoxy and the *N*1′-ureido moieties with Gln92 and His67, respectively. As for the inhibitor **20** (the most active in inhibiting the hCA I), the 2′-methoxy substituent resulted engaged in additional hydrophobic interaction with Leu198.

The main interactions for the sulfamoylphenyl head of compound **5** were the hydrophobic contacts with Leu198 and a parallel π-stacking with His200. The OH on the aromatic ring formed a water-bridged linkage with Pro201 and *N*1′ of the ureido group with Gln92. The 4′-methylphenyl moiety of the same inhibitor was also engaged by means of hydrophobic contacts with Ala135 and Pro202.

Although the tumor-associated hCA IX active site differs from the off-target hCAs I and II isoforms for the higher hydrophobic amino acidic content, it is interesting to note that the K_I values of **5** relative to these isoforms are all of the same order of magnitude, whereas compounds **15** and **20** showed high affinity for the hCA II and IX isoform, respectively. This is somehow unexpected since the crystallographic analysis revealed that the extra methylene group in **5** pushes the molecule toward the hydrophobic section of the CA cleft. Superpositions of the active sites of the complex hCA I-**5** with those of hCA II and IX also revealed that important mutations such as Val131/Phe131 (hCA IX/II), Leu91/Phe91, and Ala204/Tyr204 (hCA IX/I) are present (see Figure 6).

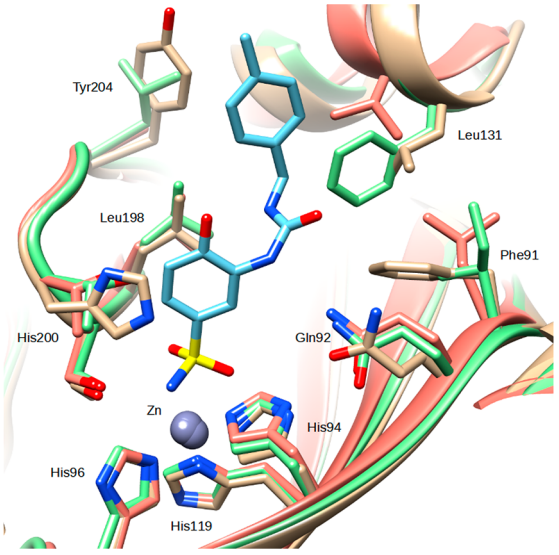

Figure 6. Superimposition of the active site of the complex hCAI-**5** (pale brown) with those of hCAII (green) and hCAIX (salmon).

Effect of Compound 11 on Primary Breast Tumor Growth and Comparison to SLC-0111. The effect of sulfonamide compound **11** on tumor growth *in vivo* was investigated using the orthotopic, syngeneic 4T1 model of breast cancer, and the obtained results were compared to treatment of similar tumors with **SLC-0111**, which shares the same 4-fluorophenylureido tail. Importantly, 4T1 mouse tumors demonstrate robust hypoxia-induced expression of CA IX, and this model has been used previously to evaluate CA IX inhibitors, including sulfonamides[13a] and glycosyl coumarins.[13b] 4T1 murine breast tumor cells were inoculated into the mammary fat pad of BALB/c mice and treatments were initiated after tumors were established (arrows in Figure 7). Both the observed tumor volumes and the volume range at which treatments were initiated are similar to those reported in published studies evaluating CA IX inhibitors in this tumor model.[13] Compounds were administered daily by intraperitoneal injection, and caliper measurements were used to monitor tumor growth. As reported below in Figure 7A, treatment of animals with increasing concentrations of **11** evoked dose-dependent inhibition of tumor growth, with a maximum effect at 100 mg/kg. A comparison of the reduction in tumor growth in animals treated with equivalent doses of **11** or **SLC-0111** showed nearly superimposable responses (Figure 7B), demonstrating that both compounds were similarly efficacious *in vivo*.

Additionally, no significant weight reduction was observed for any of the treated animals at the concentrations and the dosing schedules of compound **11** in our experiments, thus demonstrating that, in analogy to **SLC-0111**, compound **11** is well-tolerated *in vivo* at doses that are therapeutically active (Figure 8).

CONCLUSIONS

We report here a series of sulfonamides, compound **1** and its derivatives **2**−**23**, possessing aromatic/aliphatic ureido tails, and they were assayed *in vitro* on hCA I, II, IX, and XII isoforms. Almost all compounds showed selective inhibitory potencies against the tumor associated isoforms hCA IX and XII except for the compound **15**, which showed a 3.49-fold selectivity on hCA II (K_I of 94.29 nM) over IX. The most effective inhibitors (i.e., with K_Is in the low nanomolar range) of hCA IX were 4-fluorophenyl **11** (K_I of 2.59 nM), 2-nitrophenyl **13** (K_I of 2.61 nM), and 3-chlorophenyl **9** (K_I of 2.85 nM). In particular, the **SLC-0111** structural analogue (i.e., compound **11**) showed a better selectivity profile against the hCA IX, thus giving support to our design strategy for the obtainment of compounds with controlled degrees of tail flexibility. We reported the binding modes of selected compounds **5**, **15**, and **20** in adduct with hCA I, which revealed the classical coordination pattern within the hCA cavity. Superposition of the three structures showed that the **5** inhibitor tail, contrarily to compounds **15** and **20**, was oriented toward the hydrophobic part of the cavity. Finally, we investigated the antitumor activity of compound **11** *in vivo* using an orthotopic syngeneic breast tumor model that robustly expresses hypoxia-inducible CA IX. We found that compound **11** dose-dependently inhibited tumor growth to levels that matched those observed with **SLC-0111** treatment and was also associated with an absence of adverse effects similar to **SLC-0111**.

EXPERIMENTAL SECTION

Materials and Reagents. All anhydrous solvents and reagents used in this study were purchased from Alfa Aesar, TCI, and Sigma-Aldrich. The synthetic reactions involving air- or moisture-sensitive chemicals were carried out under a nitrogen atmosphere using dried glassware and syringe techniques in order to transfer the solutions. Nuclear magnetic resonance (^{1}H-, ^{13}C-, and ^{19}F-NMR) spectra were recorded using a Bruker Advance III 400 MHz spectrometer using DMSO-d_6 as solvent. The chemical shifts are reported in parts per

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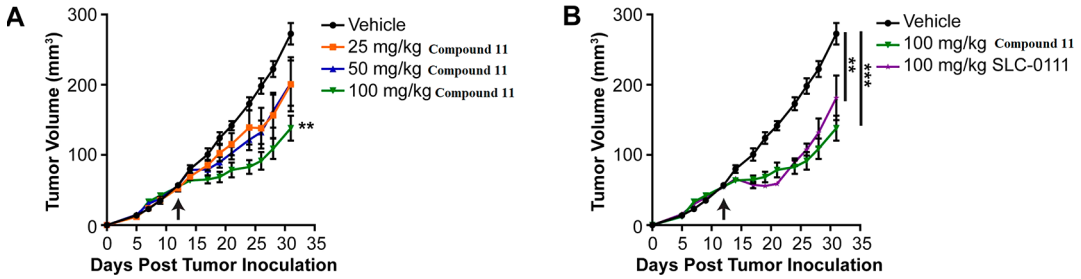

Figure 7. Inhibition of tumor growth after treatment of mice harboring 4T1 tumors with compound **11** and **SLC-0111** at diverse dosages. Data show the mean ± SEM, $N = 6$ to 8 animals/group. $^{**}P < 0.01$. (B). Data show the mean ± SEM, $N = 6$ to 7 animals/group. $^{**}P < 0.01$, $^{***}P < 0.001$.

