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Antimetastatic Effect of Sulfamate Carbonic Anhydrase IX Inhibitors in Breast Carcinoma Xenografts

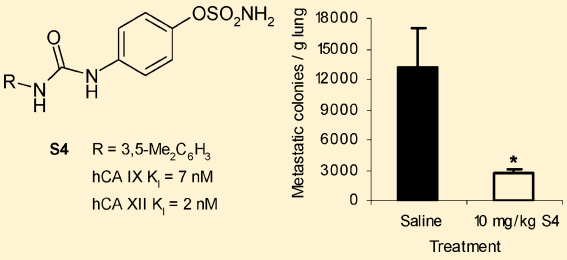
Roben G. Gieling,[†] Muhammad Babur,[†] Lupti Mamnani,[†] Natalie Burrows,[†] Brian A. Telfer,[†] Fabrizio Carta,[‡] Jean-Yves Winum,[§] Andrea Scozzafava,[‡] Claudiu T. Supuran,*[,‡] and Kaye J. Williams*[,†]

[†]Hypoxia and Therapeutics Group, School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, United Kingdom

[‡]Department of Chemistry, University of Florence, Florence, Italy

[§]Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 CNRS-UM1-UM2, Bâtiment de Recherche Max Mousseron, Ecole Nationale Supérieure de Chimie de Montpellier, 8 Rue de l'Ecole Normale, 34296 Montpellier Cedex, France

ABSTRACT: A panel of compounds belonging to the underexposed sulfamate class of carbonic anhydrase (CA, EC 4.2.1.1) inhibitors was generated that displayed high specificity at nanomolar levels for the tumor-associated CA IX/XII isoforms. Three of the specific CA IX/XII inhibitors showed a positive response in in vitro assays for tumor cell migration and spreading. One of them, 4-(3′-(3″,5″-dimethylphenyl)-ureido)phenyl sulfamate (**S4**), was taken forward into the orthotopic MDA-MB-231 (breast carcinoma) model in mice. Treatment with a 10 mg/kg maintenance dosage of **S4** given daily on a "5 days on, 2 days off" regimen reduced metastatic tumor burden in the lung while not affecting primary tumor growth or mouse condition. CA inhibitors of the sulfamate class specifically targeting the tumor-associated isoforms are potential candidates in antimetastatic therapy.



INTRODUCTION

Membrane-associated carbonic anhydrase (CA, EC 4.2.1.1) IX (CA IX) is strongly overexpressed in a broad range of tumor types, and the expression of CA IX negatively correlates with the prognosis of cancer patients.[1−3] Hypoxia and hypoxia-inducible factor (HIF) are important inducers of CA IX expression in solid tumors.[4,5] In normal tissues CA IX expression is much more restricted with abundant expression mainly present in the mucosa of the glandular stomach.[6] On the basis of these features, it is not surprising that CA IX is a pivotal target for anticancer therapy.

CA IX expression is important in metastatic dissemination by increasing the tumor cell survival and invasion in hypoxic environments.[7] CA IX supports tumor cell survival by catalysis, the reversible hydration of carbon dioxide to bicarbonate and protons, maintaining a more alkaline resting intracellular pH (pH_i 7.2−7.4) and thus enhancing tumor cell survival.[8] As a transmembrane enzyme, CA IX activity also supports the flow of protons out of the cell, which contributes to the acidification of the microenvironment in the tumor enhancing invasion.[9] The catalytic properties of CA IX in lowering the extracellular/interstitial pH (pH_e) have experimentally been tested in hypoxic tumors as for LS174Tr (colon adenocarcinoma) and MDA-MB-231 and MCF-7 (breast carcinomas).[9−11]

Carbonic anhydrase inhibitors (CAIs) derived from acetazolamide, ethoxzolamide, and benzenesulfonamides are effective at inhibiting tumor cell growth in vitro and in vivo.[8,12,13] Inhibition of the tumor-associated isoforms CA IX and CA XII explains the antitumor activity of these CAIs.[8] The development of more selective inhibitors of CA IX/XII over the past few years has been shown to be very promising in anticancer therapy. Sulfonamide-based compounds appear very effective in inhibiting specifically the tumor-associated isoform CA IX. Experiments with CA IX selective inhibitors ureidosulfonamides 25 and 104 and glycosyl coumarins 204 and 205 in mice showed that these are very effective as inhibitors of breast cancer (4T1) metastasis.[12,13] The earliest studies on sulfamates showed that they have CA inhibitory properties.[14,15] However, these early compounds were not able to distinguish between tumor-associated and more generally expressed CA isozymes (i.e., CA IX versus CA I, CA II).

Here we report the synthesis and enzyme inhibitory activity of a panel of CA IX selective inhibitors belonging to the sulfamate class, an underexplored class of CAIs. Validation in in vitro studies identified the compounds that were selectively inhibiting cell migration and spreading in the absence of oxygen, a condition commonly found in solid tumors. From this panel a lead compound (**S4**) was taken forward into in vivo studies. Here we show that **S4** was effectively inhibiting the spontaneous metastasis formation in MDA-MB-231 xenografts.

RESULTS AND DISCUSSION

Chemistry. Using the sulfonamides reported by Pacchiano et al. as lead molecules,[13,16] we report here a series of sulfamates incorporating urea/thiourea moieties of types **1A−10A**.

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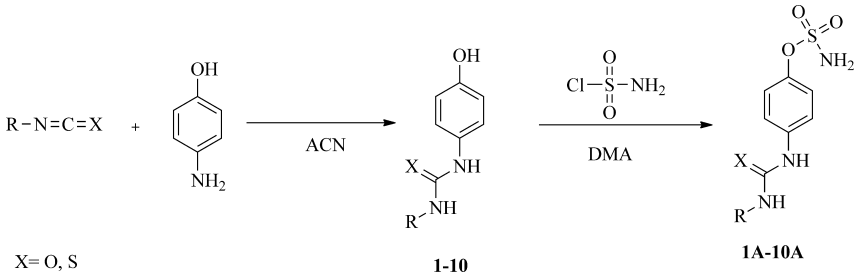
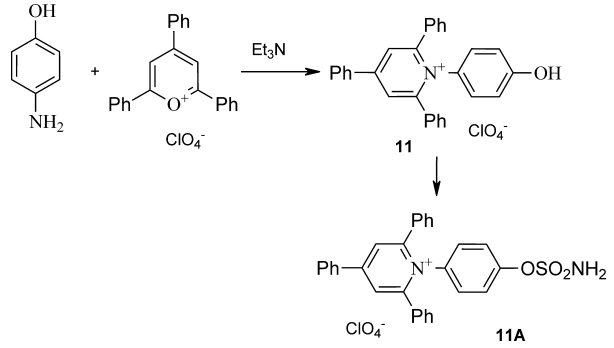
Scheme 1. Preparation of Ureido/Thioureido Sulfamates 1A−10A



The chemistry for obtaining these new compounds is outlined here (Scheme 1). Reaction of 4-aminophenol with isocyanates/isothiocyanates afforded the key intermediates **1−10**, which were sulfamoylated by reaction with sulfamoyl chloride. The corresponding sulfamates **1A−10A** have been thus obtained with high yield (see Experimental Protocols for details).

Alternatively a pyridnium sulfamate derivative (**11A**) was obtained as outlined in Scheme 2, by reaction of 4-aminophenol with 2,4,6-triphenylpyrylium perchlorate followed by sulfamoylation (Scheme 2).

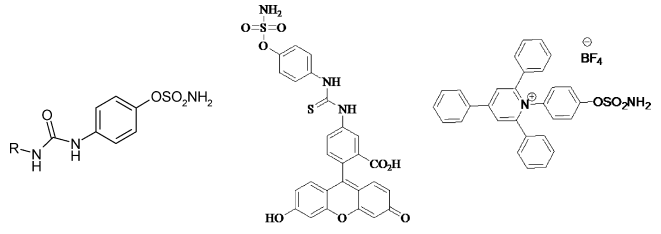
Scheme 2. Preparation of the Pyridinium Sulfamate 11A



In Vitro CA Inhibition Data. The sulfamates **1A−11A** have been assayed as inhibitors of four physiologically relevant CA isoforms, hCA I and II (cytosolic, off-target enzymes) and hCA IX and XII (transmembrane, tumor-associated CAs). All compounds inhibited hCA IX and XII at low nanomolar levels but essentially not the hCA I and II isoforms (Table 1 with individual K_I values).

