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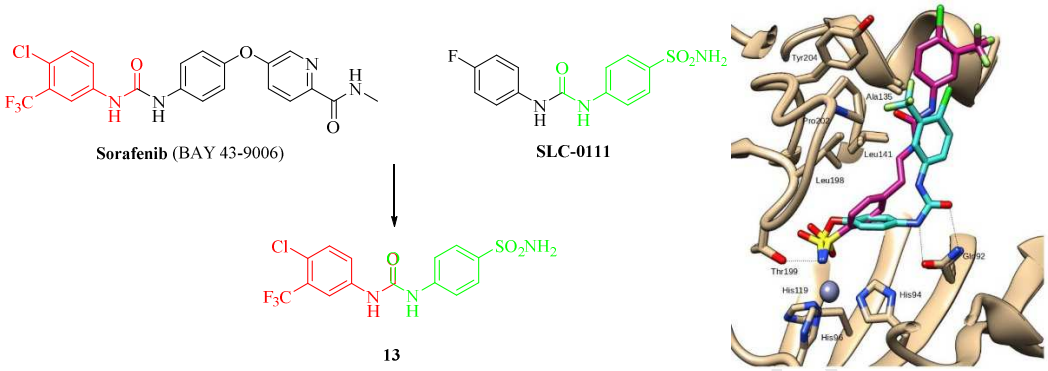
Graphical abstract



Carbonic Anhydrase Inhibitors based on sorafenib scaffold: Design, Synthesis, Crystallographic Investigation and Effects on Primary Breast Cancer Cells

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Abstract: Carbonic anhydrase inhibitors (CAIs) of the sulfonamide, sulfamate and coumarin classes bearing the phenylureido tail found in the clinically used drug Sorafenib, a multikinase inhibitor actually used for the management of hepatocellular carcinomas, are reported. All compounds were assayed on human (h) CA isoforms I, II, VII and IX, involved in various pathologies. Among the sulfonamides, several compounds were selective for inhibiting hCA IX, with K_I values in the low nanomolar ranges (i.e. 0.7-30.2 nM). We explored the binding modes of such compounds by means of X-ray crystallographic studies on isoform hCA I in adduct with one sulfonamide and a sulfamate inhibitor. Antiproliferative properties of some sulfamates on breast tumor cell lines were also investigated.

Keywords: Carbonic Anhydrase; Sorafenib; Enzyme inhibitor; sulfonamide; sulfamate; coumarin.

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1.Introduction

The 15 carbonic anhydrases (CAs, EC 4.2.1.1) expressed humans in are zinc(II) dependent metalloenzymes, differently distributed at subcellular and tissue levels [1,2]. Human (h) hCAs are involved in vital physiological processes by means of the reversible conversion of carbon dioxide to bicarbonate and protons which they catalyze [3–7]. Abnormalities in hCA expressions may lead to diseases, such as hypertension, glaucoma, epilepsy, obesity, altitude sickness, cancers and pH disequilibria [7,8]. CA inhibitors (CAIs) of the sulfonamide type or their bioisosteres, are routinely used as drugs. The most used are acetazolamide (AAZ), methazolamide (MTZ), dichlorophenamide (DCP), brinzolamide (BRZ), sulthiame (SLT), zonisamide (ZNS), the sulfamate topiramate (TPM) and various COXIBs with dual CA-cycloxygenase activity such as celecoxib and polmacoxib [7–9]. Besides the sulfonamide moiety, many new types of CAIs acting as zinc binders were discovered (i.e. dithiocarbamates [10–13] xanthates [14] and monothiocarbamates [15]). In addition, other classes of CAIs include molecules able to interact with the zinc coordinated water/hydroxide ion (i.e. phenols [16–20], carboxylic acids [20,21], polyamines [22,23], thioxocoumarins [24,25] and the sulfonic acid group formed from hydrolysis of sulfocoumarins [26–32]). Compounds occluding the entrance of the CA active site (cinnamic acid derivatives from *in situ* hydrolyzed coumarins [24,33–36]) and out of the site CAIs such as the 2-(benzylsulfonyl)-benzoic acid were also shown to act as CAIs [37]. Although many CAI chemical classes were reported, the selectivity in targeting specific enzymatic isoforms was not always granted. Therefore, any medicinal chemistry application of CAIs resulted hampered. Interestingly, the decoration of the sulfonamide CAI warhead by means of appropriate tails revealed to be a valid strategy to overcome the lack of enzymatic selectivity. In particular the arylureido-tails were particularly efficient in solving the selectivity issue among the many hCA isoforms [38–40], and in particular such a moiety led to compounds with a preferential inhibition towards the tumor associated isoforms (i.e. hCAs IX and XII) over the cytosolic off-target ones (i.e. hCAs I and II). Such results were confirmed in *in vivo* experiments using an appropriate breast cancer model [38–40], and were rationalized at molecular level by using X-ray crystallography techniques. The flexibility of the ureido-group has been claimed to be responsible for allowing the tails to better fit within the CA active site, and thus in maximizing the interactions occurring between the ligand and the amino acid residues exposed within the enzymatic cleft [38–40]. The compound (4-(3-(4-fluorophenyl)ureido)benzenesulfonamide, also known as **SLC-0111,** was obtained by application of such a strategy and is currently facing phase II clinical trials in combination with gemcitabine for the treatment of metastatic pancreatic ductal cancer [41]. **SLC-0111** is a selective inhibitor of hCA IX and XII, which are almost exclusively expressed in hypoxic tumors [42–44], being responsible

of the extracellular pH acidification [39,42–44]. Recent applications of the ureido tail strategy were reported by our group with the preparation of ureido- substituted sulfamates, coumarins, 4-hydroxy-3-(3-(phenylureido)benzenesulfonamides, *N*'-phenyl-*N*-hydroxyureas and *N*-aryl-*N*'-ureido-*O*-sulfamates [24,45–49].

Our pursuit to find more efficient ureido-bearing molecules to treat hypoxic tumors directed us to consider sorafenib (Nexavar®), an orally available multikinase inhibitor approved by FDA to treat hepatocellular carcinoma (HCC), as an interesting lead. Clinical studies showed sorafenib to exert promising effects on thyroid cancer, myeloid leukemia, mesothelioma and prostate cancers [50]. HCC is one of the most common cancers associated to high ratio of mortality [50–52]. Sorafenib was proved to extend the median time of progression in patients with advanced HCC and the life span of patients by an average of three months [50–53].

2. Results and Discussion

2.1. Drug Design and Chemistry.

The first studies on phenylureido-tailed benzenesulfonamides which revealed selective inhibition of the hypoxia related hCAs IX and XII were published by some of us [38–40] and among the series reported a molecule, known as SLC-0111, showed impressive antimetastatic effects on breast cancer *in vivo*[39]. Such a molecule currently is under phase II clinical trials in association with gemcitabine for the management of pancreatic tumors [41]. Such results allowed to validate the membrane bound hCA IX as pharmaceutical target for the treatment of hypoxic tumors. Another important study within this field was on sulfamate derivatives of SLC-0111 [54]. In vitro kinetics on the series reported showed highly potent inhibitors against the tumor associated hCAs. In particular, three CAIs were further selected for *in vitro* cellular assays and showed inhibition of cells migration and spreading in anoxic conditions. Moreover one of such inhibitors was tested on orthotopic MDA-MB-231 breast cancer model in mice and showed inhibition of metastases in lungs without relevant toxicity [54]. As expansion the same authors reported a new series of sulfamates which showed in vitro potent hCA IX inhibitory activity and antiproliferative properties on breast cancer cell lines (i.e. SKBR3, MCF10A, ZR-75-1, MDA-MB-361 in normoxia and MCF7 in hypoxia) [45]. The ureido moiety was also inserted in seleno containing compounds as CAIs and were investigated on prostate (PC3), breast (MDA-MB-231) and colon cancer (HT-29) cells in comparison with SLC-0111 [55]. Finally it is worth reporting the 2-aminophenol-4-sulfonamide ureido containing derivatives as CAIs [47]. Again the series showed isoform selective inhibition on the tumor associated hCAs, and the 4-fluorophenylureido derivative was investigated *in vivo* and

showed similar results to SLC-0111 [47]. In light of the high potency of the ureido containing CAIs on the tumor associated isoforms we decided to merge the structures of sorafenib and **SLC-0111** to obtain a novel series of CAIs potentially useful to manage such types of pathologies, as schematically represented in **Figure 1**.