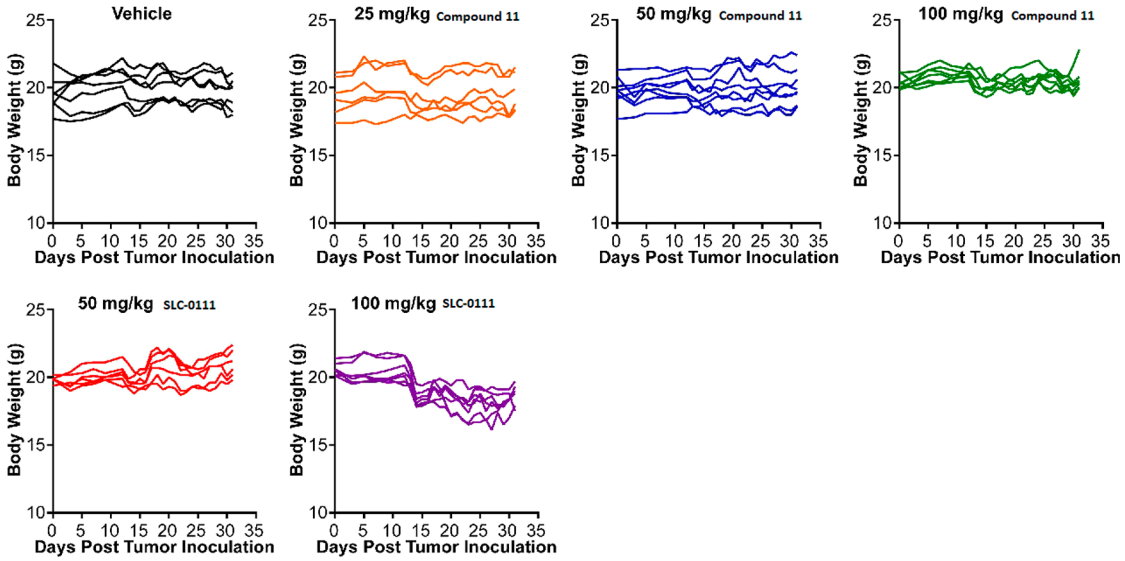

Figure 8. Spider plots showing body weights for individual animals treated with vehicle, **11**, and **SLC-0111** as indicated. Both compounds were well-tolerated by the animals at the doses tested.

million (ppm), and the coupling constants (J) are expressed in Hertz (Hz). The splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; brs, broad singlet; dd, doublet of doublets. The correct assignment of exchangeable protons (i.e., O*H* and N*H*) was carried out by means of the addition of D_2O. Analytical thin-layer chromatography (TLC) was done on Merck silica gel F-254 plates. Flash chromatography was performed on Merck Silica gel 60 (230−400 mesh ASTM) as the stationary phase, and appropriate mixtures of ethyl acetate/*n*-hexane were the eluents. Melting points (m.p.) were measured in open capillary tubes with a Gallenkamp MPD350.BM3.5 apparatus and are uncorrected. The HPLC was performed by using a Waters 2690 separation module coupled with a photodiode array detector (PDA Waters 996) using a Nova-Pak C18 4 μm 3.9 mm × 150 mm (Waters) silica-based reverse phase column. The sample was dissolved in 10% acetonitrile/H_2O and an injection volume of 45 μL. The mobile phase (flow rate 1.0 mL/min) was a gradient of H_2O + trifluoroacetic acid (TFA) 0.1% (A) and acetonitrile + TFA 0.1% (B), with steps as follows: (A%/B%), 0−10 min 90:10, 10−25 min gradient to 60:40, 26:28 min isocratic 20:80, 29−35 min isocratic 90:10. TFA 0.1% in water as well in acetonitrile was used as counterion. All compounds reported here were ≥95% HPLC pure. The solvents used in MS measures were acetone, acetonitrile (Chromasolv grade), and mQ water 18 MU. The high resolution mass spectrometry (HRMS) analysis was performed with a Thermo Finnigan LTQ Orbitrap mass spectrometer coupled with an electrospray ionization source (ESI). Analysis was carried out in positive ion mode $[M + H]^+$, and it was used a proper dwell time acquisition to achieve 60,000 units of resolution at full width at half-maximum (fwhm). Elemental composition of compounds was calculated on the basis of their measured accurate masses, accepting only results with an attribution error less than 5 ppm and a not integer RDB (double bond/ring equivalents) value.[31] Stock solutions of analytes were prepared using acetone (1.0 mg mL^{-1}) and stored at 4 °C. Then working solutions of each analyte were prepared by dilution of the stock solutions using mQ H_2O/acetonitrile 1/1 (v/v) up to a concentration of 1.0 μg mL^{-1}. The HRMS analysis was performed by introducing the analyte working solution via syringe pump at 10 μL min^{-1}.

Synthesis of Compounds. *General Procedure for the Synthesis of Compounds* ***2*****−****23***. A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3−4 mL) was treated with a proper aromatic/aliphatic isocyanate (1.0 equiv). The reaction mixture was stirred at rt until the consumption of starting materials (TLC monitoring). Reaction was quenched with H_2O and treated accordingly to afford the compounds **2**−**23**.

*Synthesis of 3-(3-Cyclopentylureido)-4-hydroxybenzenesulfonamide (**2**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (4 mL) was treated with cyclopentyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 5 mL), filtered, and dried under vacuum to afford the titled product **2** as a pale brown solid. Yield 44%; silica gel TLC R_f 0.28 (MeOH/DCM 10% v/v); mp 195−196 °C; δ_H (400 MHz, DMSO-d_6) 1.40 (2H, m, Cp), 1.59 (2H, m, Cp), 1.67 (2H, m, Cp), 1.87 (2H, m, Cp), 3.97 (1H, m, Cp), 6.90 (1H, d, J 8.4, Ar−H), 6.99 (1H, d, J 6.8, exchangeable with D_2O, N*H*), 7.07 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.25 (1H, dd, J 2.4, 8.4, Ar−H), 7.98 (1H, s, exchangeable with D_2O, N*H*), 8.59 (1H, d, J 2.4, Ar−H), 10.70 (1H, s, exchangeable with D_2O, O*H*); δ_C (100

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MHz, DMSO-d_6) 24.1, 33.7, 51.8, 114.4, 116.5, 119.9, 129.6, 135.7, 148.8, 155.6; m/z (ESI positive) 300.0 $[M + H]^+$.