Antimetastatic Effects of CA IX Inhibitors. *In Vitro.* Carbonic anhydrase IX (CA IX) is important in cell migration and adhesion, and experiments with cells expressing an inactive CA IX variant failed to support cell migration and adhesion.[17] CA IX effects cell migration and adhesion by acidification in hypoxic tumors.[18] We evaluated the ability of a panel of CA IX inhibitors to inhibit migration in MDA-MB-231 cells. Specifically we aimed to identify compounds that inhibited migration only under hypoxic conditions, in line with the physiological response to hypoxia in increased CA IX activity in the MDA-MB-231 cell line. We chose invasive eGFP-MDA-MB-231 cells that express large amounts of CA IX in response to anoxia (Figure 1). With the up-regulation of CA IX expression in eGFP-MDA-MB-231 cells in anoxia, we selected

Table 1. CA Inhibition Data with the Sulfamates 1A−11A



		K_I (nM)[a]				
compd	R	hCA I	hCA II	hCA IX	hCA XII	K_I(CA II)/K_I(CA IX)
1A	4-F-C_6H_4	2800	287	13	9	22.1
2A	4-Cl-C_6H_4	2870	291	12	5	24.3
3A	4-MeO-C_6H_4	2350	413	15	3	27.5
4A	4-PhO-C_6H_4	4360	319	27	8	11.8
5A	C_6F_5	3180	145	6	1	24.2
6A	4-$PhCH_2CH_2$	5460	213	18	7	11.8
7A	4-O_2N-C_6H_4	1230	450	6	4	75
8A	4-Me_2N-C_6H_4	4370	348	9	2	42.7
9A	3,5-$Me_2C_6H_3$	5600	546	7	2	78
10A		6125[b]	569[b]	12[b]	19	47.4
11A		12500	760	14	29	54.3

[a]Mean from three different assays, with errors in the range of ±5−10% of the reported values. [b]From Dubois et al.et al. *Radiother. Oncol.* **2011**, *99*, 424−431.

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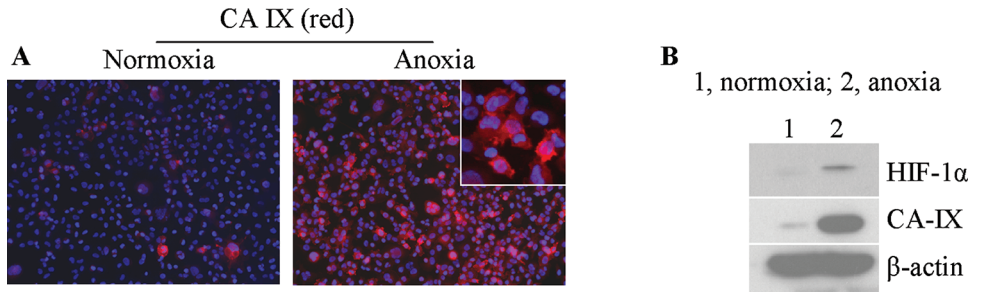

Figure 1. MDA-MB-231 cells express CA IX under anoxic conditions. (A) MDA-MB-231 cells (blue stained nuclei) express CA IX (red stain) after exposure to anoxia for 24 h. (B) Representative blot from three independent experiments with whole cell extracts from MDA-MB-231 cells in anoxia for 24 h probed for CA IX, HIF-1α, and β-actin.

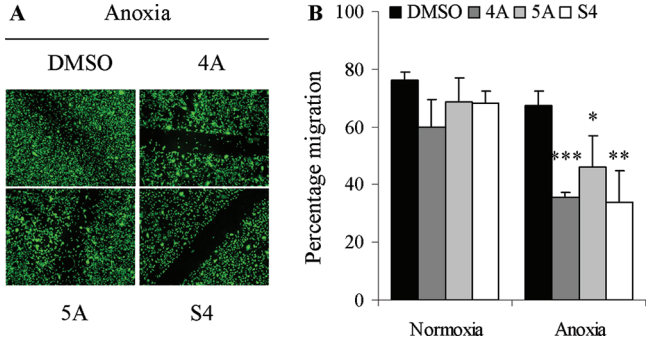

Figure 2. Sulfamate CA IX inhibitors **4A**, **5A**, and **S4** inhibit tumor cell migration. (A) Representative fluorescence images of confluent monolayers of eGFP-MDA-MB-231 cells with scratch wounds after 24 h treatment with DMSO (control) or CA IX inhibitor **4A**, **5A**, or **S4**. (B) Quantification of the difference in scratch-wound width before and after 24 h exposure to CA IX inhibitor **4A**, **5A**, or **S4**: n = 3 per compound. The inhibition for the different compounds in anoxia is as follows: **4A**, 49%; **5A**, 32%; **S4**,50%.

for CA IX inhibitors that inhibited cell migration in anoxia only. A range of phenotypes were observed. **3A**, **8A**, **10A**, and **11A** did not inhibit migration in anoxia, and **1A**, **2A**, and **7A** inhibited cell migration in both normoxia and anoxia. CA IX inhibitors that showed inhibition of eGFP-MDA-MB-231 cell migration in anoxia only were **4A** (49% decreased migration; p = 0.0005, **4A** versus DMSO), **5A** (32%, p = 0.0266), and **S4** (50%; p = 0.0018) (Figure 2). These CA IX inhibitors significantly inhibited eGFP-MDA-MB-231 cell migration when used at 33 μM. As we observed the largest inhibition in cell migration with **S4**, this CA IX inhibitor was tested in more detail in in vitro assays to validate the effectiveness on tumor cell activity. **S4** was inhibiting migration in the 3.3−33 μM range with signs of excessive cytotoxicity in eGFP-MDA-MB-231 cells when used in concentrations of around 100 μM. There was a strong correlation between the concentration of **S4** and inhibition of migration in eGFP-MDA-MB-231 cells: 3.3 μM (5%, p = 0.1663), 10 μM (21%, p = 0.5177), and 33 μM (59%, p = 0.0105) (Figure 3A,B). The effectiveness of **S4** on cell migration was confirmed in WRO and FTC-133 thyroid carcinoma cell lines which are both positive for CA IX as reported by Burrows et al[19] (WRO, 41%; p = 0.0277) (Figure 3C). In two cell lines that do not express CA IX in anoxia, **S4** either had no effect (RT112, bladder carcinoma) or stimulated cell migration (HCT116, colon carcinoma, 44%; p = 0.0381) (Figure 3C). However, **S4** treatment of mice with primary HCT116 tumors had no effect on the number of lung metastasis compared to control mice (data not shown).

