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Glycosyl Coumarin Carbonic Anhydrase IX and XII Inhibitors Strongly Attenuate the Growth of Primary Breast Tumors

Nadia Touisni,[†] Alfonso Maresca,[‡] Paul C. McDonald,[§] Yuanmei Lou,[§] Andrea Scozzafava,[‡] Shoukat Dedhar,[§,∥] Jean-Yves Winum,*[,†] and Claudiu T. Supuran*[,‡]

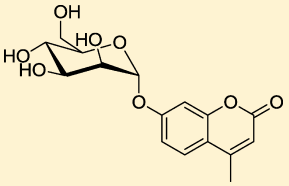
[†]Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 CNRS-UM1-UM2, Bâtiment de Recherche Max Mousseron, Ecole Nationale Supérieure de Chimie de Montpellier, 8 rue de l'Ecole Normale, 34296 Montpellier Cedex, France

[‡]Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino, Florence, Italy

[§]Department of Integrative Oncology, BC Cancer Research Centre and BC Cancer Agency, 675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada

[∥]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada

ABSTRACT: A series of 7-substituted coumarins incorporating various glycosyl moieties were synthesized and investigated for the inhibition of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1). These coumarins were very weak or ineffective as inhibitors of the housekeeping, offtarget isoforms CA I and II, but some of them inhibited tumor-associated CA IX and XII in the low nanomolar range. They also significantly inhibited the growth of primary tumors by the highly aggressive 4T1 syngeneic mouse mammary tumor cells at 30 mg/kg, constituting interesting candidates for the development of conceptually novel anticancer drugs. Because CA IX is overexpressed in hypoxic tumors and exhibits very limited expression in normal tissues, such compounds may be useful for treating cancers not responsive to classic chemo- and radiotherapy.

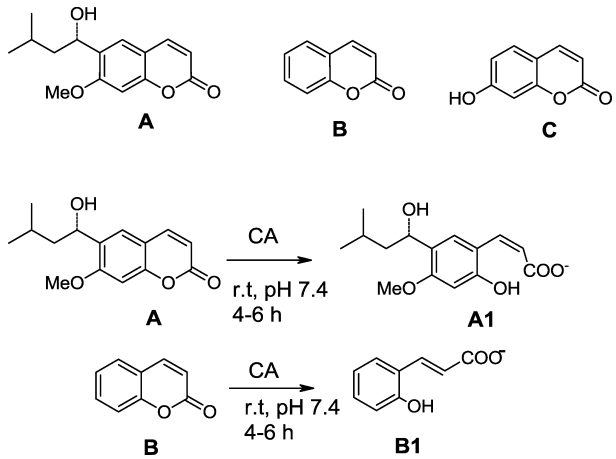


INTRODUCTION

Coumarins were recently discovered to act as inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1).[1,2] Their mechanism of action has also been elucidated, this new class of CA inhibitors (CAIs) being prodrugs and differing from all other known inhibitors of this enzyme.[1,2] Coumarins do not directly interact with the metal ion from the CA active site, which is critical both for catalysis and for inhibition with other classes of compounds, such as sulfonamides, metal-complexing anions, phenols, and polyamines.[1−7] As shown by kinetic and X-ray crystallographic studies, coumarins are mechanism-based inhibitors that undergo hydrolysis under the influence of the zinc hydroxide, nucleophilically active species of the enzyme, with generation of substituted 2-hydroxycinnamic acids.[1,2] For example, the natural product coumarin **A** or the simple nonsubstituted derivative **B** (but also many of their congeners possessing various substitution patterns at the coumarin ring)[2] acts as an effective CAI against many of the mammalian isoforms CA I−CA XV known to date, and the real enzyme inhibitor was demonstrated to be the hydrolyzed coumarins, **A1** and **B1**, formed from the original derivatives **A** and **B**, respectively (Scheme 1).[1,2]

The adducts of CA II with coumarins **A** and **B** have been characterized by X-ray crystallography.[1,2] These studies showed the 2-hydroxycinnamic acids **A1** and **B1** to be bound to the enzyme in an unprecedented way. Indeed, these inhibitors were observed at the rim of the enzyme active site cavity, plugging its entrance and thus blocking the catalytic activity of the enzyme.[1,2] Only very recently were some fullerene derivatives shown to bind in a similar manner to the CAs.[8] Occlusion of the CA active site entrance by hydrolyzed coumarins (i.e., *cis*- or *trans*-2-hydroxycinnamic acids)[1,2] or fullerenes[8] thus constitutes a totally novel mechanism of CA inhibition, which started to be exploited in the design of compounds with various applications.[2,9]