*Synthesis of 4-Hydroxy-3-(3-phenethylureido)-benzenesulfonamide (**3**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with phenethyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 5 mL), filtered, and dried under vacuum to afford the titled product **3** as a light brown solid. Yield 42%; silica gel TLC R_f 0.37 (MeOH/DCM 10% v/v); mp 199−200 °C; δ_H (400 MHz, DMSO-d_6) 2.79 (2H, t, J 6.4, CH_2), 3.38 (2H, m, CH_2), 6.91 (1H, d, J 8.4, Ar−H), 6.99 (1H, t, J 6.4, exchangeable with D_2O, N*H*), 7.07 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.33 (6H, m, Ar−H), 8.13 (1H, s, exchangeable with D_2O, N*H*), 8.60 (1H, d, J 2.4, Ar−H), 10.68 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 36.7, 41.5, 114.6, 116.8, 120.1, 127.0, 129.3, 129.5, 129.6, 135.7, 140.5, 148.9, 156.0; m/z (ESI negative) 334.0 $[M - H]^-$.

*Synthesis of 3-(3-Benzylureido)-4-hydroxybenzenesulfonamide (**4**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with benzyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 5 mL), filtered, and dried under vacuum to afford the titled product **4** as a pale brown solid. Yield 76%; silica gel TLC R_f 0.26 (MeOH/DCM 10% v/v); mp 196−197 °C; δ_H (400 MHz, DMSO-d_6) 4.34 (2H, d, J 6.0, CH_2), 6.91 (1H, d, J 8.4. Ar−H), 7.09 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.26−7.40 (6H, m, Ar−H), 7.44 (1H, t, J 6.0, exchangeable with D_2O, N*H*), 8.21 (1H, s, exchangeable with D_2O, N*H*), 8.62 (1H, d, J 2.4, Ar−H), 10.74 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 43.6, 114.5, 116.7, 120.2, 127.7, 128.1, 129.3, 129.4, 135.7, 141.1, 149.0, 156.1; m/z (ESI positive) 322.0 $[M + H]^+$.

*Synthesis of 4-Hydroxy-3-(3-(4-methylbenzyl)ureido)-benzenesulfonamide (**5**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 4-methylbenzyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 5 mL), filtered, and dried under vacuum to afford the titled product **5** as a pale brown solid. Yield 75%; silica gel TLC R_f 0.28 (MeOH/DCM 10% v/v); mp 190−191 °C; δ_H (400 MHz, DMSO-d_6) 2.32 (3H, s, CH_3), 4.29 (2H, d, J 5.6, CH_2), 6.91 (1H, d, J 8.4, Ar−H), 7.09 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.18 (2H, d, J 8.0, Ar−H), 7.22 (2H, d, J 8.0, Ar−H), 7.26 (1H, dd, J 2.4, 8.4, Ar−H), 7.38 (1H, t, J 5.6, exchangeable with D_2O, N*H*), 8.19 (1H, s, exchangeable with D_2O, N*H*), 8.61 (1H, d, J 2.4, Ar−H), 10.74 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 21.6, 43.4, 114.5, 116.8, 120.2, 128.1, 129.4, 129.8, 135.7, 136.7, 138.0, 149.0, 156.0; m/z (ESI negative) 334.0 $[M - H]^-$.

*Synthesis of 4-Hydroxy-3-(3-phenylureido)benzenesulfonamide (**6**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (4 mL) was treated with phenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 5 mL), filtered, and dried under vacuum to afford the titled product **6** as a pale brown solid. Yield 67%; silica gel TLC R_f 0.25 (MeOH/DCM 10% v/v); mp 223−224 °C (dec); δ_H (400 MHz, DMSO-d_6) 6.99 (2H, m, Ar−H), 7.13 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.32 (3H, m, Ar−H), 7.50 (2H, d, J 8.0, Ar−H), 8.39 (1H, s, exchangeable with D_2O, N*H*), 8.68 (1H, d, J 2.4, Ar−H), 9.41 (1H, s, exchangeable with D_2O, N*H*), 10.87 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 114.5, 116.9, 118.9, 120.8, 122.8, 128.8, 129.8, 135.8, 140.6, 149.2, 153.2; m/z (ESI negative) 306.0 $[M - H]^-$.

*Synthesis of 4-Hydroxy-3-(3-(o-tolyl)ureido)benzenesulfonamide (**7**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 2-methylphenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 5 mL), filtered, and dried under vacuum to afford the titled product **7** as a beige solid. Yield 85%; silica gel TLC R_f 0.33 (MeOH/DCM 10% v/v); mp 201−202 °C; δ_H (400 MHz, DMSO-d_6) 2.29 (3H, s, CH_3), 6.99 (2H, m, Ar−H), 7.18 (4H, m, 2H exchangeable with D_2O, SO_2NH_2), 7.32 (1H, dd, J 2.4, 8.4, Ar−H), 7.87 (1H, d, J 8.0, Ar−H), 8.64 (1H, s, exchangeable with D_2O, N*H*), 8.69 (1H, d, J 2.4, Ar−H), 8.84 (1H, s, exchangeable with D_2O, N*H*), 10.85 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 19.0, 114.6, 117.2, 120.7, 122.4, 123.7, 127.0, 128.8, 129.0, 131.1, 135.7, 138.2, 149.3, 153.6; m/z (ESI positive) 321.9 $[M + H]^+$.

*Synthesis of 3-(3-(4-Chlorophenyl)ureido)-4-hydroxybenzenesulfonamide (**8**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 4-chlorophenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 5 mL), filtered, and dried under vacuum to afford the titled product **8** as a beige solid. Yield 82%; silica gel TLC R_f 0.29 (MeOH/DCM 10% v/v); mp 223−224 °C (dec); δ_H (400 MHz, DMSO-d_6) 6.98 (1H, d, J 8.4, Ar−H), 7.15 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.33 (1H, dd, J 2.4, 8.4, Ar−H), 7.37 (2H, d, J 8.8, Ar−H), 7.52 (2H, d, J 8.8, Ar−H), 8.42 (1H, s, exchangeable with D_2O, N*H*), 8.67 (1H, d, J 2.4, Ar−H), 9.55 (1H, s, exchangeable with D_2O, N*H*), 10.93 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 114.6, 116.9, 120.4, 121.0, 126.3, 128.6, 129.7, 135.8, 139.6, 149.3, 153.1; m/z (ESI negative) 339.9 $[M - H]^-$.

*Synthesis of 3-(3-(3-Chlorophenyl)ureido)-4-hydroxybenzenesulfonamide (**9**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 3-chlorophenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **9** as a pale brown solid. Yield 58%; silica gel TLC R_f 0.28 (MeOH/DCM 10% v/v); mp 235−236 °C (dec); δ_H (400 MHz, DMSO-d_6) 6.99 (1H, d, J 8.4, Ar−H), 7.06 (1H, m, Ar−H), 7.16 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.25 (1H, m, Ar−H), 7.35 (2H, m, Ar−H), 7.80 (1H, t, J 2.0, Ar−H), 8.45 (1H, s, exchangeable with D_2O, N*H*), 8.67 (1H, d, J 2.4, Ar−H), 9.62 (1H, s, exchangeable with D_2O, N*H*), 10.96 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 114.6, 117.0, 117.3, 118.2, 121.1, 122.4, 128.5, 131.4, 134.2, 135.8, 142.1, 149.3, 153.1; m/z (ESI positive) 341.9 $[M + H]^+$.