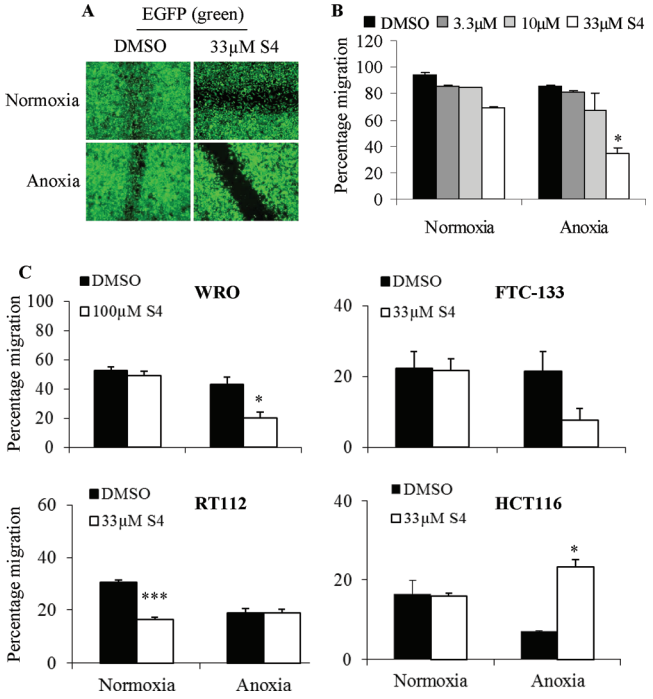

Figure 3. S4 inhibits tumor cell migration in a CA IX-dependent manner. (A) Representative fluorescence images of confluent monolayers of eGFP-MDA-MB-231 cells treated with DMSO or 33 μM **S4**. (B) Quantification of the inhibition by **S4** at 3.3, 10, or 33 μM of migration of eGFP-MDA-MB-231 cells. (C) **S4** inhibits migration of WRO and FTC-133 thyroid carcinoma cells which express CA IX in response to anoxia. **S4** either had no effect (RT112) or increased migration (HCT116) of cell lines which do not express CA IX in response to anoxia. For each cell line, n = 3 per concentration of **S4**.

It is well established that CA IX is enhancing cell migration by acidification of extracellular pH and destabilization of intercellular contacts.[20,21] CA IX is interacting with bicarbonate transporters in lamellipodia which facilitates ion transport and controls pH, allowing cells to move.[17] It is for this reason that we tested the effectiveness of **S4** in inhibiting CA IX dependent cell movement. We validated the cell spreading in vitro after seeding MDA-MB-231 cells (wild type) in dishes on coverslips either uncoated or fibronectin-coated. Cells were fixed in formalin and stained with phalloidin-594, and average cell surface area was measured at the end of each test. Data from a time−response test (0, 15, 30, 45, and 60 min) revealed that at the 60 min point optimal mesenchymal spreading in control cell population was reached (data not shown). The 60 min time

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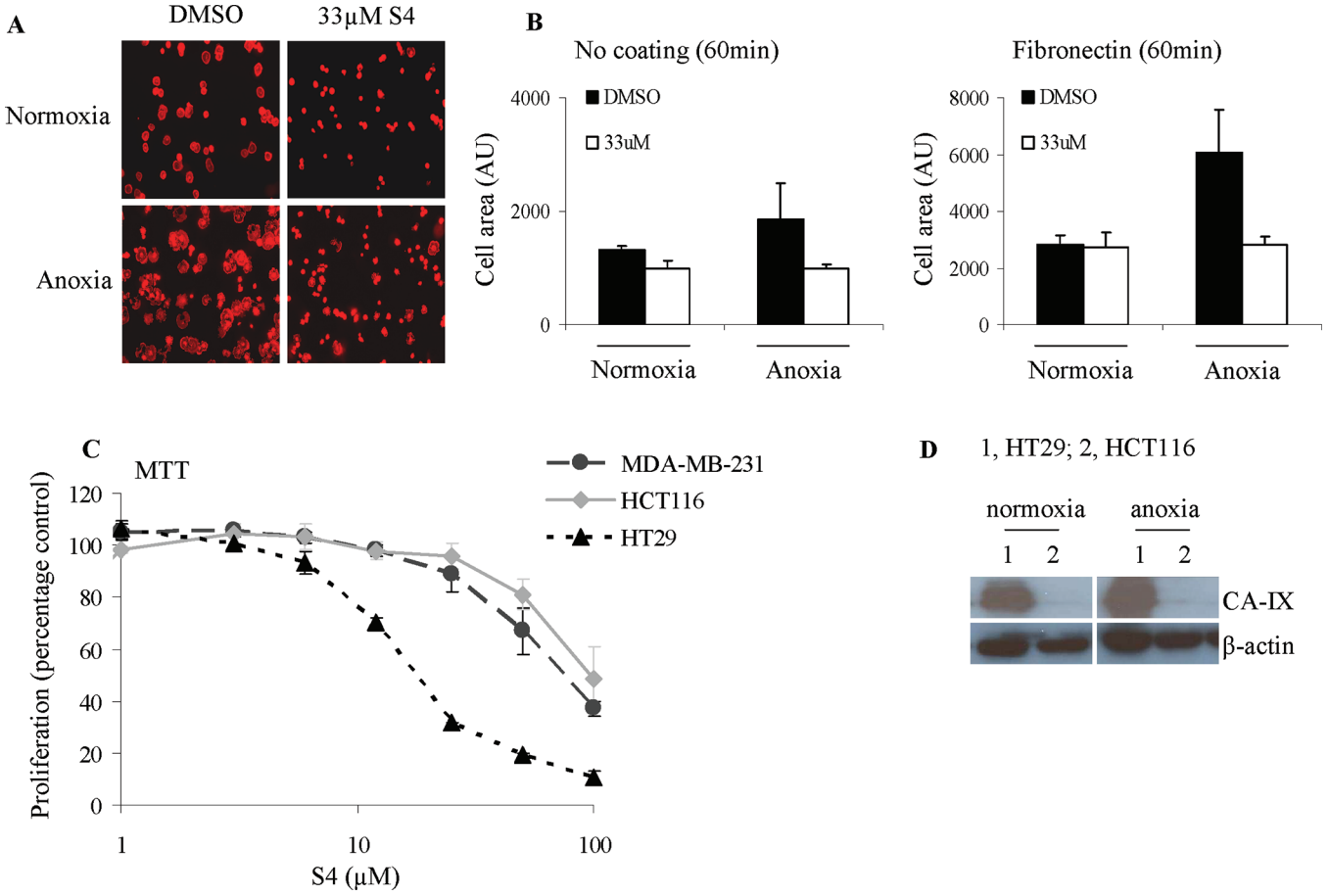

Figure 4. Inhibition of tumor cell spreading and survival by **S4** in anoxia. (A) Representative images of MDA-MB-231 cells grown on fibronectin-coated coverslips stained with phalloidin (red) for each experimental condition. (B) Graphs on cell area measurements of MDA-MB-231 cells grown on glass coverslips coated with or without fibronectin. (C) MTT assay to validate the proliferation of MDA-MB-231, HCT116, and HT29 cells 4 days after incubation with increasing concentrations of **S4** for 24 h.

point was repeated for the different conditions in three independent tests. Representative images of phalloidin-594 stained MDA-MB-231 cells on fibronectin-coated coverslips are shown for each experimental condition (Figure 4A). Cell spreading of MDA-MB-231 cells increased in anoxia compared to that in normoxia (Figure 4B). **S4** treatment delayed the cell spreading of MDA-MB-231 cells in anoxia but essentially not in normoxia. The effects of **S4** on cell spreading were enhanced by a supportive layer of fibronectin (Figure 4B).

Cell proliferation was detected by MTT assay, and the results show that **S4** inhibited the proliferation of MDA-MB-231 cells in a dose-dependent manner (Figure 4C). **S4** also inhibited proliferation of other cells lines, i.e., HCT116 and HT29 cells. The IC_{50} values from the dose−response curves were for MDA-MB-231 (481 μM **S4**), HCT116 (>1000 μM **S4**), and HT29 (20 μM **S4**) (Figure 4C). The level of inhibition of proliferation, by increasing concentrations of **S4**, correlates well with the level of CA IX expression, with MDA-MB-231 expression low, HCT116 expression not detectable, and HT29 expressing high amounts of CA IX in normoxia (Figure 1B, Figure 4D).

In Vivo. Orthotopic xenograft tumors of the human breast cancer line MDA-MB-231 are widely used for the ability to study spontaneous metastases development to the lung.[22] From the panel of CA IX inhibitors we validated in vitro, **S4** appeared to be very effective in inhibiting cell migration and spreading and was taken forward into in vivo studies. The general health of the mice was inspected daily with no signs of intolerance for **S4** at 10 mg/kg (n = 8 per group). The average body weights between vehicle and **S4** treated mice were similar throughout the experiments (Figure 5A). The average growth rate of the primary orthotopic tumors of eGFP-MDA-MB-231 was not affected by a daily dose of **S4** (Figure 5B). We noticed an increase in CA IX activity expressed in MDA-MB-231 tumors near areas of necrosis, indicating that these areas were under hypoxic stress (Figure 5C). The effect of **S4** on metastatic dissemination was based on counting the number of tumor cell colonies growing out of the cell suspension isolated from lung (Figure 5D). Treatment with **S4** significantly reduced the metastatic tumor burden in lungs of mice bearing orthotopic eGFP-MDA-MB-231 tumors (Figure 5D, p = 0.0284).