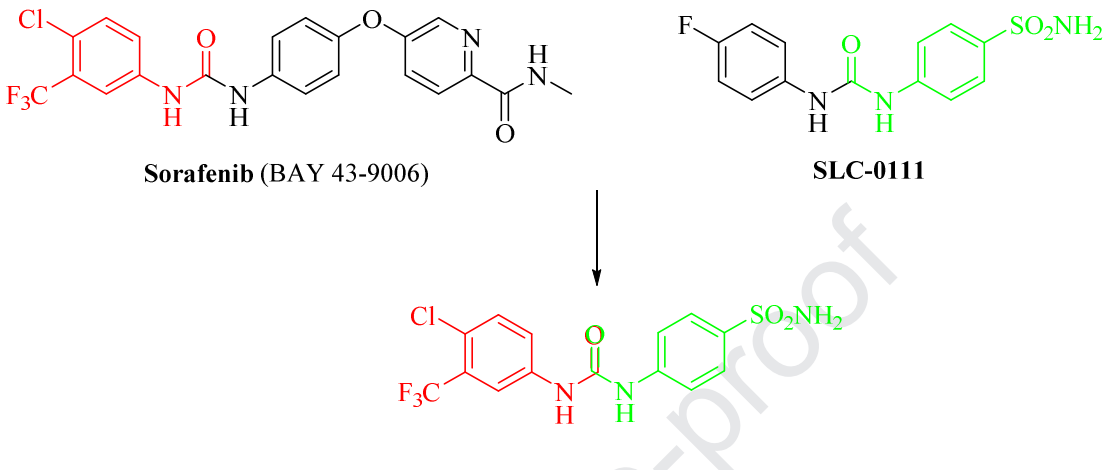
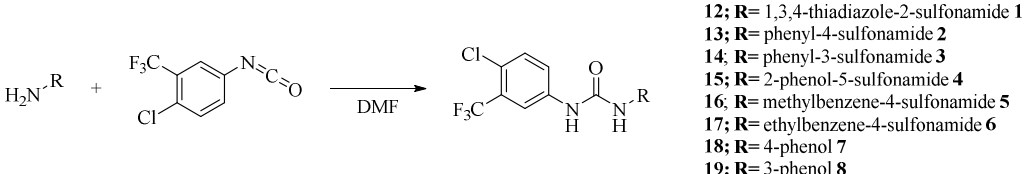


Figure 1. Sorafenib, **SLC-0111** and **13** obtained by our synthetic strategy.

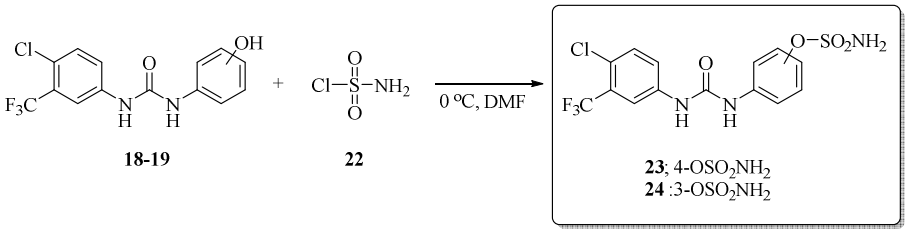
Our design strategy was further extended to include series of compounds bearing the sulfonamides, sulfamates and coumarin moieties as warheads.

As reported in **Schemes 1-3**, compounds **12-17** and **21** were obtained by coupling the amino containing derivatives **1-6** and **10** with the commercially available 4-chloro-3-(trifluoromethyl)phenyl isocyanate **11**. The same reaction was applied to the synthesis of intermediates **18-20.** In particular **18** and **19** were further decorated with the sulfonamide bioisoster sulfamate, in order to afford **23** and **24** (scheme 2). On the other hand **20** was activated by means of its NHS ester **25** which was used to obtain elongated CAIs of the sulfonamide (i.e. **26, 27, 29, 31**) [56] and coumarin type (i.e. **33** and **35**) [36].

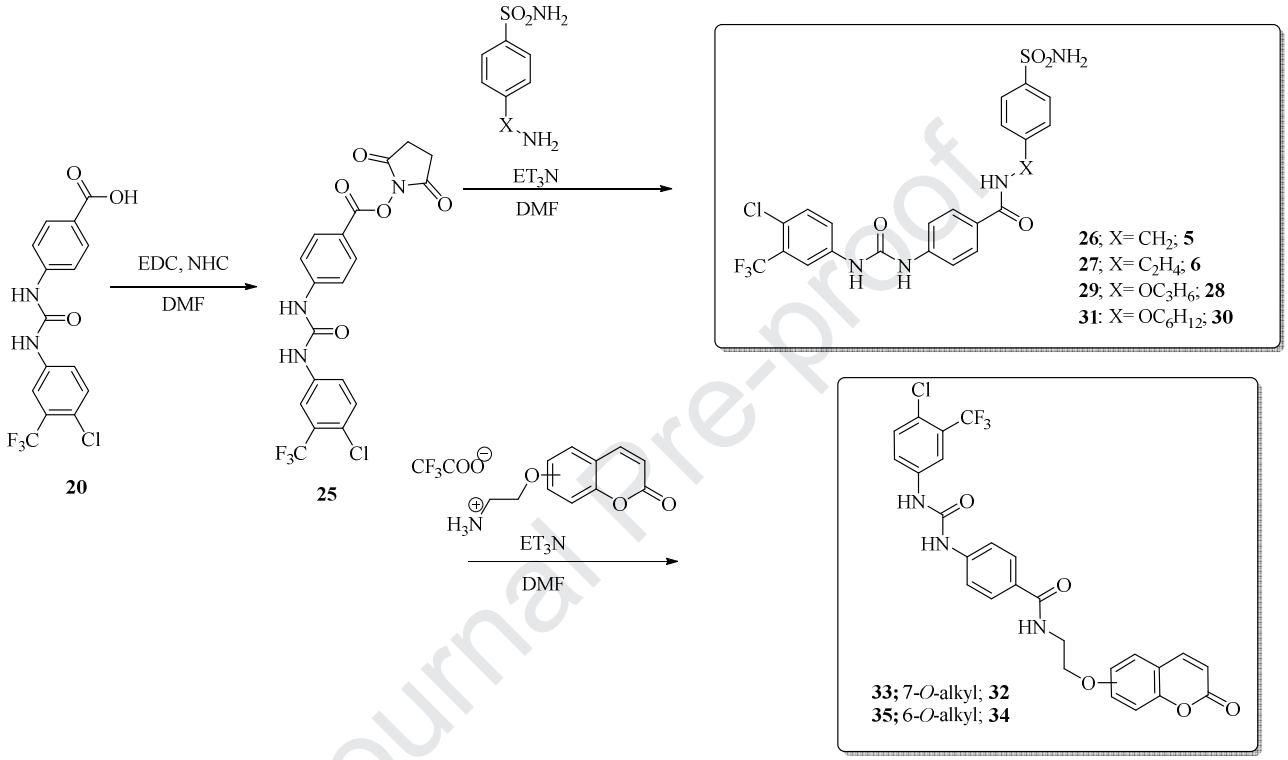


12; R= 1,3,4-thiadiazole-2-sulfonamide **1**
13; R= phenyl-4-sulfonamide **2**
14; **R=** phenyl-3-sulfonamide **3**
15; R= 2-phenol-5-sulfonamide **4**
16; **R=** methylbenzene-4-sulfonamide **5**
17; R= ethylbenzene-4-sulfonamide **6**
18; R= 4-phenol **7**
19; R= 3-phenol **8**
20; R= 4-benzoic acid **9**
21; R= 4-methyl-2-oxo-2*H*-chromen **10**

Scheme 1. General synthesis of ureido derivatives **12-21**



Scheme 2. General synthesis of sulfamates **23-24**



Scheme 3. General synthesis of carbamides **26-27, 29, 31, 33, 35**

All compounds were characterized by means of ^{1}H-, ^{13}C-, ^{19}F-NMR and ESI mass spectroscopy techniques.