*Synthesis of 3-(3-(2-Chlorophenyl)ureido)-4-hydroxybenzenesulfonamide (**10**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (4 mL) was treated with 2-chlorophenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **10** as a beige solid. Yield 80%; silica gel TLC R_f 0.33 (MeOH/DCM 10% v/v); δ_H (400 MHz, DMSO-d_6) 6.99 (1H, d, J 8.4; Ar−H), 7.08 (1H, dt, J 1.2, 8.0, Ar−H), 7.15 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.34 (2H, m, Ar−H), 7.49 (1H, dd, J 1.2, 8.0, Ar−H), 8.15 (1H, dd, J 1.2, 8.0, Ar−H), 8.66 (1H, d, J 2.4, Ar−H), 9.10 (1H, s, exchangeable with D_2O, N*H*), 9.22 (1H, exchangeable with D_2O, N*H*), 10.88 (1H, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 114.8, 117.7, 121.3, 123.2, 123.6, 124.6, 128.4, 128.6, 130.3, 135.7, 137.0, 149.8, 153.4; m/z (ESI negative) 339.9 $[M - H]^-$.

*Synthesis of 3-(3-(4-Fluorophenyl)ureido)-4-hydroxybenzenesulfonamide (**11**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 4-fluorophenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) and extracted with ethyl acetate (3 × 15 mL). The combined organic layers were washed with H_2O (3 × 20 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to give the titled product **11** as a beige solid. Yield 86%; silica gel TLC R_f 0.28 (MeOH/DCM 10% v/v); mp 230−231 °C (dec); δ_H (400 MHz, DMSO-d_6) 6.97 (1H, d, J 8.4, Ar−H), 7.16 (4H, m, 2H exchangeable with D_2O, SO_2NH_2), 7.32 (1H, dd, J 2.4,

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8.4, Ar−H), 7.50 (2H, m, Ar−H), 8.37 (1H, s, exchangeable with D_2O, N*H*), 8.66 (1H, d, *J* 2.4, Ar−H), 9.46 (1H, s, exchangeable with D_2O, N*H*), 10.91 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 114.6, 116.3 (d, $^2J_{C-F}$ 22), 116.9, 120.5 (d, $^3J_{C-F}$ 8), 120.9, 128.8, 135.8, 137 (d, $^4J_{C-F}$ 2), 149.2, 153.3, 158.3 (d, $^1J_{C-F}$ 237); δ_F (376 MHz, DMSO-d_6) −121.4 (1F, s); *m*/*z* (ESI negative) 324.0 $[M - H]^-$.

*Synthesis of 4-Hydroxy-3-(3-(4-nitrophenyl)ureido)-benzenesulfonamide (**12**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (4 mL) was treated with 4-nitrophenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **12** as a pale brown solid. Yield 75%; silica gel TLC R_f 0.33 (MeOH/DCM 10% v/v); mp 258−259 °C (dec); δ_H (400 MHz, DMSO-d_6) 6.99 (1H, d, *J* 8.4, Ar−H), 7.17 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.37 (1H, dd, *J* 2.4, 8.4, Ar−H), 7.74 (2H, d, *J* 9.2, Ar−H), 8.24 (2H, d, *J* 9.2, Ar−H), 8.62 (1H, s, exchangeable with D_2O, N*H*), 8.68 (1H, d, *J* 2.4, Ar−H), 10.13 (1H, s, exchangeable with D_2O, N*H*), 11.04 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 114.8, 117.2, 118.2, 121.6, 126.2, 128.1, 135.8, 142.0, 147.2, 149.5, 152.7; *m*/*z* (ESI negative) 350.9 $[M - H]^-$.

*Synthesis of 4-Hydroxy-3-(3-(2-nitrophenyl)ureido)-benzenesulfonamide (**13**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 2-nitrophenyl isocyanate (1.0 equiv) according to the reported general procedure previously reported. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **13** as a yellow solid. Yield 77%; silica gel TLC R_f 0.31 (MeOH/DCM 10% v/v); δ_H (400 MHz, DMSO-d_6) 7.00 (1H, d, *J* 8.4, Ar−H), 7.16 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.29 (1H, t, *J* 7.2, Ar−H), 7.37 (1H, dd, *J* 2.4, 8.4, Ar−H), 7.72 (1H, t, *J* 7.2, Ar−H), 8.06 (2H, m, Ar−H), 8.56 (1H, d, *J* 2.4, Ar−H), 9.27 (1H, s, exchangeable with D_2O, N*H*), 9.87 (1H, s, exchangeable with D_2O, N*H*), 10.90 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 115.0, 118.2, 121.8, 124.0, 124.9, 126.1, 128.1, 134.4, 135.3, 135.7, 140.6, 150.1, 153.0; *m*/*z* (ESI negative) 350.9 $[M - H]^-$.

*Synthesis of 3-(3-(3,5-Dimethylphenyl)ureido)-4-hydroxybenzenesulfonamide (**14**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 3,5-dimethlyphenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **14** as a pale brown solid. Yield 81%; silica gel TLC R_f 0.39 (MeOH/DCM 10% v/v); mp 228−229 °C (dec); δ_H (400 MHz, DMSO-d_6) 2.27 (6H, s, 2 x CH_3), 6.66 (1H, s, Ar−H), 6.97 (1H, d, *J* 8.4, Ar−H), 7.13 (4H, m, 2H exchangeable with D_2O, SO_2NH_2), 7.32 (1H, dd, *J* 2.4, 8.4, Ar−H), 8.38 (1H, s, exchangeable with D_2O, N*H*), 8.69 (1H, d, *J* 2.4, Ar−H), 9.27 (1H, s, exchangeable with D_2O, N*H*), 10.88 (1H, brs; exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 22.1, 114.5, 116.6, 116.8, 120.7, 124.4, 128.9, 135.8, 138.7, 140.5, 149.2, 153.2; *m*/*z* (ESI positive) 336.0 $[M + H]^+$.

*Synthesis of 3-(3-(2,5-Dimethylphenyl)ureido)-4-hydroxybenzenesulfonamide (**15**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 2,5-dimethylphenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **15** as a beige solid. Yield 95%; silica gel TLC R_f 0.22 (MeOH/DCM 10% v/v); mp 245−246 °C (dec); δ_H (400 MHz, DMSO-d_6) 2.24 (3H, s, CH_3), 2.29 (3H, s, CH_3), 6.80 (1H, d, *J* 8.0, Ar−H), 6.98 (1H, d, *J* 8.4, Ar−H), 7.08 (1H, d, *J* 8.0, Ar−H), 7.13 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.31 (1H, dd, *J* 2.4, 8.4, Ar−H), 7.73 (1H, s, Ar−H), 8.58 (1H, s, exchangeable with D_2O, N*H*), 8.70 (1H, d, *J* 2.4, Ar−H), 8.83 (1H, s, exchangeable with D_2O, N*H*), 10.83 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 18.6, 21.9, 114.6, 117.1, 120.7, 122.9, 124.3, 125.6, 129.0, 131.0, 135.8, 135.9, 138.1, 149.3, 153.6; *m*/*z* (ESI negative) 334.0 $[M - H]^-$.