■ CONCLUSIONS

Sulfamates 4-(3′-(4″-phenoxyphenyl)ureido)phenyl sulfamate (**4A**), 4-(3′-(perfluorophenyl)ureido)phenyl sulfamate (**5A**), and 4-(3′-(3″,5″-dimethylphenyl)ureido)phenyl sulfamate (**9A** = **S4**) inhibited migration and spreading of tumor cells under oxygen-decreased conditions as found in solid tumors. In addition, low dose maintenance therapy with **S4** strongly inhibited the development of MDA-MB-231 metastases in lung with no signs of toxicity. **S4** had no effect on the primary

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A

Body weights (g)		Start	Finish
MDA-MB-231	Saline	25 ± 0.6	25 ± 0.5
	10 mg/kg S4	24 ± 0.4	25 ± 0.4

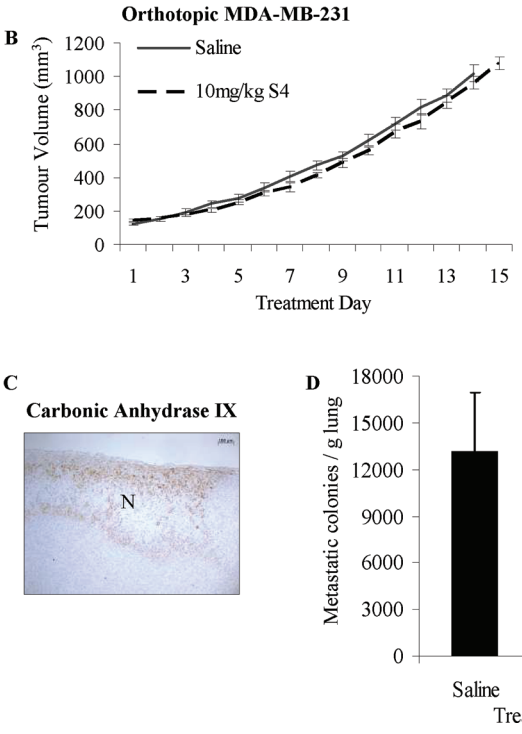


Figure 5. S4 inhibits metastatic tumor burden in MDA-MB-231 model while having no effect on primary tumor growth or mouse condition. (A) Table with the average body weights after treatment with **S4**. (B) Growth curve of the primary tumor volumes from the day of treatment. (C) Representative image of section from primary MDA-MB-231 tumor stained for human carbonic anhydrase IX (CA IX) (N = necrosis). (D) Assessment of the metastatic tumor burden in lung by calculating numbers of colonies per gram of lung established after 10 days in vitro (N = 8 per group).

MDA-MB-231 tumor growth. **S4** is an excellent new candidate as an antimetastatic drug in breast cancer therapy.

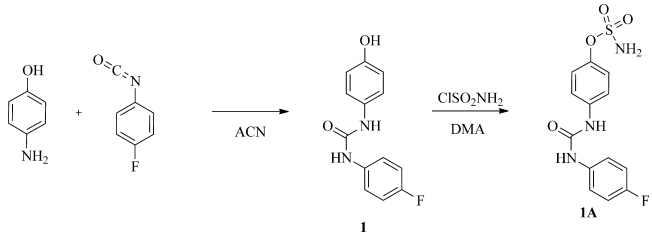
EXPERIMENTAL PROTOCOLS

Chemistry. Anhydrous solvents and all reagents were purchased from Sigma-Aldrich, Alfa Aesar, and TCI. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringe techniques to transfer solutions. NaH, 60% in oil dispersion, was washed with *n*-hexane until a homogeneous white solid was obtained, which was dried and stored under a nitrogen atmosphere prior to use. Infrared (IR) spectra were recorded as KBr plates and are expressed in ν (cm^{-1}). Nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR, DEPT-135, DEPT-90, HSQC, HMBC) spectra were recorded using a Bruker Avance III 400 MHz spectrometer with $CDCl_3$, MeOH-d_4, or DMSO-d_6. Chemical shifts are reported in parts per million (ppm), and the coupling constants (J) are expressed in hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; sept, septet; t, triplet; q, quadruplet; m, multiplet; brs, broad singlet; dd, double of doubles; appt, apparent triplet; appq, apparent quartet. The assignment of exchangeable protons (O*H* and N*H*) was confirmed by the addition of D_2O. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel F-254 plates. Flash chromatography purifications were performed on Merck silica gel 60 (230−400 mesh ASTM) as the stationary phase, and ethyl acetate/*n*-hexane or MeOH/DCM was used as eluent. Melting points (mp) were measured in open capillary tubes and are uncorrected. All compounds were >99% pure as assessed by HPLC.

General Procedure for the Synthesis of Compounds 1−10.[13,16,23] A 1.0 M solution of the appropriate isocyanate (1.0 equiv) was dissolved in dry ACN and treated with 4-aminophenol (1.0 equiv). The reaction mixture was stirred vigorously at room temperature until no starting material was left (TLC monitoring). The solid formed was separated by filtration, washed with H_2O, dried under vacuum, and purified to produce the corresponding ureidophenols **1−10**.

Freshly prepared chlorosulfanilamide (2.0 equiv) was added to a 2.0 M solution of ureidophenols **1−10** in dry DMA under a nitrogen atmosphere until no starting material was left (TLC monitoring). Then the solution was quenched with slush and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H_2O (4 × 20 mL), brine (3 × 20 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to give a sticky residue that was purified to produce the desired sulfamates **1A−10A**.

Synthesis of 4-(3′-(4″-Fluorophenyl)ureido)phenyl Sulfamate (1A).

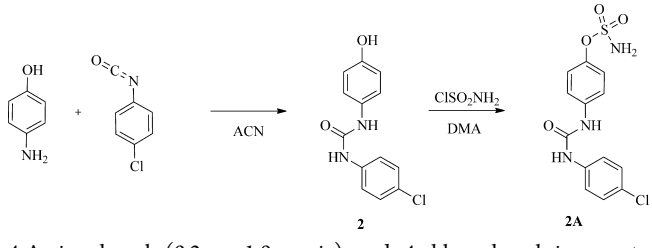


4-Aminophenol (0.2 g, 1.0 equiv) and 4-fluorophenyl isocyanate (1.0 equiv) in dry ACN (10 mL) were treated according to the general procedure previously described. The residue obtained was purified by silica gel column chromatography, eluting with 50% ethyl acetate/*n*-hexane to afford **1** as a white solid which was treated with freshly prepared sulfamyl chloride in dry DMA. The crude residue was purified by silica gel column chromatography, eluting with 50% ethyl acetate/*n*-hexane to produce **1A** as a white solid.

1-(4″-Fluorophenyl)-3′-(4-hydroxyphenyl)urea (**1**): 92% yield; silica gel TLC R_f = 0.24 (ethyl acetate/*n*-hexane, 50% v/v); δ_H (400 MHz, DMSO-d_6) 6.71 (2H, d, J = 8.8, Ar-H), 7.13 (2H, t, J = 8.8, Ar-H), 7.23 (2H, d, J = 8.8, Ar-H), 7.46 (2H, m, Ar-H), 8.33 (1H, s, exchange with D_2O, N*H*), 8.59 (1H, s, exchange with D_2O, N*H*), 9.08 (1H, s, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 158.1 (d, J^1_{C-F} = 236.2, C-4″), 153.8, 153.5, 137.2 (d, J^4_{C-F} = 2.3, C-1″), 132.0, 121.5, 120.7 (d, J^3_{C-F} = 7.6, C-2″), 116.1 (d, J^2_{C-F} = 22.0, C-3″), 116.0; δ_F (376 MHz, DMSO-d_6) −122.0 (1F, s).