2.2. Carbonic Anhydrase Inhibition

The reported compounds having the sulfonamides (i.e. **12-17**, **26, 27**, **29**, **31)**, the sulfamates (i.e. **23, 24**) and the coumarin (i.e. **21, 33**, **35**) moieties were tested *in vitro* for their inhibitory properties against the physiologically relevant hCA isoforms I, II, VII, IX in comparison with sorafenib and the clinically used drug **AAZ,** by means of the stopped-flow carbon dioxide assay [57] (Table 1).

	K_I (nM)*			
Cmp	hCA I	hCA II	hCA VII	hCA IX

12	0.63	0.24	0.38	0.13
13	84.5	1.8	198.5	3.9
14	6422.9	71.5	916.3	1.8
15	4042.3	744.5	893.0	30.2
16	513.6	51.4	801.2	0.7
17	913.4	385.6	911.3	3972
21	>10000	>10000	>10000	1588.1
23	48.7	3.7	5.7	1.3
24	39.3	4.0	3.5	0.8
26	432.1	90.0	210.1	4.2
27	4545.1	2660.2	488.1	3298
29	4601.0	932.9	363.3	188.3
31	6348.3	5761.4	584.9	102.4
33	>10000	>10000	>10000	3412.4
35	>10000	>10000	>10000	2254.4
Sorafenib	>10000	>10000	8136.4	>10000
SLC-0111**	5080	960	8550	45.1
AAZ	250.0	12.1	6.0	25.8

* Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5-10 % of the reported values). ** From reference **[38,58]**.

The inhibition data are showed in Table 1 and the following SARs are below discussed:

(i) The off target cytosolic isoform hCA I was inhibited by all compounds bearing the zinc binding functionality of the sulfonamide and sulfamate type **12-17**, **23-24**, **26-27**, **29**, **31** with a variety of potencies. The derivative of **AAZ 12** acted as sub-nanomolar hCA I inhibitor with a K_I of 0.63 nM. The sulfanilamide derivative **13** and two sulfamates **23-24** were potent hCA I inhibitors with K_Is ranging between 39.3 to 84.5 nM. The remaining sulfonamides **14-17**, **26-27**, **29** and **31** were medium to weak potency inhibitors of hCA I with K_Is in the range of 432.1-6422.9 nM and they acted as weaker inhibitors compare to **AAZ** (K_I of 250 nM). The coumarins **21, 33**, **35** did not inhibit the slow cytosolic isoform

(hCA I) with inhibition constants > 10 µM. This result has been observed before by many coumarin derivatives [24,33–35].

(ii) The second abundant cytosolic isoform hCA II was inhibited by sulfonamides and sulfamates with K_Is in a range of 0.24-5761.4 nM. Compounds **12-13** and **23-24** were potent hCA II inhibitors with K_Is of 0.24-4.0 nM. The **AAZ** derivative **12** was the most potent inhibitor of cytosolic isoform hCA II with a K_I of 0.24 nM, the second most potent inhibitor was the sulfanilamide derivative **13** with a K_I of 1.8 nM. Two sulfamates **23-24** were also acted as potent inhibitor of this isoform with K_Is of 3.7 and 4.0, respectively. The sulfonamide derivatives **14-17** were medium to less potent inhibitors of this isoform with a variety of K_Is between 51.4-744.5 nM. We observed a dramatic change of K_I (39.7 fold) by switching sulfonamide group from para- position **13** to meta- position **14** which had an inhibition constant of 71.5 nM. When a hydroxy group was appended at the para- position towards the sulfonamide of **14** to afford **15** resulted decrease of the inhibition constant (K_I) to 744.5 nM (10.4 fold). Very recently we investigated inhibition properties of ureido-derivatives of **4** which are same chemotype of **15** which were also weak inhibitors of hCA II isoform [47]. The introduction of aliphatic linker between the ring bearing zinc binding group (ZBG) and urea group (**16-17**) was showed weaker hCA II inhibitory properties, with K_Is 51.4 for **16** and 385.6 for **17** (28.6, 214.2 fold, respectively compared to **13**). When a benzamide group was inserted between the phenylureido tail and inhibitor heads to investigate inhibition properties of longer compounds, **26-27**, **29**, **31** were resulted as weak inhibitors of hCA II isoform with K_Is in range of 90-5761 nM. The coumarins **21, 33**, **35** did not inhibit the fast cytosolic isoform (hCA II) with inhibition constants > 10 µM. This result has been observed before by many coumarin derivatives [24,33–35].

(iii) Compounds investigated here also showed a variety of different inhibition constants on the cytosolic isoform hCA VII. The sulfonamides and the sulfamates were inhibited hCA VII with K_Is in the range of 0.38-916.3 nM. Again, the **AAZ** derivative **12** was the most potent inhibitor with a K_I of 0.38 nM. Followed by two potent sulfamates **23-24** with K_Is 5.7, 3.5 nM, respectively. These three compounds were more effective inhibitors compare to **AAZ** (K_I of 6 nM). Remaining compounds were weak inhibitors of hCA VII isoform K_Is in the range of 198.5-916.3 nM. The coumarins did not inhibit hCA VII as well with K_Is > 10 µM.

(iv) The sulfonamides and the sulfamates reported here showed a variety of inhibitory effects on the tumor associated extracellular isoform hCA IX (K_Is spanning from 0.13 to 3972 nM). **AAZ** derivative **12** was a sub-nanomolar inhibitor, and the most potent inhibitor of the series (with a K_I of 0.13 nM) followed by methylbenzenesulfonamide derivative **16** (K_I of

0.7 nM). Ethylbenzene sulfonamide derivative **17** was a weak inhibitor with a K_I of 3972 nM, that showed a dramatic decrease on inhibition effect by extension of linker between the ureido and sulfonamide possessing phenyl ring of inhibitor (5674-fold ineffective compare to **16**). Whereas, direct attachment of ureido group to sulfonamide possessing phenyl ring, **13** (the derivative of **SLC-0111**) showed potent inhibitory effect on hCA IX with a K_I of 3.9 nM (5.6-fold weaker inhibitor compare to **16)**. Manipulation of the sulfonamide group from 4-position to 3-position resulted **14** which enhanced the inhibitory profile with a K_I of 1.8 nM (2.16-fold more potent compare to **13**). Whereas **15**, a derivative of **14** possessing -OH moiety at the para position of sulfonamide showed dramatic decrease of inhibition with a K_I of 30.2 nM (16.78-fold compare to **14**). Two sulfamates **23-24** were excellent inhibitor of this isoform with K_Is of 1.3 and 0.8 nM, respectively. Similar results were reported by previously investigated ureido-possessing sulfamates [54]. The methylene benzenesulfonamide head possessing derivative **26** showed excellent inhibitory effect with a K_I of 4.2 nM whereas ethylbenzenesulfonamide derivative **27** was ineffective inhibitor with a K_I of 3298 nM, again a carbon chain extention shows a dramatic effect on inhibition capacity between **26** and **27**. **29** and **31** were medium potency inhibitors with K_Is of 188.3 and 102.4 nM, respectively. **12-14**, **16**, **23-24**, **26** (K_Is between 0.13-4.2 nM) were revealed more potent inhibitory effects on hCA IX compare to clinically used drug **AAZ** (K_I of 25.8). Coumarins **21, 33**, **35** inhibited hCA IX with low efficiency (K_Is 1.59-3.41 µM), whereas did not inhibit any of cytosolic isoforms (CA I, II, VII), (Table 1).

Overall the inhibition data showed a preferential activity of the sulfonamides **14-16** and **26** and the sulfamates **23-24** on hCA IX, with sub-to low nanomolar K_I values for many such derivatives.

2.3. X-Ray crystallography.

We assessed the binding modes of compounds **17** and **23** within the active site of hCA I by means of X-ray crystallographic experiments. Our intention was to investigate a hCA isoform usually less investigated, since the majority of CA X-ray crystal adducts reported are related to the ubiquitous hCA II or to the tumor associated hCA IX mimic [20]. hCA I is abundant in red blood cells (at 1 µM concentration level), and it might influence the pharmacokinetics of drugs interacting with it or other CA isoforms of pharmacological interest.