*Synthesis of 4-Hydroxy-3-(3-(2-isopropylphenyl)ureido)-benzenesulfonamide (**16**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 2-isopropylphenyl isocyanate (1.0 equiv) according to the general procedure previously reported. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **16** as a beige solid. Yield 11%; silica gel TLC R_f 0.42 (MeOH/DCM 10% v/v); mp 218−219 °C; δ_H (400 MHz, DMSO-d_6) 1.23 (6H, d, *J* 6.8, 2 × CH_3), 3.25 (1H, m, CH), 6.98 (1H, d, *J* 8.4, Ar−H), 7.14 (4H, m, 2H exchangeable with D_2O, SO_2NH_2), 7.32 (2H, m, Ar−H), 7.67 (1H, dd, *J* 1.6, 8.0, Ar−H), 8.66 (1H, s, exchangeable with D_2O, N*H*), 8.68 (1H, d, *J* 2.4, Ar−H), 8.77 (1H, s, exchangeable with D_2O, N*H*), 10.86 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 24.2, 27.6, 114.6, 117.1, 120.7, 125.0, 125.0, 126.2, 126.6, 129.1, 135.7, 136.3, 140.8, 149.3, 154.1; *m*/*z* (ESI negative) 348.0 $[M - H]^-$.

*Synthesis of 4-Hydroxy-3-(3-(3-(methylthio)phenyl)ureido)-benzenesulfonamide (**17**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 3-(methylthio)phenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **17** as a pale brown solid. Yield 56%; silica gel TLC R_f 0.20 (MeOH/DCM 10% v/v); mp 220−221 °C (dec); δ_H (400 MHz, DMSO-d_6) 2.50 (3H, s, CH_3), 6.90 (1H, d, *J* 8.0, Ar−H), 6.98 (1H, d, *J* 8.4, Ar−H), 7.15 (3H, m, 2H exchangeable with D_2O, SO_2NH_2), 7.26 (1H, t, *J* 8.0, Ar−H), 7.33 (1H, dd, *J* 2.4, 8.4, Ar−H), 7.55 (1H, m, Ar−H), 8.40 (1H, s, exchangeable with D_2O, N*H*), 8.67 (1H, d, *J* 2.4, Ar−H), 9.47 (1H, s, exchangeable with D_2O, N*H*), 10.90 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 15.5, 114.6, 115.4, 115.8, 116.9, 120.2, 120.9, 128.7, 130.3, 135.8, 139.7, 141.2, 149.2, 153.1; *m*/*z* (ESI negative) 352.0 $[M - H]^-$.

*Synthesis of 3-[3-(4-Ethyl-phenyl)-ureido]-4-hydroxy-benzenesulfonamide (**18**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 4-ethylphenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **18** as a beige solid. Yield 78%; silica gel TLC R_f 0.29 (MeOH/DCM 10% v/v); mp 210−211 °C; δ_H (400 MHz, DMSO-d_6) 1.20 (3H, t, *J* 7.6, CH_3), 2.59 (2H, m, CH_2), 6.96 (1H, d, *J* 8.4, Ar−H), 7.14 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.16 (2H, d, *J* 8.4, Ar−H), 7.32 (1H, dd, *J* 2.4, 8.4, Ar−H), 7.40 (2H, d, *J* 8.4, Ar−H), 8.36 (1H, s, exchangeable with D_2O, N*H*), 8.68 (1H, d, *J* 2.4, Ar−H), 9.33 (1H, s, exchangeable with D_2O, N*H*), 10.87 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 16.7, 28.4, 114.5, 116.8, 119.0, 120.7, 128.9, 129.0, 135.8, 138.2, 138.3, 149.2, 153.3; *m*/*z* (ESI negative) 334.0 $[M - H]^-$.

*Synthesis of 3-(3-(4-Butylphenyl)ureido)-4-hydroxybenzenesulfonamide (**19**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (4 mL) was treated with 4-butylphenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **19** as a beige solid. Yield 65%; silica gel TLC R_f 0.74 (ethyl acetate 100% v/v); mp 204−205 °C (dec); δ_H (400 MHz, DMSO-d_6) 0.93 (3H, t, *J* 7.3, CH_3), 1.33 (2H, m, CH_2), 1.56 (2H, quint, *J* 7.3, CH_2), 2.56 (2H, m, CH_2), 6.96 (1H, d, *J* 8.4, Ar−H), 7.13 (4H, m, 2H exchangeable with D_2O, SO_2NH_2), 7.31 (1H, dd, *J* 2.4, 8.4, Ar−H), 7.39 (2H, d, *J* 8.4, Ar−H), 8.36 (1H, s, exchangeable with D_2O, N*H*), 8.68 (1H, d, *J* 2.4, Ar−H), 9.33 (1H, s, exchangeable with D_2O, N*H*), 10.89 (1H, brs, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 14.7,

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22.6, 34.2, 35.1, 114.5, 116.8, 119.0, 120.7, 128.9, 129.5, 135.8, 136.7, 138.3, 149.2, 153.3; m/z (ESI negative) 362.0 $[M - H]^-$.

*Synthesis of 4-Hydroxy-3-(3-(2-methoxyphenyl)ureido)-benzenesulfonamide (**20**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 2-methoxyphenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **20** as a beige solid. Yield 44%; silica gel TLC R_f 0.28 (MeOH/DCM 10% v/v); mp 209−210 °C (dec); δ_H (400 MHz, DMSO-d_6) 3.90 (3H, s, OCH_3), 6.90−7.05 (4H, m, Ar−H), 7.13 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.32 (1H, dd, J 2.4, 8.4, Ar−H), 8.15 (1H, dd, J 1.2, 8.0, Ar−H), 8.66 (1H, d, J 2.4, Ar−H), 9.03 (1H, s, exchangeable with D_2O, N*H*), 9.10 (1H, s, exchangeable with D_2O, N*H*), 10.75 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 56.6, 111.8, 114.7, 117.7, 120.0, 120.9, 121.4, 123.0, 128.9, 129.6, 135.7, 149.2, 149.8, 153.6; m/z (ESI negative) 336.0 $[M - H]^-$.

*Synthesis of 3-(3-(2-Ethoxyphenyl)ureido)-4-hydroxybenzenesulfonamide (**21**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (4 mL) was treated with 2-ethoxyphenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **21** as a beige solid. Yield 80%; silica gel TLC R_f 0.72 (ethyl acetate 100% v/v); mp 208−209 °C (dec); δ_H (400 MHz, DMSO-d_6) 1.45 (3H, t, J 7.0, CH_3), 4.16 (2H, q, J 7.0, OCH_2), 6.91 (1H, dt, J 1.6, 8.0, Ar−H), 6.97 (2H, m, Ar−H), 7.03 (1H, dd, J 1.6, 8.0, Ar−H), 7.13 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.33 (1H, dd, J 2.4, 8.4, Ar−H), 8.13 (1H, dd, J 1.6, 8.0, Ar−H), 8.64 (1H, d, J 2.4, Ar−H), 8.83 (1H, s, exchangeable with D_2O, N*H*), 9.11 (1H, s, exchangeable with D_2O, N*H*), 10.77 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 15.7, 64.8, 112.9, 114.8, 118.0, 120.4, 121.0, 121.3, 123.1, 128.8, 129.8, 135.7, 148.3, 149.9, 153.6; m/z (ESI negative) 350.0 $[M - H]^-$.