4-(3′-(4″-Fluorophenyl)ureido)phenyl sulfamate (**1A**): 90% yield; silica gel TLC R_f = 0.12 (ethyl acetate/*n*-hexane, 50% v/v); δ_H (400 MHz, DMSO-d_6) 7.12 (2H, t, J = 8.8, Ar-H), 7.19 (2H, d, J = 8.8, Ar-H), 7.47 (4H, m, Ar-H), 7.89 (2H, s, exchange with D_2O, SO_2NH_2), 8.71 (2H, brs, exchange with D_2O, N*H*), 8.78 (2H, brs, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 158.0 (d, J^1_{C-F} = 236.6, C-4″), 153.5, 145.4, 139.0, 136.8 (d, J^4_{C-F} = 2.7, C-1″), 123.4, 120.9 (d, J 3_{C-F} = 7.5, C-2″), 120.1, 116.2 (d, J^2_{C-F} = 22.2, C-3″); δ_F (376 MHz, DMSO-d_6) −121.4 (1F, s).

Synthesis of 4-(3′-(4″-Chlorophenyl)ureido)phenyl Sulfamate (2A).



4-Aminophenol (0.2 g, 1.0 equiv) and 4-chlorophenyl isocyanate (1.0 equiv) in dry ACN (10 mL) were treated according to the general procedure previously described. The residue obtained was purified by silica gel column chromatography, eluting with 50% ethyl

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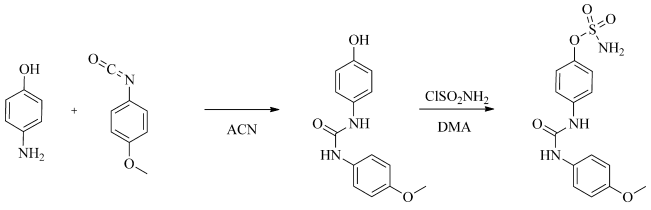
acetate/*n*-hexane to afford **2** as a white solid which was treated with freshly prepared sulfamyl chloride in dry DMA. The crude residue was purified by silica gel column chromatography, eluting with 50% ethyl acetate/*n*-hexane to produce **2A** as a white solid.

1-(4″-Chlorophenyl)-3′-(4-hydroxyphenyl)urea (**2**): 89% yield; silica gel TLC R_f = 0.28 (ethyl acetate/*n*-hexane, 50% v/v); δ_H (400 MHz, DMSO-d_6) 6.71 (2H, d, *J* = 8.8, Ar-H), 7.24 (2H, d, *J* = 8.8, Ar-H), 7.32 (2H, d, *J* = 8.8, Ar-H), 7.49 (2H, d, *J* = 8.8, Ar-H), 8.47 (1H, s, exchange with D_2O, N*H*), 8.81 (1H, s, exchange with D_2O, N*H*), 9.09 (1H, s, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 153.6, 140.0, 131.8, 129.5, 125.9, 123.1, 121.5, 120.4, 116.1.

4-(3′-(4″-Chlorophenyl)ureido)phenyl sulfamate (**2A**): 95% yield; silica gel TLC R_f = 0.17 (ethyl acetate/*n*-hexane, 50% v/v); δ_H (400 MHz, DMSO-d_6) 7.22 (2H, d, *J* = 8.8, Ar-H), 7.37 (2H, d, *J* = 8.8, Ar-H), 7.53 (4H, m, Ar-H), 7.94 (2H, s, exchange with D_2O, SO_2NH_2), 8.86 (2H, brs, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 153.3, 145.6, 139.5, 138.8, 129.5, 126.3, 123.5, 120.7, 120.2.

Synthesis of 4-(3′-(4″-Methoxyphenyl)ureido)phenyl Sulfamate (3A).



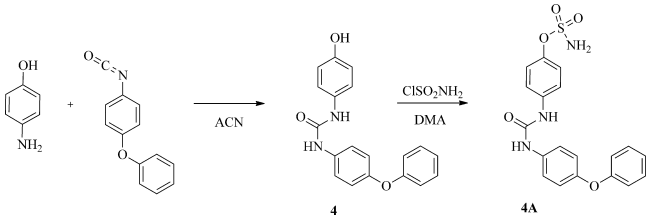
3 3A

4-Aminophenol (0.2 g, 1.0 equiv) and 4-methoxyphenyl isocyanate (1.0 equiv) in dry ACN (10 mL) were treated according to the general procedure previously described. The residue obtained was purified by silica gel column chromatography, eluting with ethyl acetate to produce **3**, a white solid that was treated with freshly prepared sulfamyl chloride in dry DMA. The crude residue was purified by silica gel column chromatography, eluting with ethyl acetate to produce **3A** as a white solid.

1-(4-Hydroxyphenyl)-3′-(4″-methoxyphenyl)urea (**3**): 85% yield; silica gel TLC R_f = 0.76 (ethyl acetate); δ_H (400 MHz, DMSO-d_6) 3.74 (2H, s, CH_3), 6.70 (2H, d, *J* = 8.8, Ar-H), 6.88 (2H, t, *J* = 8.8, Ar-H), 7.23 (2H, d, *J* = 8.8, Ar-H), 7.36 (2H, m, Ar-H), 8.27 (1H, s, exchange with D_2O, N*H*), 8.38 (1H, s, exchange with D_2O, N*H*), 9.04 (1H, s, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 155.2, 153.9, 153.3, 134.0, 132.2, 121.2, 120.7, 116.1, 114.9, 56.1.

4-(3′-(4″-Methoxyphenyl)ureido)phenyl sulfamate (**3A**): 86% yield; silica gel TLC R_f = 0.88 (ethyl acetate); δ_H (400 MHz, DMSO-d_6) 3.75 (3H, s, CH_3), 6.90 (2H, d, *J* = 8.8, Ar-H), 7.21 (2H, d, *J* = 8.8, Ar-H), 7.38 (2H, d, *J* = 8.8, Ar-H), 7.52 (2H, d, *J* = 8.8, Ar-H), 7.92 (2H, s, exchange with D_2O, SO_2NH_2), 8.51 (1H, s, exchange with D_2O, N*H*), 8.73 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 153.6, 139.2, 133.5, 128.2, 123.5, 121.0, 119.9, 114.9, 55.7.

Synthesis of 4-(3′-(4″-Phenoxyphenyl)ureido)phenyl Sulfamate (4A).



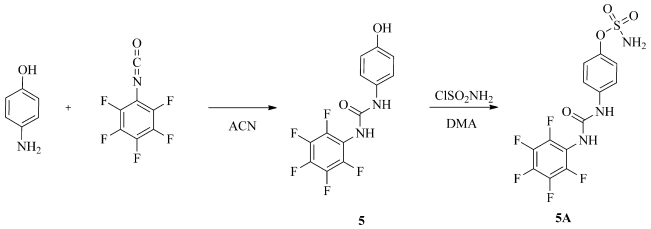
4 4A

4-Aminophenol (0.2 g, 1.0 equiv) and 4-phenoxyphenyl isocyanate (1.0 equiv) in dry ACN (10 mL) were treated according to the general procedure previously described. The residue obtained was purified by silica gel column chromatography, eluting with 50% ethyl acetate/*n*-hexane to produce **4** as a white solid which was treated with freshly prepared sulfamyl chloride in dry DMA. The crude residue was purified by silica gel column chromatography, eluting with 70% v/v ethyl acetate/*n*-hexane to produce **4A** as a white solid.