The difference |Fo Fc| electron density maps of adducts of hCA I with inhibitors showed well ordered structures within the active site of hCA I and clearly assignable to compounds **17** and **23** bound by means of their sulfonamide and sulfamate group to the active site metal ion (**Figure 3**).

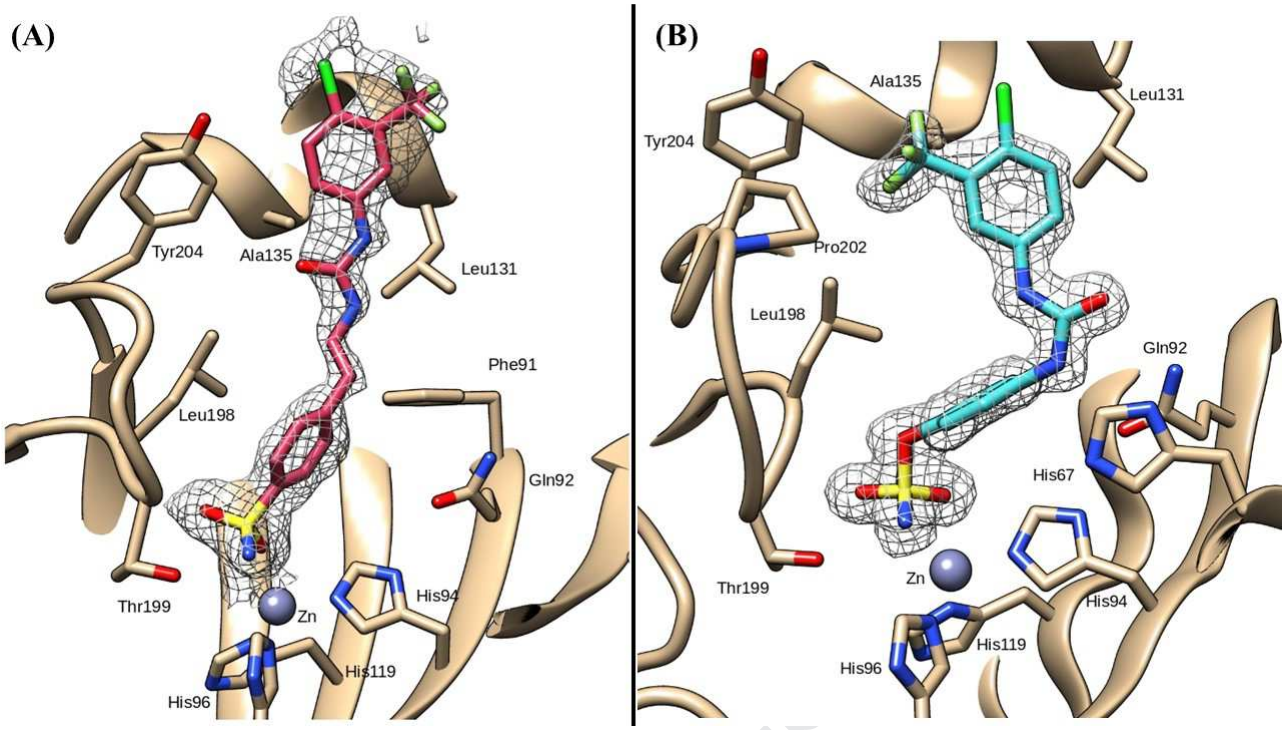


Figure 2. hCA I complexes of compound **17 (A;** PDB accession code 6I0L**)** and **23 (B;** PDB accession code 6I0J**)** at 1.4 and 1.35 Å resolution respectively**.**

A further look into the structural features revealed **17** and **23** coordinated to the Zn(II) ion by means of the deprotonated sulfonamide or sulfamate moieties (SO_2NH^-, OSO_2NH^-), which in turn are also involved in a strong H-bond with the OH of Thr199 residue. One of the sulfonamide/sulfamate oxygen was also involved in a hydrogen bond interaction with the amide nitrogen of Thr199. This coordination pattern, which is typical for all primary sulfonamides/sulfamates investigated so far, is respected also in these cases [20]. The two inhibitors have a similar orientation inside the cavity, but **23** presents a bended conformation. This could be the result of the strong H-bond involvement of the ureido-moiety of **23** with Gln92 residue, further reinforced by a perpendicular π-stacking between its phenyl ring and the imidazole of His94 at the bottom of the active site cavity site (**Figure 3**).

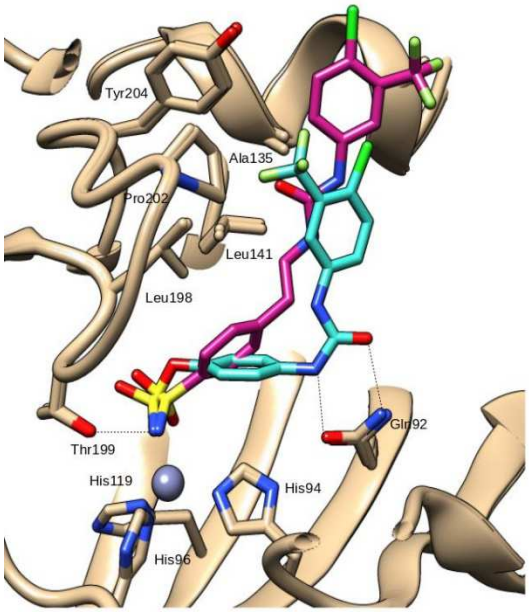


Figure 3. Superimposition of hCA I-**17** (purple) and **23** (cyan) adducts.

The 3-(trifluoromethyl)-4-chlorophenyl tail of **17** is involved in hydrophobic interactions with Ala135 and Tyr204 whereas the same tail moiety of **23** is slightly retained within the enzymatic cleft and makes hydrophobic interactions with Leu131 and Ala135. The additional interactions of **23** when compared to **17**, within the catalytic cavity site (i.e. π stacking and the hydrogen bond interaction with Gln92) are reflected into the inhibition results on the same CA isoform. As reported in **Table 1** and above discussed, compound **17** is 18.8 fold less effective on hCA I when compared to **23** which is bound tighter.

2.4. Effects of Compounds 23 and 24 on Primary Breast Tumor Growth.

The *in vitro* obtained results led us to select less investigated sulfamate type inhibitors **23** and **24** (which are excellent inhibitors of hCA IX with K_Is between 0.8-1.3 nM, Table 1) for their effects on primary breast tumor HBL100, MDA-MB-231 and MCF7 cell lines in normoxic and hypoxic conditions.