*Synthesis of 4-Hydroxy-3-(3-(4-phenoxyphenyl)ureido)-benzenesulfonamide (**22**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 4-phenoxyphenyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **22** as a beige solid. Yield 70%; silica gel TLC R_f 0.20 (MeOH/DCM 10% v/v); mp 225−226 °C; δ_H (400 MHz, DMSO-d_6) 7.00 (5H, m, Ar−H), 7.14 (3H, m, 2H exchangeable with D_2O, SO_2NH_2), 7.33 (1H, dd, J 2.4, 8.4, Ar−H), 7.40 (2H, m, Ar−H), 7.52 (2H, d, J 8.8, Ar−H), 8.37 (1H, s, exchangeable with D_2O, N*H*), 8.68 (1H, d, J 2.4, Ar−H), 9.44 (1H, s, exchangeable with D_2O, N*H*), 10.89 (1H, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 114.6, 116.9, 118.7, 120.6, 120.8, 120.9, 123.8, 128.9, 130.9, 135.8, 136.7, 149.2, 151.7, 153.3, 158.6; m/z (ESI negative) 398.0 $[M - H]^-$.

*Synthesis of 4-Hydroxy-3-(3-(naphthalen-1-yl)ureido)-benzenesulfonamide (**23**).* A solution of 2-aminophenol-4-sulfonamide **1** (0.2 g, 1.0 equiv) in dry acetonitrile (3 mL) was treated with 1-naphthyl isocyanate (1.0 equiv) according to the reported general procedure. The reaction was quenched with H_2O (1.0 mL) to give precipitates that were washed with diethyl ether (3 × 10 mL), filtered, and dried under vacuum to afford the titled product **23** as a beige solid. Yield 84%; silica gel TLC R_f 0.35 (MeOH/DCM 10% v/v); mp 235−236 °C (dec); δ_H (400 MHz, DMSO-d_6) 7.01 (1H, d, J 8.4, Ar−H), 7.16 (2H, s, exchangeable with D_2O, SO_2NH_2), 7.35 (1H, dd, J 2.4, 8.4, Ar−H), 7.51 (1H, t, J 8.0, Ar−H), 7.60 (2H, m, Ar−H), 7.67 (1H, d, J 8.0, Ar−H), 7.96 (1H, d, J 8.0, Ar−H), 8.11 (1H, d, J 8.0, Ar−H), 8.26 (1H, d, J 8.0, Ar−H), 8.75 (1H, d, J 2.4, Ar−H), 9.00 (1H, s, exchangeable with D_2O, N*H*), 9.44 (1H, s, exchangeable with D_2O, N*H*), 10.93 (1H, s, exchangeable with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 114.9, 117.3, 118.5, 121.1, 122.6, 124.1, 126.7, 126.9, 127.0, 129.1, 129.5, 134.8, 135.4, 135.9, 149.6, 154.0; m/z (ESI negative) 356.0 $[M - H]^-$.

In Vitro Enzyme Assays. *Carbonic Anhydrase Inhibition.* The CA-catalyzed CO_2 hydration activity was performed on an Applied Photophysics stopped-flow instrument using phenol red (at a concentration of 0.2 mM) as a pH indicator with 20 mM Hepes (pH 7.5) as the buffer, 20 mM Na_2SO_4, and following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10−100 s and working at the maximum absorbance of 557 nm.[29] The CO_2 concentrations ranged from 1.7 to 17 mM. For each inhibitor six traces of the initial 5−10% of the reaction have been used in order to determine the initial velocity. The uncatalyzed reaction rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled water, and dilutions up to 0.01 nM were prepared. Solutions containing inhibitor and enzyme were preincubated for 15 min at room temperature prior to assay in order to allow the formation of the E−I complex. The inhibition constants were obtained as nonlinear least-squares protocols using PRISM 3[31−33] and are the mean from at least three different measurements. All hCAs were recombinant ones and were obtained in house.[31−33]

X-ray Crystallography. *Crystallization and Data Collection.* Crystals of hCA I complexed with compounds **5**, **15**, and **20** were obtained using the sitting drop vapor diffusion method. Two microliters of 10 mg/mL solution of hCA I in Tris-HCl 20 mM pH 9.0 were mixed with 2 μL of a solution of 28−31% PEG4000, 0.2 M sodium acetate, and 0.1 M Tris pH 8.5−9.0 and were equilibrated against the same solution at 296 K. Crystals of the protein grew in 15 days. Afterward, hCA I crystals were soaked in 5 mM inhibitor solutions for 1 day. The crystals were flash-frozen at 100 K using a solution obtained by adding 15% (v/v) glycerol to the mother liquor solution as cryoprotectant. Data on crystals of the complexes were collected using synchrotron radiation at the ID30B beamline at ESRF (Grenoble, France) with a wavelength of 0.973 Å and a PILATUS3 6 M Dectris CCD detector. Data were integrated and scaled using the program XDS.[34] Data processing statistics are shown in Supporting Information Table S1.

Structure Determination. The crystal structure of hCA I (PDB accession code: 1JV0) without solvent molecules and other heteroatoms was used to obtain initial phases for the structures using Refmac5.[35] Five percent of the unique reflections were selected randomly and excluded from the refinement data set for the purpose of R_{free} calculations. The initial |Fo − Fc| difference electron density maps unambiguously showed the inhibitor molecules. Atomic models for inhibitors were calculated and energy minimized using the program JLigand 1.0.40.[36] Refinements proceeded using normal protocols of positional, isotropic atomic displacement parameters alternating with manual building of the models using COOT.[37] Solvent molecules were introduced automatically using the program ARP.[38] The quality of the final models were assessed with COOT and RAMPAGE.[39] Crystal parameters and refinement data are summarized in Table S1 in the Supporting Information. Atomic coordinates were deposited in the Protein Data Bank (PDB accession code: 6F3B, 6FAF, and 6FAG). Graphical representations were generated with Chimera.[40]

Biological Assays. *Cell Culture.* The 4T1 murine breast cancer cell line was cultured as previously described.[13,41] Briefly, cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose (Gibco cat # 11995−065, Burlington, Ontario) and supplemented with 10% fetal bovine serum (FBS; Gibco, Burlington, Ontario) and nonessential amino acids (1× NEAA). Cells were routinely tested for mycoplasma contamination using the LookOut Mycoplasma PCR detection kit (Sigma; Cat. No. MP0035). The 4T1 cells have been authenticated using short tandem repeat DNA profiling (DNA fingerprinting) by a commercial testing facility (Genetica, Burlington, NC, USA). In addition, the cell lines were routinely tested for viability, morphology, hypoxia-induced endogenous CA IX expression, and *in vivo* tumor growth.

Orthotopic Syngeneic Breast Tumor Model. All animal studies and procedures were performed in accordance with protocols approved by the Institutional Animal Care Committee at the BC Cancer Research Centre and University of British Columbia

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(Vancouver, BC, Canada). 4T1 murine mammary tumor cells (1.0×10^6 cells/animal) were implanted orthotopically into the mammary fat pad of BALB/c mice (Simonsen Laboratories, Gilroy, CA) as described previously.[13,42] Tumors were measured 3×/week using digital calipers, and volumes were calculated using the modified ellipsoid formula ((length × width × width)π/6). Immediately prior to initiation of treatment, mice were randomized and sorted into groups of similar average tumor volume. Treatment was initiated when mean tumor volumes reached approximately 60 mm^3.