1-(4-Hydroxyphenyl)-3′-(4″-phenoxyphenyl)urea (**4**): 88% yield; silica gel TLC R_f = 0.59 (ethyl acetate/*n*-hexane, 70% v/v); δ_H (400 MHz, DMSO-d_6) 6.71 (2H, d, *J* = 6.8, Ar-H), 6.98 (4H, m, Ar-H), 7.12 (1H, t, *J* = 6.8, Ar-H), 7.24 (2H, d, *J* = 6.8, Ar-H), 7.41 (2H, t, *J* = 6.8, Ar-H), 7.47 (2H, d, *J* = 6.8, Ar-H), 8.33 (1H, s, exchange with D_2O, N*H*), 8.56 (1H, s, exchange with D_2O, N*H*), 9.08 (1H, s, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 158.6, 153.8, 153.5, 151.3, 137.0, 132.0, 130.8, 123.6, 121.3, 120.7, 120.6, 118.4, 116.1.

4-(3′-(4″-Phenoxyphenyl)ureido)phenyl sulfamate (**4A**): 90% yield; silica gel TLC R_f = 0.65 (ethyl acetate/*n*-hexane, 70% v/v); δ_H (400 MHz, DMSO-d_6) 6.98 (4H, m, Ar-H), 7.12 (1H, t, *J* = 7.2, Ar-H), 7.23 (2H, d, *J* = 7.2, Ar-H), 7.39 (2H, d, *J* = 7.2, Ar-H), 7.52 (4H, m, Ar-H), 7.93 (2H, s, exchange with D_2O, SO_2NH_2), 8.72 (1H, s, exchange with D_2O, N*H*), 8.79 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 158.5, 153.5, 151.7, 145.4, 139.0, 136.5, 130.8, 123.7, 123.5, 120.9, 120.7, 120.0, 118.5.

Synthesis of 4-(3′-(Perfluorophenyl)ureido)phenyl Sulfamate (5A).



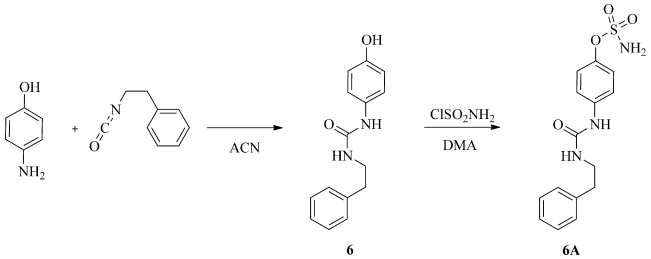
5 5A

4-Aminophenol (0.2 g, 1.0 equiv) and pentafluorophenyl isocyanate (1.0 equiv) in dry ACN (10 mL) were treated according to the general procedure previously described. The obtained residue was purified by silica gel column chromatography, eluting with 50% ethyl acetate/*n*-hexane to produce **5** as a white solid which was treated with freshly prepared sulfamyl chloride in dry DMA. The crude product was purified by silica gel column chromatography, eluting with 50% ethyl acetate/*n*-hexane to produce **5A** as a pale yellow solid.

1-(4-Hydroxyphenyl)-3′-(perfluorophenyl)urea (**5**): 94% yield; silica gel TLC R_f = 0.38 (50% v/v ethyl acetate/*n*-hexane); δ_H (400 MHz, DMSO-d_6) 6.72 (2H, d, *J* = 8.7, Ar-H), 7.24 (2H, d, *J* = 8.7, Ar-H), 8.38 (1H, s, exchange with D_2O, N*H*), 8.73 (1H, s, exchange with D_2O, N*H*), 9.15 (1H, s, exchange with D_2O, O*H*); *m*/*z* (ESI) 341 [M + Na].

4-(3′-(Perfluorophenyl)ureido)phenyl sulfamate (**5A**): 78% yield; silica gel TLC R_f = 0.25 (50% v/v ethyl acetate/*n*-hexane); δ_H (400 MHz, DMSO-d_6) 7.23 (2H, d, *J* = 9.1, Ar-H), 7.53 (2H, d, *J* = 9.1, Ar-H), 7.94 (2H, s, exchange with D_2O, SO_2NH_2), 8.56 (1H, s, exchange with D_2O, N*H*), 9.20 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 153.0, 145.9, 143.9 (dt, J^1_{CF} = 238, J^3_{CF} = 15), 139.4 (dt, J^1_{CF} = 247, J^3_{CF} = 15), 138.2, 137.8 (dt, J^1_{CF} = 247, J^3_{CF} = 15), 123.5, 120.5, 115.0 (t, J^3_{CF} = 15, C-1″),

Synthesis of 4-(3′-Phenethylureido)phenyl Sulfamate (6A).



6 6A

4-Aminophenol (0.2 g, 1.0 equiv) and phenethyl isocyanate (1.0 equiv) in dry ACN (10 mL) were treated according to the general procedure previously described. The residue obtained was purified by silica gel column chromatography, eluting with ethyl acetate to produce **6** as a white solid which was treated with freshly prepared sulfamyl chloride in dry DMA. The crude residue was triturated from DCM to produce **6A** as a white solid.

1-(4-Hydroxyphenyl)-3′-phenethylurea (**6**): 91% yield; silica gel TLC R_f = 0.63 (ethyl acetate); δ_H (400 MHz, DMSO-d_6) 2.76 (2H, t,

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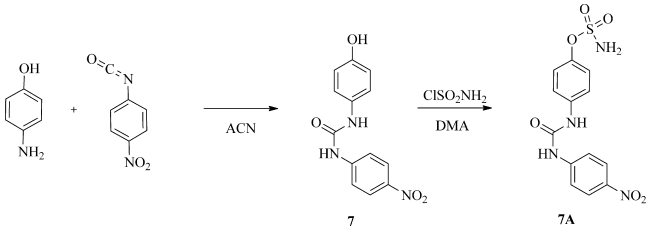
J = 7.2, 5′-H_2), 3.35 (2H, q, J = 7.2, 6′-H_2), 5.98 (1H, t, J = 7.2, exchange with D_2O, 3′-N*H*), 6.65 (2H, d, J = 8.8, Ar-H), 7.16 (2H, d, J = 8.8, Ar-H), 7.28 (5H, m, Ar-H), 8.15 (1H, s, exchange with D_2O, N*H*), 8.95 (1H, s, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 156.4, 152.8, 140.6, 132.9, 129.6, 129.2, 126.9, 120.7, 116.0, 41.6, 36.9.

4-(3′-Phenethylureido)phenyl sulfamate (**6A**): 92% yield; silica gel TLC R_f = 0.75 (ethyl acetate); δ_H (400 MHz, DMSO-d_6) 2.80 (2H, t, J = 6.4, 5′-H_2), 3.38 (2H, q, J = 6.4, 4′-H_2), 6.15 (1H, t, J = 6.4, exchange with D_2O, 3′-N*H*), 7.15 (2H, d, J = 8.8, Ar-H), 7.27 (3H, m, Ar-H), 7.35 (2H, d, J = 8.8, Ar-H), 7.47 (2H, d, J = 8.8, Ar-H), 7.88 (2H, s, exchange with D_2O, SO_2NH_2), 8.62 (1H, s, exchange with D_2O, N*H*), 10.31 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 156.0, 144.9, 140.4, 139.9, 129.6, 129.3, 127.0, 123.4, 119.3, 41.6, 36.7.

Synthesis of 4-(3′-(4″-Nitrophenyl)ureido)phenyl Sulfamate (7A).



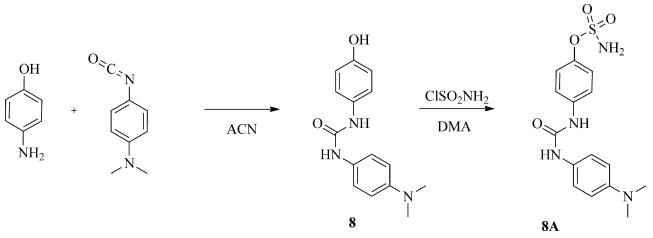
7 7A

4-Aminophenol (0.2 g, 1.0 equiv) and 4-nitrophenyl isocyanate (1.0 equiv) in dry ACN (10 mL) were treated according to the general procedure previously described. The residue obtained was purified by silica gel column chromatography, eluting with 50% ethyl acetate/*n*-hexane to produce **7** as a white solid which was treated with freshly prepared sulfamyl chloride in dry DMA. The residue was filtered-off, washed several times with water, and dried under vacuum to produce **7A** as a yellow solid.