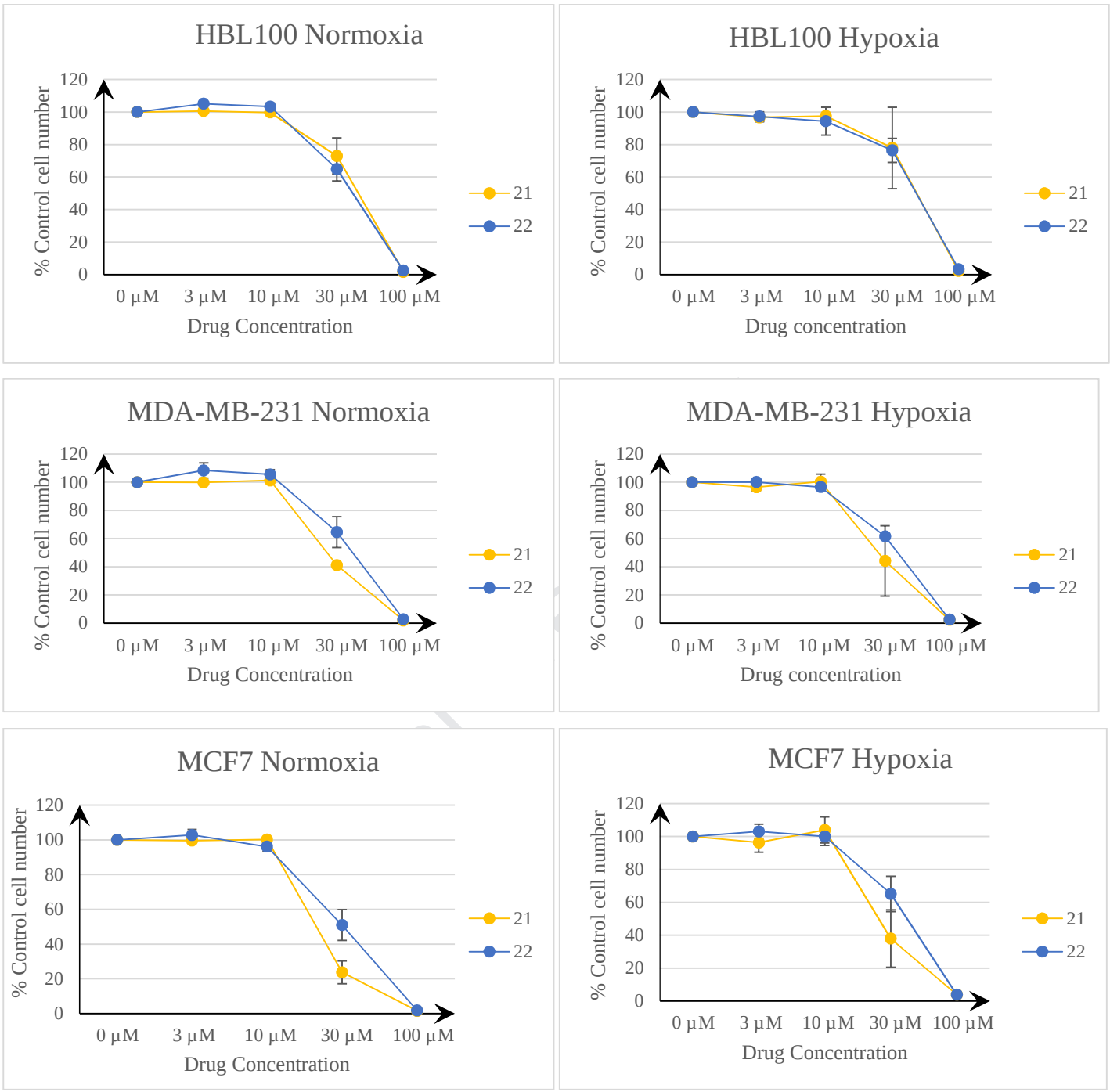


Figure 4. The effect of sulfamate type inhibitors **23** and **24** on proliferation of breast cancer cells in normoxic and hypoxic conditions.

HBL100, MDA-MB-231 and MCF7 breast cancer cells (1 x 10^3)/well, were allowed to adhere for 24h. Cells were either cultured in normoxia (21% O_2), or hypoxia (0.5% O_2), for a further 24 h before the addition of inhibitors at the concentrations indicated above and cultured in these conditions for a further 5 days. Proliferation was assessed using SRB assays. Results shown mean ± SEM (n=6; 6 replicates per experiment).

Figure 4 demonstrates that the sulfamate inhibitors **23** and **24** decreased the proliferation of all three breast cancer cell lines over the time period examined at concentration greater than 10 μM. At 30 μM, both sulfamates reduced proliferation by approximately 50%. This occurred regardless of the oxygenation status of the cells, with similar concentration responses to both inhibitors, in normoxic or hypoxic conditions and in all cell lines studied.

3. Conclusion.

Herein we reported a series of CAIs obtained by merging the multikinase inhibitor Sorafenib with the benzene sulfonamide hCA IX inhibitor **SLC-0111**. All compounds here share the same tail moiety of Sorafenib (i.e. 4-chloro-3-(trifluoromethyl)phenyl ureido group) and were assayed *in vitro* as inhibitors of four hCA isoforms of pharmacological relevance, the cytosolic isoforms hCA I, II and VII, and the tumor associated transmembrane isoform hCA IX. Interestingly the *in vitro* results showed a preferential inhibition of the tumor associated isoform with K_I values spanning between the low to medium nanomolar concentration. We determined the binding modes of compounds **17** and **23** on the hCA I isoform by means of X-ray crystallographic studies of their adducts with hCA I. Moreover, two sulfamates **23** and **24** were investigated for their antiproliferative activities on HBL100, MDA-MB-231 and MCF7 cell lines at normoxic and hypoxic conditions. Both sulfamates were found to decrease proliferation of these breast cancer cell lines at concentrations above 10 μM regardless of oxygenation status.

4. Experimental Section

4.1. Chemistry

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 Avance III 400 MHz spectrometer using DMSO-d_6 as solvent. The chemical shifts are reported in parts per 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. 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. 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/ACN 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}.

4.2. Synthesis of reported compounds

4.2.1. General synthetic procedure **a** to synthesis of urea derivatives **12-21**

A solution of amine **1-10** (0.2g, 1 equiv) was treated with 4-chloro-3-(trifluoromethyl)phenyl isocyanate **11** (1.0 equv) in dry acetonitrile (3-4 mL). The reaction mixture was stirred at rt until the consumption of starting materials (TLC monitoring). Reaction was quenched with H_2O to give a precipitate that was filtered-off and washed with diethyl ether (3 x 5 mL) and dried under vacuum to afford the products **12-21**.

4.2.2. General synthetic procedure **b** to synthesis of sulfamates **23-24**

A solution of phenols **18-19** (0.2 g, 1.0 equiv) was treated with freshly prepared sulfamoylchloride **22** (6.0 equiv, portion-wise) in dry DMA at 0 °C. The reaction mixture was warmed to rt and stirred for 15 min then quenched with slush. The obtained precipitate was filtered-off, washed with DCM (3 x 5 mL) and dried under vacuum to afford desired products **23-24**.

4.2.3 Synthesis of 2,5-dioxopyrrolidin-1-yl4-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido) benzoate *(**25**):* A solution of **20** (1.0 g, 1.0 equiv) was treated with NHS (1.5 equiv) at 0 ˚C in dry DMF (5.0 ml), followed by addition of EDCI (1.5 equiv). The reaction mixture was stirred until the consumption of starting materials (TLC monitoring). The reaction was quenched with H_2O to obtain a precipitate which was filtered-off, washed with H_2O (3 x 5 mL) and dried under vacuum to obtain compound **25**.

4.2.4. General synthetic method **c** for the synthesis of compounds **26-27**, **29**, **31**, **33**, **35**: A solution of amine **5-6**, **28**, **30**, **32** and **34** (50-150 mg, 1.0 equiv) in dry DMF (3-4 mL) was treated with activated ester **25** (1.0 equiv), followed by addition of Et_3N (2.5 equiv). The reaction mixture was stirred until the consumption of the starting materials, then quenched with H_2O (2-3 mL) and acidified by 1M aqueous solution of HCl. The obtained precipitate was filtered-off, washed with diethyl ether (3 x 5 mL) and dried under vacuum to obtain desired products **26-27**, **29, 31**, **33** and **35**.

4.2.5. *5-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)-1,3,4-thiadiazole-2-sulfonamide (**12**):* white solid; yield 65%; silica gel TLC R_f 0.35 (MeOH/DCM 10% *v/v*); mp 242-243 °C; δ_H (400 MHz, DMSO-d_6) 7.72 (1H, d, *J* 8.8), 7.83 (1H, m), 8.13 (1H, s), 8.35 (2H, exchange with D_2O, SO_2NH_2), 9.72 (1H, s, exchange with D_2O, N*H*), 11.88 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 118.9 (q, $^3J_{C\text{-}F}$ 6), 123.6 (1, $^1J_{C\text{-}F}$ 271), 125.0, 125.1, 127.7 (q, $^2J_{C\text{-}F}$ 30), 133.1, 138.8, 153.3, 164.1, 164.5; δ_F (376 MHz, DMSO-d_6) -61.5; *m/z* (ESI negative) 399.9 $[M\text{-}H]^-$.

4.2.6. *4-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)benzenesulfonamide* (***13***): White solid, yield 60%; silica gel TLC R_f 0.55 (MeOH/DCM 20% *v/v*); mp 260-261 °C; δ_H (400 MHz, DMSO-d_6) 7.26 (2H, s, exchange with D_2O, SO_2NH_2), 7.66 (4H, m), 7.77 (2H, d, *J* 8.0), 8.16 (1H, m), 9.28

(1H, s, exchange with D_2O, N*H*), 9.31 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 117.9 (q, $^3J_{C\text{-}F}$6), 118.9, 123.7 (q, $^4J_{C\text{-}F}$ 2), 123.8 (q, $^1J_{C\text{-}F}$ 271.3), 124.2, 127.7 (q, $^2J_{C\text{-}F}$ 30.4), 127.8, 133.0, 138.3, 139.9, 143.3, 153.2; δ_F (376 MHz, DMSO-d_6) -61.4; *m/z* (ESI negative) 392.0 $[M\text{-}H]^-$.