Drug Treatment. Compound **11** and **SLC-0111** were solubilized at the indicated concentrations in the same vehicle (37.5% PEG-400/12.5% ethanol/50%PBS), divided into daily aliquots and frozen at −80 °C until use. Compounds were administered by intraperitoneal injection daily until the experimental end point. Control animals were administered the vehicle alone in a similar fashion. The investigators were not blinded to the treatment groups.

Statistical Analysis. For tumor growth data, statistical analyses were performed using GraphPad Prism 7. Data are reported as the mean ± SEM. Outliers were identified using Grubb's test (alpha = 0.05) and excluded from further analysis. For comparison of three or more data sets, a one-way ANOVA was performed and a Holms-Sidak's test was used to correct for multiple comparisons. Statistical significance was set at $P < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.8b00770.

Summary of data collection and atomic model refinement statistics (PDF)

SMILES representation for compounds (CSV)

Accession Codes

Coordinates and structure factors for hCA I complexes with **5**, **15**, and **20** have been deposited in the Protein Data Bank (PDB) accession codes: 6F3B, 6FAF, and 6FAG.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

CAI, carbonic anhydrase inhibitor; TLC, thin layer chromatography; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; HPLC, high-pressure liquid chromatography; DCM, dichloromethane; TFA, trifluoroacetic acid; MeOH, methanol; DMSO, dimethyl sulfoxide; SAR, structure−activity relationship; Dec, decomposition; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Cp, Cyclopentyl

■ REFERENCES

(1) Supuran, C. T. Structure and function of carbonic anhydrases. *Biochem. J.* **2016**, *473*, 2023−2032.

(2) Supuran, C. T. Carbonic anhydrases. *Bioorg. Med. Chem.* **2013**, *21*, 1377−1378.

(3) Supuran, C. T.; Capasso, C. An overview of the bacterial barbonic anhydrases. *Metabolites* **2017**, *7*, 56.

(4) Akdemir, A.; Vullo, D.; De Luca, V.; Scozzafava, A.; Carginale, V.; Rossi, M.; Supuran, C. T.; Capasso, C. The extremo-α-carbonic anhydrase (CA) from Sulfurihydrogenibium azorense, the fastest CA known, is highly activated by amino acids and amines. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 1087−1090.

(5) Luca, V. D.; Vullo, D.; Scozzafava, A.; Carginale, V.; Rossi, M.; Supuran, C. T.; Capasso, C. An α-carbonic anhydrase from the thermophilic bacterium Sulphurihydrogenibium azorense is the fastest enzyme known for the CO2 hydration reaction. *Bioorg. Med. Chem.* **2013**, *21*, 1465−1469.

(6) Supuran, C. T.; Capasso, C. Carbonic anhydrase from Porphyromonas Gingivalis as a drug target. *Pathogens* **2017**, *6*, E30.

(7) Del Prete, S.; Vullo, D.; Fisher, G. M.; Andrews, K. T.; Poulsen, S. A.; Capasso, C.; Supuran, C. T. Discovery of a new family of carbonic anhydrases in the malaria pathogen Plasmodium falciparum−the η-carbonic anhydrases. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 4389−4396.

(8) Moya, A.; Tambutté, S.; Bertucci, A.; Tambutté, E.; Lotto, S.; Vullo, D.; Supuran, C. T.; Allemand, D.; Zoccola, D. Carbonic anhydrase in the scleractinian coral Stylophora pistillata: characterization, localization, and role in biomineralization. *J. Biol. Chem.* **2008**, *283*, 25475−25484.

(9) Supuran, C. T. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat. Rev. Drug Discovery* **2008**, *7*, 168−181.

(10) Neri, D.; Supuran, C. T. Interfering with pH regulation in tumours as a therapeutic strategy. *Nat. Rev. Drug Discovery* **2011**, *10*, 767−777.

(11) Supuran, C. T. Carbonic anhydrase inhibitors and activators for novel therapeutic applications. *Future Med. Chem.* **2011**, *3*, 1165−1180.

(12) Krishnamurthy, V. M.; Kaufman, G. K.; Urbach, A. R.; Gitlin, I.; Gudiksen, K. L.; Weibel, D. B.; Whitesides, G. M. Carbonic anhydrase as a model for biophysical and physical-organic studies of proteins and protein-ligand binding. *Chem. Rev.* **2008**, *108*, 946−1051.

(13) (a) Lou, Y.; McDonald, P. C.; Oloumi, A.; Chia, S.; Ostlund, C.; Ahmadi, A.; Kyle, A.; Auf dem Keller, U.; Leung, S.; Huntsman, D.; Clarke, B.; Sutherland, B. W.; Waterhouse, D.; Bally, M.; Roskelley, C.; Overall, C. M.; Minchinton, A.; Pacchiano, F.; Carta, F.; Scozzafava, A.; Touisni, N.; Winum, J. Y.; Supuran, C. T.; Dedhar, S. Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. *Cancer Res.* **2011**, *9*, 3364−3376. (b) Touisni, N.; Maresca, A.; McDonald, P. C.; Lou, Y.; Scozzafava, A.; Dedhar, S.; Winum, J. Y.; Supuran, C. T. Glycosyl coumarin carbonic anhydrase IX and XII inhibitors strongly attenuate the growth of primary breast tumors. *J. Med. Chem.* **2011**, *54*, 8271−8277.

(14) Chia, S. K.; Wykoff, C. C.; Watson, P. H.; Han, C.; Leek, R. D.; Pastorek, J.; Gatter, K. C.; Ratcliffe, P.; Harris, A. L. Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. *J. Clin. Oncol.* **2001**, *19*, 3660−3668.

(15) Choi, S. W.; Kim, J. Y.; Park, J. Y.; Cha, I. H.; Kim, J.; Lee, S. Expression of carbonic anhydrase IX is associated with postoperative recurrence and poor prognosis in surgically treated oral squamous cell carcinoma. *Hum. Pathol.* **2008**, *39*, 1317−1322.

(16) Kon-no, H.; Ishii, G.; Nagai, K.; Yoshida, J.; Nishimura, M.; Nara, M.; Fujii, T.; Murata, Y.; Miyamoto, H.; Ochiai, A. Carbonic anhydrase IX expression is associated with tumor progression and a poor prognosis of lung adenocarcinoma. *Lung Cancer* **2006**, *54*, 409−418.

(17) Tan, E. Y.; Yan, M.; Campo, L.; Han, C.; Takano, E.; Turley, H.; Candiloro, I.; Pezzella, F.; Gatter, K. C.; Millar, E. K.; O'Toole, S. A.; McNeil, C. M.; Crea, P.; Segara, D.; Sutherland, R. L.; Harris, A. L.; Fox, S. B. The key hypoxia regulated gene CAIX is upregulated in basal-like breast tumours and is associated with resistance to chemotherapy. *Br. J. Cancer* **2009**, *100*, 405−411.