1-(4-Hydroxyphenyl)-3′-(4″-nitrophenyl)urea (**7**): 90% yield; silica gel TLC R_f = 0.18 (ethyl acetate/*n*-hexane, 50% v/v); δ_H (400 MHz, DMSO-d_6) 6.74 (2H, d, J = 8.8, Ar-H), 7.28 (2H, d, J = 8.8, Ar-H), 7.70 (2H, d, J = 8.8, Ar-H), 8.20 (2H, d, J = 8.8, Ar-H), 8.66 (1H, s, exchange with D_2O, O*H*), 9.09 (2H, s, exchange with D_2O, 2 x N*H*); δ_C (100 MHz, DMSO-d_6) 154.0, 153.0, 147.6, 141.7, 131.2, 126.0, 121.9, 118.1, 116.2.

4-(3′-(4″-Nitrophenyl)ureido)phenyl sulfamate (**7A**): 88% yield; silica gel TLC R_f = 0.12 (ethyl acetate/*n*-hexane, 50% v/v); δ_H (400 MHz, DMSO-d_6) 7.26 (2H, d, J = 8.8, Ar-H), 7.56 (2H, d, J = 8.8, Ar-H), 7.75 (2H, d, J = 8.8, Ar-H), 7.96 (2H, s, exchange with D_2O, SO_2NH_2), 8.23 (2H, d, J = 8.8, Ar-H), 9.70 (2H, brs, exchange with D_2O, N*H*), 10.20 (2H, brs, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 153.0, 147.3, 146.0, 141.9, 138.4, 126.0, 123.6, 120.2, 118.1.

Synthesis of 4-(3′-(4″-(Dimethylamino)phenyl)ureido)-phenyl Sulfamate (8A).



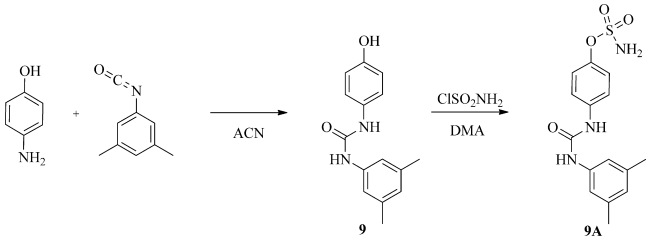
8 8A

4-Aminophenol (0.2 g, 1.0 equiv) and 4-(dimethylamino)phenyl isocyanate (1.0 equiv) in dry ACN (10 mL) were treated according to the general procedure previously described. The residue obtained was purified by silica gel column chromatography, eluting with ethyl acetate to produce **8** as a white solid which was treated with freshly prepared sulfamyl chloride in dry DMA. The crude residue was purified by silica gel column chromatography, eluting with ethyl acetate to produce **8A** as a white solid.

1-(4″-(Dimethylamino)phenyl)-3′-(4-hydroxyphenyl)urea (**8**): 87% yield; silica gel TLC R_f = 0.59 (ethyl acetate); δ_H (400 MHz, DMSO-d_6) 2.89 (6H, s, 2 × CH_3), 6.69 (2H, d, J = 8.8, Ar-H), 6.71 (2H, d, J = 8.8, Ar-H), 7.22 (2H, d, J = 8.8, Ar-H), 7.26 (2H, d, J = 8.8, Ar-H), 8.19 (1H, s, exchange with D_2O, N*H*), 8.21 (1H, s, exchange with D_2O, N*H*), 9.03 (1H, s, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 154.0, 153.2, 147.2, 132.4, 130.8, 121.1, 120.9, 116.1, 114.1, 41.7.

4-(3′-(4″-(Dimethylamino)phenyl)ureido)phenyl sulfamate (**8A**): 88% yield; silica gel TLC R_f = 0.65 (ethyl acetate); δ_H (400 MHz, DMSO-d_6) 2.87 (6H, s, 2 × CH_3), 6.73 (2H, d, J = 8.8, Ar-H), 7.20 (2H, d, J = 8.8, Ar-H), 7.28 (2H, d, J = 8.8, Ar-H), 7.51 (2H, d, J = 8.8, Ar-H), 7.93 (2H, s, exchange with D_2O, SO_2NH_2), 8.30 (2H, brs, exchange with D_2O, N*H*), 8.66 (2H, brs, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 153.2, 147.1, 145.6, 130.2, 126.1, 122.9, 122.1, 117.0, 115.3, 39.6.

Synthesis of 4-(3′-(3″,5″-Dimethylphenyl)ureido)phenyl Sulfamate (9A = S4).



9 9A

4-Aminophenol (0.2 g, 1.0 equiv) and 3,5-dimethylphenylphenyl isocyanate (1.0 equiv) in dry ACN (10 mL) were treated according to the general procedure previously described. The residue was purified by silica gel column chromatography, eluting with 50% ethyl acetate/*n*-hexane to produce **9** as a white solid which was treated with freshly prepared sulfamyl chloride in dry DMA. The crude residue was purified by silica gel column chromatography, eluting with 50% ethyl acetate/*n*-hexane to produce **9A** (=**S4**) as a white solid.

1-(3,5-Dimethylphenyl)-3-(4-hydroxyphenyl)urea (**9**): 89% yield; silica gel TLC R_f = 0.30 (ethyl acetate/*n*-hexane, 50% v/v); δ_H (400 MHz, DMSO-d_6) 2.23 (6H, s, 2 × CH_3), 6.61 (1H, s, 4-H), 6.72 (2H, d, J = 7.2, Ar-H), 7.08 (2H, s, 2 × 2-H), 7.23 (2H, d, J = 7.2, Ar-H), 8.31 (1H, s, exchange with D_2O, N*H*), 8.37 (1H, s, exchange with D_2O, N*H*), 9.09 (1H, s, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 153.7, 153.4, 140.7, 138.5, 132.1, 124.0, 121.2, 116.7, 116.1, 22.0.

4-(3-(3,5-Dimethylphenyl)ureido)phenyl sulfamate (**9A**): 89% yield; silica gel TLC R_f = 0.25 (ethyl acetate/*n*-hexane, 50% v/v); δ_H (400 MHz, DMSO-d_6) 2.27 (6H, s, 2 × CH_3), 6.65 (1H, s, 4-H), 7.11 (2H, s, 2 × 2-H), 7.22 (2H, d, J = 7.2, Ar-H), 7.53 (2H, d, J = 7.2, Ar-H), 7.93 (2H, s, exchange with D_2O, SO_2NH_2), 8.54 (1H, s, exchange with D_2O, N*H*), 8.77 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 153.4, 145.4, 140.3, 139.1, 138.6, 124.4, 123.5, 119.9, 116.9, 22.0.

Synthesis of 2-(3-Hydroxy-6-oxo-6*H*-xanthen-9-yl)-5-(3-(4-(sulfamoyloxy)phenyl)thioureido)benzoic Acid (FC11-489A bis = 10A). The synthesis of **10A** has been reported by Dubois et al.[24]

2-(3-Hydroxy-6-oxo-6*H*-xanthen-9-yl)-5-(3-(4-hydroxyphenyl)-thioureido)benzoic acid (**10**): 57% yield; silica gel TLC R_f = 0.11 (ethyl acetate); δ_H (400 MHz, DMSO-d_6) 6.49 (1H, dd, J = 7.5, 6′-H), 6.65 (4H, brs), 6.70 (2H, s), 6.80 (2H, d, J = 8.8), 7.22 (2H, d, J = 8.8), 8.83 (1H, d, J = 7.5, 5′-H), 8.18 (1H, s, 2′-H), 9.49 (1H, s, exchange with D_2O, O*H*), 9.87 (1H, s, exchange with D_2O, N*H*), 9.95 (1H, s, exchange with D2O, N*H*), 10.18 (1H, s, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 180.9 (*C*═S), 169.6, 160.5, 156.2, 152.9, 148.6, 142.5, 131.9, 131.2, 131.0, 130.1, 127.4, 127.38, 125.0, 118.7, 116.7, 116.6, 116.2, 113.7, 110.7, 103.3, 84.1.