4.2.7. *3-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)benzenesulfonamide (**14**)*: White solid, yield 76%; silica gel TLC R_f 0.45 (MeOH/DCM 10% *v/v*); mp 245-246 ºC; δ_H (400 MHz, DMSO-d_6) 7.40 (2H, s, exchange with D_2O, SO_2NH_2), 7.51 (2H, m), 7.60 (1H, dt, *J* 2.0, 7.6), 7.68 (2H, m), 8.12 (1H, m), 8.17 (1H, d, *J* 2.0), 9.23-9.25 (2H, m, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 116.5, 117.9 (q, $^3J_{C\text{-}F}$ 22), 120.3, 122.5, 123.6 (q, $^4J_{C\text{-}F}$ 2), 123.8 (q, $^1J_{C\text{-}F}$ 271), 124.2, 127.7 (q, $^3J_{C\text{-}F}$ 30), 130.4, 132.9, 140.1, 140.7, 145.7, 153.3; δ_F (376 MHz, DMSO-d_6) -61.5; *m/z* (ESI positive) 392.0 $[M\text{-}H]^-$.

4.2.8. *3-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)-4-hydroxybenzenesulfonamide (**15**)*: White solid, yield 62%; silica gel TLC R_f 0.12 (MeOH/DCM 10% *v/v*); mp 254-255 ºC; δ_H (400 MHz, DMSO-d_6) 6.99 (1H, d, *J* 8.4), 7.16 (2H, s, exchange with D_2O, SO_2NH_2), 7.36 (1H, dd, *J* 2.4, 8.4), 7.60 (1H, dd, *J* 2.0, 8.8), 7.66 (1H, d, *J* 8.8), 8.20 (1H, d, *J* 2.0), 8.48 (1H, s, exchange with D_2O, N*H*), 8.66 (1H, d, *J* 2.4), 9.89 (1H, s, exchange with D_2O, N*H*), 10.96 (1H, s, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 114.7, 117.1, 117.3 (q, $^3J_{C\text{-}F}$ 6), 121.4, 123.2 (q, $^1J_{C\text{-}F}$ 273), 123.4 (q, $^4J_{C\text{-}F}$ 2), 123.6, 127.8 (q, $^2J_{C\text{-}F}$ 30), 128.3, 133.1, 135.9, 140.2, 149.5, 153.1; δ_F (376 MHz, DMSO-d_6) -61.5; *m/z* (ESI negative) 408.0 $[M\text{-}H]^-$.

4.2.9. *4-((3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)methyl)benzenesulfonamide (**16**)*: White solid, yield 20%; mp 158-159 °C; δ_H (400 MHz, DMSO-d_6) 4.40 (2H, d, *J* 5.6), 7.00 (1H, t, *J* 5.6, exchange with D_2O, N*H*), 7.34 (2H, s, exchange with D_2O, SO_2NH_2), 7.50 (2H, d, *J* 8.4), 7.58 (1H, d, *J* 8.8), 7.64 (1H, dd, *J* 2.4, 8.8), 7.81 (2H, d, *J* 8.4), 8.11 (1H, d, *J* 2.4), 9.25 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 43.4, 117.1 (q, $^3J_{C\text{-}F}$ 6), 122.4 (q, $^4J_{C\text{-}F}$ 2), 123.3, 123.8 (q, $^1J_{C\text{-}F}$ 271), 126.7, 127.6 (q, $^2J_{C\text{-}F}$ 30), 128.2, 132.7, 141.0, 143.5, 145.2, 155.9; δ_F (376 MHz, DMSO-d_6) -61.4; *m/z* (ESI positive) 452.0 $[M+HCOO]^-$.

4.2.10. *4-(2-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)ethyl)benzenesulfonamide (**17**)*: White solid, yield 78%; silica gel TLC R_f 0.67 (MeOH/DCM 20% *v/v*); mp 229-230 °C; δ_H (400 MHz, DMSO-d_6) 2.88 (2H, t, *J* 6.8), 3.41 (2H, q, *J* 6.8), 6.39 (1H, t, *J* 6.8, exchange with D_2O, N*H*), 7.33 (2H, s, exchange with D_2O, SO_2NH_2), 7.46 (2H, d, *J* 8.2), 7.58 (2H, m), 7.79 (2H, d, *J* 8.2), 8.10 (1H, m), 9.03 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 36,3, 41.3, 117.1 (q, $^3J_{C\text{-}F}$ 6), 122.4 (q, $^4J_{C\text{-}F}$ 2), 123.2, 123.8 (q, $^1J_{C\text{-}F}$ 271), 126.7, 127.6 (q, $^2J_{C\text{-}F}$ 30), 130.1, 132.7, 141.0, 143.1, 144.7, 155.8; δ_F (376 MHz, DMSO-d_6) -61.4; *m/z* (ESI positive) 422.0 $[M+H]^+$.

4.2.11. *1-(4-chloro-3-(trifluoromethyl)phenyl)-3-(4-hydroxyphenyl)urea (**18**)*: White solid, yield 55%; silica gel TLC R_f 0.33 (MeOH/DCM 10% *v/v*); δ_H (400 MHz, DMSO-d_6) 6.73 (2H, d, *J* 8.8), 7.26 (2H, d, *J* 8.8), 7.64 (2H, m), 8.13 (1H, d, *J* 2.4), 8.51 (1H, exchange with D_2O, N*H*), 9.05 (1H, exchange with D_2O, N*H*), 9.15 (1H, s, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 116.2, 117.5 (q, $^3J_{C\text{-}F}$ 6), 122.0, 122.9 (q, $^4J_{C\text{-}F}$ 2), 123.7, 123.8 (q, $^1J_{C\text{-}F}$ 271), 127.8 (q, $^2J_{C\text{-}F}$ 30), 131.5, 132.8, 140.6, 153.6, 154.0; δ_F (376 MHz, DMSO-d_6) -61.5.

4.2.12. *1-(4-chloro-3-(trifluoromethyl)phenyl)-3-(3-hydroxyphenyl)urea (**19**)*: White solid, yield 60%; silica gel TLC R_f 0.47 (MeOH/DCM 10% *v/v*); δ_H (400 MHz, DMSO-d_6) 6.44 (1H, m), 6.84 (1H, m), 7.08 (2H, m), 7.64 (1H, d, *J* 1.2), 8.15 (1H, t, *J* 1.2), 8.75 (1H, exchange with D_2O, N*H*), 9.10 (1H, exchange with D_2O, N*H*), 9.37 (1H, s, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 106.6, 110.2, 110.4, 117.6 (q, $^3J_{C\text{-}F}$ 6), 123.1 (q, $^4J_{C\text{-}F}$ 2), 123.7 (q, $^1J_{C\text{-}F}$ 271), 123.9, 127.5 (q, $^2J_{C\text{-}F}$ 30), 130.4, 132.9, 140.3, 141.1, 153.2, 158.7; δ_F (376 MHz, DMSO-d_6) -61.5.

4.2.13. *4-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)benzoic acid (**20**)*: White solid, yield 77%; silica gel TLC R_f 0.45 (EtOAc/*n*-hexane 80% *v/v*); δ_H (400 MHz, DMSO-d_6) 7.62 (2H, d, *J* 8.8), 7.68 (2H, m), 7.91 (2H, d, *J* 8.8), 8.15 (1H, m), 9.26 (1H, exchange with D_2O, N*H*), 9.30 (1H, s, exchange with D_2O, N*H*), 12.68 (1H, s, exchange with D_2O, COO*H*); δ_C (100 MHz, DMSO-d_6) 117.9 (q, $^3J_{C\text{-}F}$ 6), 118.6, 123.6 (q, $^4J_{C\text{-}F}$ 2), 123.7 (q, $^1J_{C\text{-}F}$ 271), 124.2, 125.1, 127.7 (q, $^2J_{C\text{-}F}$ 30), 131.5, 133.0, 140.0, 144.5, 153.1, 167.9; δ_F (376 MHz, DMSO-d_6) -61.5.