DOI: 10.1021/acs.jmedchem.8b00770
J. Med. Chem. 2018, 61, 6328−6338

(18) Lau, J.; Lin, K. S.; Bénard, F. Past, present, and future: development of theranostic agents targeting carbonic anhydrase IX. *Theranostics* **2017**, *17*, 4322−4339.
(19) Pacchiano, F.; Carta, F.; McDonald, P. C.; Lou, Y.; Vullo, D.; Scozzafava, A.; Dedhar, S.; Supuran, C. T. Ureido-substituted benzenesulfonamides potently inhibit carbonic anhydrase IX and show antimetastatic activity in a model of breast cancer metastasis. *J. Med. Chem.* **2011**, *54*, 1896−1902.
(20) Pacchiano, F.; Aggarwal, M.; Avvaru, B. S.; Robbins, A. H.; Scozzafava, A.; McKenna, R.; Supuran, C. T. Selective hydrophobic pocket binding observed within the carbonic anhydrase II active site accommodate different 4-substituted-ureido-benzenesulfonamides and correlate to inhibitor potency. *Chem. Commun. (Cambridge, U. K.)* **2010**, *46*, 8371−8373.
(21) Safety Study of SLC-0111 in Subjects With Advanced Solid Tumours. https://clinicaltrials.gov/ct2/show/NCT02215850 (accessed Jun 12, 2018).
(22) Lomelino, C. L.; Mahon, B. P.; McKenna, R.; Carta, F.; Supuran, C. T. Kinetic and X-ray crystallographic investigations on carbonic anhydrase isoforms I, II, IX and XII of a thioureido analog of SLC-0111. *Bioorg. Med. Chem.* **2016**, *24*, 976−981.
(23) Gieling, R. G.; Babur, M.; Mamnani, L.; Burrows, N.; Telfer, B. A.; Carta, F.; Winum, J. Y.; Scozzafava, A.; Supuran, C. T.; Williams, K. J. Antimetastatic effect of sulfamate carbonic anhydrase IX inhibitors in breast carcinoma xenografts. *J. Med. Chem.* **2012**, *55*, 5591−5600.
(24) Angeli, A.; Tanini, D.; Peat, T. S.; Di Cesare Mannelli, L.; Bartolucci, G.; Capperucci, A.; Ghelardini, C.; Supuran, C. T.; Carta, F. Discovery of new selenoureido analogues of 4-(4-fluorophenylureido)benzenesulfonamide as carbonic anhydrase Inhibitors. *ACS Med. Chem. Lett.* **2017**, *8*, 963−968.
(25) Pan, J.; Lau, J.; Mesak, F.; Hundal, N.; Pourghiasian, M.; Liu, Z.; Bénard, F.; Dedhar, S.; Supuran, C. T.; Lin, K. S. Synthesis and evaluation of 18F-labeled carbonic anhydrase IX inhibitors for imaging with positron emission tomography. *J. Enzyme Inhib. Med. Chem.* **2014**, *29*, 249−255.
(26) Asakawa, C.; Ogawa, M.; Kumata, K.; Fujinaga, M.; Yamasaki, T.; Xie, L.; Yui, J.; Kawamura, K.; Fukumura, T.; Zhang, M. R. Radiosynthesis of three [11C]ureido-substituted benzenesulfonamides as PET probes for carbonic anhydrase IX in tumors. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 7017−7020.
(27) Supuran, C. T.; Shoukat, D.; McDonald, P. C.; Carta, F. Novel Sulfonamide Compounds for Inhibition of Metastatic Tumor Growth. WO2012021963 (A1).
(28) Carta, F.; Vullo, D.; Osman, S. M.; AlOthman, Z.; Supuran, C. T. Synthesis and carbonic anhydrase inhibition of a series of SLC-0111 analogs. *Bioorg. Med. Chem.* **2017**, *9*, 2569−2576.
(29) Khalifah, R. G. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. *J. Biol. Chem.* **1971**, *246*, 2561−2573.
(30) Alterio, V.; Di Fiore, A.; D'Ambrosio, K.; Supuran, C. T.; De Simone, G. Multiple binding modes of inhibitors to carbonic anhydrases: how to design specific drugs targeting 15 different isoforms? *Chem. Rev.* **2012**, *112*, 4421−4468.
(31) Carta, F.; Maresca, A.; Scozzafava, A.; Supuran, C. T. 5- and 6-membered (thio)lactones are prodrug type carbonic anhydrase inhibitors. *Bioorg. Med. Chem. Lett.* **2012**, *1*, 267−270.
(32) Di Cesare Mannelli, L.; Micheli, L.; Carta, F.; Cozzi, A.; Ghelardini, C.; Supuran, C. T. Carbonic anhydrase inhibition for the management of cerebral ischemia: in vivo evaluation of sulfonamide and coumarin inhibitors. *J. Enzyme Inhib. Med. Chem.* **2016**, *6*, 894−899.
(33) Grandane, A.; Tanc, M.; Di Cesare Mannelli, L.; Carta, F.; Ghelardini, C.; Žalubovskis, R.; Supuran, C. T. 6-Substituted sulfocoumarins are selective carbonic anhydrase IX and XII inhibitors with significant cytotoxicity against colorectal cancer cells. *J. Med. Chem.* **2015**, *9*, 3975−3883.
(34) Leslie, A. G. W.; Powell, H. R. Processing Diffraction Data with Mosflm. In *Evolving Methods for Macromolecular Crystallography*; Read, R. J., Sussman, L. J., Eds.; Springer: Netherlands, 2007; Vol. *245*, pp 41−51.
(35) Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1997**, *D53*, 240−255.
(36) Lebedev, A. A.; Young, P.; Isupov, M. N.; Moroz, O. V.; Vagin, A. A.; Murshudov, G. N. JLigand: a graphical tool for the CCP4 template-restraint library. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2012**, *68*, 431−440.
(37) Emsley, P.; Lohkamp, B.; Scott, W.; Cowtan, K. Features and development of Coot. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2010**, *D66*, 486−501.
(38) Lamzin, V. S.; Perrakis, A.; Wilson, K. S.; Crystallography of Biological Macromolecules. In *Int. Tables for Crystallography*. Rossmann, M. G., Arnold, E., Eds.; Kluwer Academic Publishers, Dordrecht, The Netherlands, 2001; Vol *F*, pp 720−722.
(39) Lovell, S. C.; Davis, I. W.; Arendall, W. B., III; de Bakker, P. I. W.; Word, J. M.; Prisant, M. G.; Richardson, J. S.; Richardson, D. C. Structure validation by Calpha geometry: phi,psi and Cbeta deviation. *Proteins: Struct., Funct., Genet.* **2003**, *50*, 437−450.
(40) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera−a visualization system for exploratory research and analysis. *J. Comput. Chem.* **2004**, *13*, 1605−1612.
(41) Lou, Y.; Preobrazhenska, O.; auf dem Keller, U.; Sutcliffe, M.; Barclay, L.; McDonald, P. C.; Roskelley, C.; Overall, C. M.; Dedhar, S. Epithelial-mesenchymal transition (EMT) is not sufficient for spontaneous murine breast cancer metastasis. *Dev. Dyn.* **2008**, *237*, 2755−2768.
(42) Lock, F. E.; McDonald, P. C.; Lou, Y.; Serrano, I.; Chafe, S. C.; Ostlund, C.; Aparicio, S.; Winum, J. Y.; Supuran, C. T.; Dedhar, S. Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche. *Oncogene* **2013**, *32*, 5210−5219.

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