2-(3-Hydroxy-6-oxo-6*H*-xanthen-9-yl)-5-(3-(4-(sulfamoyloxy)-phenyl)thioureido)benzoic acid (**10A**): 56% yield; silica gel TLC R_f = 0.07 (ethyl acetate); δ_H (400 MHz, DMSO-d_6) 7.06–7.38 (8H, m), 7.40 (2H, s), 7.62 (2H, d, J = 8.8), 7.93 (1H, d, J = 7.5), 8.10 (2H, s, exchange with D_2O, SO_2NH_2), 8.26 (4H, s), 8.32 (1H, s), 10.20 (1H, s, exchange with D_2O, N*H*), 10.31 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 180.8 (*C*═S), 169.1, 162.0, 152.5, 151.7, 148.3, 147.8, 145.8, 142.7, 138.9, 138.3, 131.8, 130.5, 127.5, 126.6, 126.1, 125.0, 123.7, 123.3, 119.8, 119.7, 118.8, 117.9, 111.5, 81.6.

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1-(4-Hydroxyphenyl)-2,4,6-triphenylpyridinium **11**: 70% yield; ^{1}H NMR (400 MHz, DMSO) δ ppm 9.89 (s, 1H), 8.62 (s, 2H), 8.33 (d, 2H, J = 4 Hz), 7.67 (m, 3H), 7.40 (m, 10H), 7.19 (d, 2H, J = 8 Hz), 6.48 (d, 2H, J = 8 Hz). MS ESI$^+$: m/z 400.00 (M)$^+$.

1-(4-*O*-Sulfamoylphenyl)-2,4,6-triphenylpyridinium **11A**: 80% yield; ^{1}H NMR (400 MHz, DMSO) δ ppm 8.70 (s, 2H), 8.4 (d, 2H, J = 7 Hz), 8.2 (s, 2H), 7.70 (m, 3H), 7.55 (d, 2H, J = 9 Hz), 7.40 (m, HH), 7.15 (d, 2H, J = 9 Hz), 5.75 (s, 2H). MS ESI$^+$: m/z 479.18 (M)$^+$.

In Vitro CA Inhibition Data. An Applied Photophysics stop-flow instrument was used for assaying the CA catalyzed CO_2 hydration activity.[25] Phenol red (at 0.2 mM) was 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 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 were 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 made thereafter with distilled−deionized water. Inhibitor and enzyme solutions were preincubated together 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 by nonlinear least-squares methods using PRISM 3, as reported earlier,[26] and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in house as reported earlier.[27]

Cell Culture. MDA-MB-231 (breast carcinoma), HCT116 and HT29 (colon carcinomas), WRO and FTC-133, (thyroid carcinomas), and RT112 (bladder carcinoma) cells were maintained in RMPI 1640 medium with the addition of 10% fetal calf serum (Biosera, East Sussex, U.K.) and 2 mM glutamine (Gibco, Invitrogen Ltd., U.K.). MDA-MB-231, WRO, and FTC-133 cells were tagged with enhanced green fluorescence protein (eGFP). The eGFP MDA-MB-231 stable cell line was generated by cloning the coding sequence of eGFP into pEF IRESp[28] and transfecting the construct into MDA-MB-231 cells using the method previously described.[29] Data are plotted as average values ± standard error of the mean (SEM) and analyzed with the Student's t test (GraphPad Software, CA, U.S.). In the graphs, the symbols *, **, and *** denote $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Scratch-Wound Migration Assay. The procedure was performed as previously described.[30] Cells were incubated under normoxia or anoxia for 24 h with or without increasing concentrations of the carbonic anhydrase inhibitors **1A**−**11A**.

Cell Spreading Assay. Cells (2.5×10^4) were seeded in six-well plates on top of glass coverslips uncoated or coated with 10 μg/mL human fibronectin (Millipore, Watford, U.K.). Cells in normoxia or anoxia were incubated in medium containing 33 μM **S4** or DMSO for 0, 15, 30, 45, or 60 min. The cell spreading was analyzed as previously described.[30]

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay. Cells (7500 cells per well for HT29, 1800 cells per well for HCT116 cells, and 2250 cells per well for MDA-MB-231) were seeded in 96-well plates, and 24 h later the medium was replaced with medium containing **S4** (1−100 μM) or DMSO. Medium was replaced again 24 h later with fresh medium, and the cells were allowed to grow for an additional 96 h after which cell proliferation was assessed in MTT assay as previously described.[31]

Spontaneous Metastasis Model. eGFP-MDA-MB-231 cells were prepared at 5×10^7 cells/mL in a 1:1 mix of serum-free RPMI, and Matrigel (BD Biosciences, Erembodegem, Belgium) eGFP-MDA-MB-231 cells were injected into the fourth inguinal area nipple (orthotopic) of female *nu/nu* CBA mice aged 10−12 weeks old (8 mice per group). eGFP-MDA-MB-231 tumors formed 5−7 days after implantation, and the eGFP-MDA-MB-231 tumor volume (length × breadth × height) was measured 3 times a week using callipers. eGFP-MDA-MB-231 (150 mm^3) carrying mice were treated with **S4** (10 mg/kg made up in saline with 4% DMSO) daily intraperitoneally on a "5 days on, 2 days off" dosing regimen. Mice were culled when tumor volume reached 1000 mm^3. Ex vivo lung clonogenic assays were performed as previously described.[30] Experiments were ethically approved and carried out according to the Scientific Procedure Act 1986 and Guidelines for the Welfare and Use of Animals in Cancer 2010 under the Home Office License PPL40/3112 (held by K.J.W.).

Immunohistochemistry. Sections (2 μm) of paraffin-embedded tissue samples were deparaffinized and rehydrated. Heat-induced antigen retrieval with citrate buffer (pH 6.0) was used. Sections were incubated in mouse monoclonal antibody M75 (human specific CA IX antibody, gift from Dr. Pastorek, Bratislava, Slovakia) in the ARK (Animal Research Kit) peroxidase assay according to the manufacturer's instructions (Dako, Ely, U.K.). The protein was visualized with 3,3′-diaminobenzidine (DAB), and sections were counterstained with Mayer's hematoxylin.

Western Blotting. Western blotting was performed as previously described.[19] Antibodies against human CA IX, HIF-1α (BD Transduction Laboratories, Oxford, U.K.), and β-actin (Sigma, Gillingham, U.K.) were used.

AUTHOR INFORMATION

Corresponding Author

*For C.T.S.: phone, +39-055-4573005; e-mail, claudiu.supuran@unifi.it. For K.J.W.: phone, +44 (0) 161 275 2428; e-mail, kaye.williams@manchester.ac.uk.

Notes

The authors declare the following competing financial interest(s): Drs. Supuran and Williams are authors of a patent (Carbonic Anhydrase Inhibitors, PCT/EP2011/052156).

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ABBREVIATIONS USED

aq, aqueous; Ar-H, aromatic proton; bs, broad singlet; °C, temperature in degrees Celsius; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; dec, decomposition; DMA, dimethylacetamide; DMAP, *N*,*N*-dimethyl-4-aminopyridine; DMF, *N*,*N*-dimethylformamide; EDCI·HCl, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; equiv, equivalent; g, gram; h, hour; HOBt, 1-hydroxybenzotriazole; Hz, hertz; IR, infrared; J, coupling constant in Hz; ν_{max}, wavenumber; min, minute; MHz, megahertz; ppm, parts per million; Pyr, pyridine; rt, room temperature; δ_C, ^{13}C chemical shift reported in ppm; δ_F, ^{19}F chemical shift reported in ppm; δ_H, ^{1}H chemical shift reported in ppm; THF, tetrahydrofuran; TLC, thin layer chromatography

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