4.2.14. *1-(4-chloro-3-(trifluoromethyl)phenyl)-3-(4-methyl-2-oxo-2H-chromen-7-yl)urea (**21**)*: White solid; yield 70%; mp>300°C; δ_H (400 MHz, DMSO-d_6) 2.44 (3H, s), 6.27 (1H, s), 7.40 (1H, d, *J* 8.6), 7.64-7.74 (4H, m), 8.13 (1H, m), 9.39 (1H, s, exchange with D_2O, N*H*), 9.42 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 18.8, 105.6, 112.6, 115.0, 115.4, 117.8 (q, $^3J_{C\text{-}F}$ 6), 123.5 (q, $^4J_{C\text{-}F}$ 2), 123.6 (q, $^1J_{C\text{-}F}$ 270), 124.2, 126.7, 127.7 (q, $^2J_{C\text{-}F}$ 30), 132.9, 139.8, 143.8, 153.0, 154.0, 154.8, 160.9; δ_F (376 MHz, DMSO-d_6) -61.5; *m/z* (ESI negative) 395.0 $[M\text{-}H]^-$.

4.2.15. *4-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)phenyl sulfamate (**23**)*: White solid, yield 77%; silica gel TLC R_f 0.70 (MeOH/DCM 10% *v/v*); mp 175-176°C (dec); δ_H (400 MHz, DMSO-d_6) 7.24 (2H, d, *J* 9.0), 7.55 (2H, d, *J* 9.0), 7.66 (2H, m), 7.95 (2H, s, exchange with D_2O, SO_2NH_2), 8.14 (1H, d, *J* 2.2), 9.00 (1H, exchange with D_2O, N*H*), 9.21 (1H, exchange with D_2O, N*H*);δ_C (100 MHz, DMSO-d_6) 117.7 (q, $^3J_{C\text{-}F}$ 6), 120.6, 123.3 (q, $^4J_{C\text{-}F}$ 3), 123.6, 123.8 (q, $^1J_{C\text{-}F}$ 271), 124.0, 127.7 (q, $^2J_{C\text{-}F}$ 30), 132.9, 138.6, 140.2, 145.9, 153.4; *m/z* (ESI negative) 408.0 $[M\text{-}H]^-$.

4.2.16. *3-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)phenyl sulfamate (**24**)*: White solid, yield 65%; silica gel TLC R_f 0.55 (MeOH/DCM 10% *v/v*); mp 190-191°C (dec); δ_H (400 MHz, DMSO-

d$_6$) 6.95 (1H, m), 7.39 (2H, m), 7.57 (1H, m), 7.67 (2H, m), 8.03 (2H, exchange with D_2O, SO_2NH_2), 8.15 (1H, d, *J* 2.0), 9.11 (1H, exchange with D_2O, N*H*), 9.21 (1H, s, exchange with D_2O, N*H*);δ_C (100 MHz, DMSO-*d*$_6$) 113.2, 116.6, 117.4, 117.8 (q, $^3J_{C-F}$ 6), 123.4 (q, $^4J_{C-F}$ 2), 123.7 (q, $^1J_{C-F}$ 271), 124.1, 127.7 (q, $^2J_{C-F}$ 30), 130.7, 132.9, 140.1, 141.4, 151.4, 153.2; δ_F (376 MHz, DMSO-*d*$_6$) -61.5; *m/z* (ESI negative) 408.0 $[M-H]^-$.

4.2.17. *2,5-dioxopyrrolidin-1-yl 4-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)benzoate (**25**):* White solid; yield 92%; δ_H (400 MHz, DMSO-*d*$_6$) 2.92 (4H, s), 7.70 (2H, m), 7.77 (2H, d, *J* 9.0), 7.99 (1H, s), 8.07 (2H, d, *J* 9.0), 8.17 (1H, d, *J* 2.2), 9.45 (1H, exchange with D_2O, N*H*), 9.57 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-*d*$_6$) 26.4, 118.1, 118.1 (q, $^3J_{C-F}$ 6), 119.0, 123.8 (q, $^4J_{C-F}$ 2), 123.7 (q, $^1J_{C-F}$ 271), 124.4, 127.7 (q, $^2J_{C-F}$ 30), 132.4, 132.9, 139.7, 146.8, 152.9, 162.2, 171.3; δ_F (376 MHz, DMSO-*d*$_6$) -61.5.

4.2.18. *4-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)-N-(4-sulfamoylbenzyl)benzamide (**26**)*: White solid; yield 60%; silica gel TLC R_f 0.26 (MeOH/DCM 10% *v/v*); mp195-196 °C; δ_H (400 MHz, DMSO-*d*$_6$) 4.56 (2H, d, *J* 6.0), 7.35 (2H, s, exchange with D_2O, SO_2NH_2), 7.52 (2H, d, *J* 8.3), 7.59 (2H, d, *J* 8.8), 7.68 (2H, m), 7.81 (2H, d, *J* 8.3), 7.90 (2H, d, *J* 8.8), 8.16 (1H, m), 9.04 (1H, t, *J* 6.0, exchange with D_2O, N*H*), 9.18 (1H, s, exchange with D_2O, N*H*), 9.30 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-*d*$_6$) 43.2, 117.8 (q, $^3J_{C-F}$ 6), 118.5, 123.5 (q, $^4J_{C-F}$ 2), 123.7 (q, $^1J_{C-F}$ 270), 124.2, 126.6, 127.7 (q, $^2J_{C-F}$ 30), 128.4, 128.6, 129.2, 133.0, 140.1, 143.1, 143.5, 144.9, 153.2, 166.8; δ_F (376 MHz, DMSO-*d*$_6$) -61.5; *m/z* (ESI negative) 525.1 $[M-H]^-$.

4.2.19. *4-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)-N-(4-sulfamoylphenethyl)benzamide (**27**)*: White solid; yield 62%; silica gel TLC R_f 0.22 (EtOAc/*n*-hexane 80% *v/v*); mp 269-270 °C; δ_H (400 MHz, DMSO-*d*$_6$) 2.96 (2H, t, *J* 7.2), 3.54 (2H, q, *J* 7.2), 7.32 (2H, s, exchange with D_2O, SO_2NH_2), 7.47 (2H, d, *J* 8.4), 7.57 (2H, d, *J* 8.8), 7.67 (2H, m), 7.80 (4H, m), 8.15 (1H, m), 8.49 (1H, t, *J* 7.2, exchange with D_2O, N*H*), 9.14 (1H, s, exchange with D_2O, N*H*), 9.29 (1H, s, exchange with D_2O, N*H*);δ_C (100 MHz, DMSO-*d*$_6$) 35.8, 41.3, 117.8 (q, $^3J_{C-F}$ 6), 118.5, 123.5 (q, $^4J_{C-F}$ 2), 123.7 (q, $^1J_{C-F}$ 270), 124.1, 126.6, 127.7 (q, $^2J_{C-F}$ 30), 129.0, 129.0, 130.1, 133.0, 140.1, 142.8, 143.0, 144.8, 153.2, 166.7; δ_F (376 MHz, DMSO-*d*$_6$) -61.5; *m/z* (ESI negative) 539.1 $[M-H]^-$.

4.2.20. *4-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)-N-(3-(4-sulfamoylphenoxy)propyl)benzamide (**29**):* White solid, yield 64%; silica gel TLC R_f 0.25 (MeOH/DCM 10% *v/v*); mp 231-232 °C; δ_H (400 MHz, DMSO-*d*$_6$) 2.03 (2H, pent, *J* 6.5), 3.45 (2H, q, *J* 6.5), 4.15 (2H, t, *J* 6.5), 7.12 (2H, d, *J* 8.9), 7.23 (2H, s, exchange with D_2O, SO_2NH_2), 7.57 (2H, d, *J* 8.8), 7.67 (2H, m), 7.78 (2H, d, *J* 8.9), 7.84 (2H, d, *J* 8.8), 8.15 (1H, m), 8.46 (1H, t, *J* 6.5, exchange with D_2O, N*H*), 9.14 (1H, s, exchange with D_2O, N*H*), 9.27 (1H, s, exchange with D_2O,

N*H*); δ_C (100 MHz, DMSO-d_6) 29.8, 37.1, 66.8, 115.4, 117.8 (q, $^3J_{C-F}$ 6), 118.4, 123.5 (q, $^4J_{C-F}$ 2), 123.7 (q, $^1J_{C-F}$ 270), 124.1, 127.7 (q, $^2J_{C-F}$ 30), 127.8, 128.6, 129.0, 129.1, 132.9, 137.0, 140.1, 142.8, 153.2, 162.0, 166.8; δ_F (376 MHz, DMSO-d_6) -61.5; *m/z* (ESI negative) 569.1 $[M-H]^-$.

4.2.21. *4-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)-N-(6-(4-sulfamoylphenoxy)hexyl)benzamide **(31)**:* White solid; yield 55%; mp 237-238°C; δ_H (400 MHz, DMSO-d_6) 1.44 (4H, m), 1.58 (2H, pent, *J* 7.0), 1.77 (2H, pent, *J* 7),3.28 (2H, q, *J* 7.0), 4.08 (2H, t, *J* 7.0), 7.10 (2H, d, *J* 8.8), 7.23 (2H, s, exchange with D_2O, SO_2NH_2), 7.57 (2H, d, *J* 8.6), 7.66 (2H, m), 7.78 (2H, d, *J* 8.8), 7.83 (2H, d, *J* 8.6), 8.16 (1H, s), 8.36 (1H, t, *J* 7.0, exchange with D_2O, N*H*), 9.15 (1H, s, exchange with D_2O, N*H*), 9.30 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 26.2, 27.2, 29.4, 30.1, 68.8, 115.3,117.8 (q, $^3J_{C-F}$ 6), 118.4, 123.5 (q, $^4J_{C-F}$ 2), 123.7 (q, $^1J_{C-F}$ 270), 124.1, 127.7 (q, $^2J_{C-F}$ 30), 127.8, 128.6, 129.0, 129.2, 133.0, 136.9, 140.1, 142.7, 153.2, 162.0, 166.5; δ_F (376 MHz, DMSO-d_6) -61.5; *m/z* (ESI negative) 611.2 $[M-H]^-$.

4.2.22. 4-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)-N-(2-((2-oxo-2H-chromen-7-yl)oxy)ethyl) benzamide ***(33)***: White solid; yield 35%; mp 217-218 °C; δ_H (400 MHz, DMSO-d_6) 3.67 (2H, q, *J* 5.6), 4.26 (2H, t, *J* 5.6), 6.32 (1H, d, *J* 9.5), 7.00 (1H, dd, *J* 2.4, 8.6), 7.08 (1H, d, *J* 2.4), 7.57 (2H, d, *J* 8.8), 7.66 (3H, m), 7.86 (2H, d, *J* 8.8), 8.02 (1H, d, *J* 9.5), 8.15 (1H, m), 8.65 (1H, t, *J* 5.6, exchange with D_2O, N*H*), 9.21 (1H, s, exchange with D_2O, N*H*), 9.36 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 39.5, 67.8, 102.2, 113.4, 113.5, 113.7, 117.7 (q, $^3J_{C-F}$ 6), 118.4, 123.5 (q, $^4J_{C-F}$ 2), 123.8 (q, $^1J_{C-F}$ 270), 124.1, 127.6 (q, $^2J_{C-F}$ 30), 127.8, 128.6, 128.6, 129.2, 130.5, 133.0, 140.1, 143.1, 145.3, 153.2, 156.4, 161.3, 162.6, 167.0; δ_F (376 MHz, DMSO-d_6) -61.5; *m/z* (ESI negative) 544.1 $[M-H]^-$.

4.2.23. *4-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)-N-(2-((2-oxo-2H-chromen-6-yl)oxy)ethyl) benzamide **(35)***: White solid, yield 42%; mp 219-220 °C; δ_H (400 MHz, DMSO-d_6) 3.68 (2H, q, *J* 5.6), 4.20 (2H, t, *J* 5.6), 6.53 (1H, d, *J* 9.6), 7.27 (1H, dd, *J* 2.4, 8.8), 7.38 (2H, m), 7.58 (2H, d, *J* 8.8), 7.67 (2H, m), 7.86 (2H, d, *J* 8.8), 8.04 (1H, d, *J* 9.6), 8.15 (1H, m), 8.66 (1H, t, *J* 5.6,exchange with D_2O, N*H*), 9.29 (1H, s, exchange with D_2O, N*H*), 9.45 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 39.7, 67.7, 112.5, 117.5, 117.7 (q, $^3J_{C-F}$ 6), 118.3, 118.4, 120.2, 120.8, 123.4 (q, $^4J_{C-F}$ 2), 124.0, 124.2 (q, $^1J_{C-F}$ 270), 127.6 (q, $^2J_{C-F}$ 30), 128.6, 129.2, 133.0, 140.1, 143.0, 145.0, 148.8,153.2, 155.7, 161.1, 167.0 ; δ_F (376 MHz, DMSO-d_6) -61.5; *m/z* (ESI negative) 544.1 $[M-H]^-$.

4.3. CA inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity [57]. Phenol red (at a concentration of 0.2 mM) has been used as indicator,

working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10-100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5-10% of the reaction have been used for determining the initial velocity. The uncatalyzed 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-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together at room temperature (15 min) prior to assay, in order to allow for the formation of the E-I complex. Data from Table 1 were obtained after 15 minutes incubation of enzyme and inhibitor, as for the sulfonamides reported earlier [9,45,59,60]. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation, as reported earlier [9,45,59,60] and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier [9,45,59,60].

4.4. Crystallization and X-ray data collection

Crystals of hCA I complexed with compounds **17** and **23** were obtained using the sitting drop vapor diffusion method. 2 µl 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, 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 fifteen days. Afterwards hCAI crystals were soaked in 5mM inhibitor solutions for 3 days.

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 ID30A-1 beamline at ESRF (Grenoble, France) with a wavelength of 0.966 Å and a PILATUS3 2M Dectris CCD detector. Data were integrated and scaled using the program XDS [61]. Data processing statistics are shown in Supporting Information Table S1.

4.4.1. Structure determination

The crystal structure of hCA I (PDB accession code 1JV0) without solvent molecules and other heteroatoms was used to obtain initial phases of the structures using Refmac5 [62]. 5% 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 map unambiguously showed the inhibitor molecules. Atomic models for inhibitors were calculated and energy

minimized using the program JLigand 1.0.40 [63]. Refinements proceeded using normal protocols of positional, isotropic atomic displacement parameters alternating with manual building of the models using COOT [64]. Solvent molecules were introduced automatically using the program ARP [65]. The quality of the final models were assessed with COOT and RAMPAGE [66]. 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: 6I0L and 6I0J). Graphical representations were generated with Chimera [67].

4.5. Cell Lines and Culture

The breast cancer cell lines, HBL100, MCF7 and MDA.MB.231 were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in 21% O_2. Hypoxic cells were grown at 0.5% O_2 in a Don Whitley H35 Hypoxystation. All cell lines used were tested and authenticated using STR profiling by Public Health England, Porton Down, Salisbury, UK.

4.5.1. Sulforhodamine B assay

Cells (1 x 10^3/well) were seeded into 96-well flat-bottomed plates and incubated for 48 h before the sulfamates were added using concentrations between 0 – 300 μM. After 5 days treatment, plates were fixed by the addition of 50 μl 25% trichloroacetic acid solution per well for 1 h at 4 °C. Plates were washed with H_2O and air dried before the addition of 50 μl 0.4% sulforhodamine B solution in 1% acetic acid for 30 min. Plates were washed in 1% acetic acid and air dried before the addition of 150 μl 10 mM Tris solution (pH 10.5). After 30 min, the absorbance of each well was measured at 540 nm on a BP900 biohit plate reader.

Disclosure Statement

The authors declare no competing financial interest.

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- CAIs obtained by merging the multikinase inhibitor sorafenib with the benzene sulfonamide hCA IX inhibitor **SLC-0111** are reported
- Sulfamates **23** and **24** decrease proliferation breast cancer cell lines at concentrations above 10 μM.
- The binding modes of compounds **17** and **23** on the hCA I isoform by means of X-ray crystallographic studies are reported.