Scheme 1. Formation of 2-Hydroxycinnamic Acids A1 and B1 by the CA-Mediated Hydrolysis of Coumarins A and B



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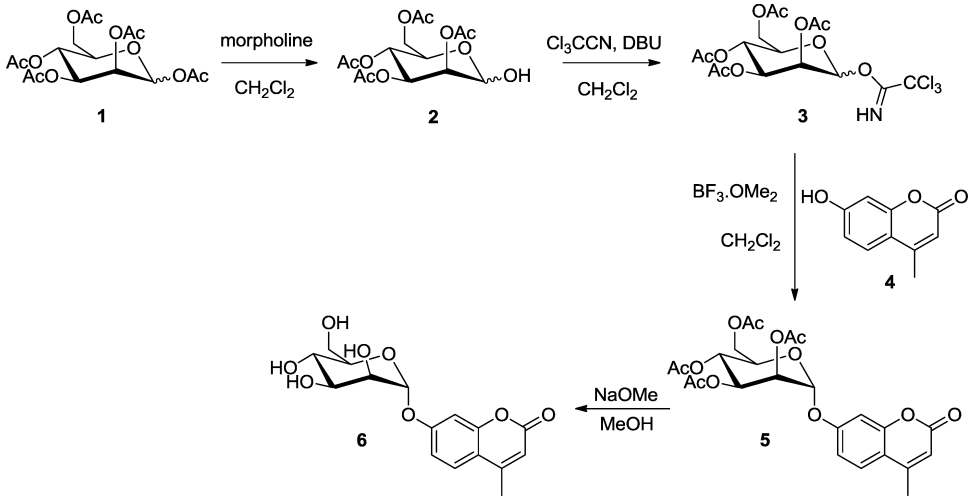
Scheme 2. Example of the Synthesis of One of the Glycosyl Coumarins Investigated in This Study (6), Starting from Umbelliferone [7-hydroxy coumarin (4)] and Pentaacetylated Sugars



CAIs of the sulfonamide type have been used clinically for decades,[3] for various classes of diuretics and systemically acting antiglaucoma agents, but their main drawback is their potent inhibition of CA I and II, ubiquitous enzymes playing important physiological roles.[3,4,10] Thus, critical barriers to the use of CAIs as therapeutic agents are related to the large number of isoforms in humans (i.e., 15 CAs, of which 12 have catalytic activity), their diffuse localization in many tissues and organs, and the lack of isozyme selectivity for many of the presently available inhibitors of the sulfonamide and/or sulfamate type.[3,4,10−13] Thus, there is a stringent need for CAIs with an inhibition profile more selective than those of the classical sulfonamides and their isosteres, and the coumarins represent an interesting such class because of the fact that several isoform-selective CAIs targeting CA isoforms IX, XII, and XIII have been reported recently by our group.[1,2,9]

Recently, we have also demonstrated[14,15] that potent sulfonamide CAIs (with selectivity for the tumor-associated isoforms IX and XII over the cytosolic ones I and II) inhibit the growth of the primary tumors and formation of metastases in several mouse and human breast cancer cell lines. Furthermore, the antimetastatic effects (but not the effects on primary tumors) have also been observed with coumarin-based CAIs.[15]

Here we report the discovery of 7-glycosyl coumarins as potent and CA IX/XII-selective inhibitors. Furthermore, some of these compounds show significant primary tumor growth inhibitory effects in a mouse breast cancer model. Correlated with the previous demonstration[15] of their antimetastatic effects, we claim that members of this class of compounds possess important anticancer activity through a novel mechanism of action that takes advantage of the fact that CA IX and XII are overexpressed only in hypoxic tumors (which do not respond to classical chemo- and radiotherapy) and exhibit very limited expression in normal tissues. Thus, tumor CA IX/XII inhibition may lead to significantly fewer side effects compared to classical anticancer agents in clinical use.

RESULTS AND DISCUSSION

Chemistry. 7-Hydroxy coumarin (umbelliferone) **C** and some of its derivatives were shown recently[9] to be selective, though not very potent (submicromolar), inhibitors of tumor-associated isoforms CA IX and XII, whereas they did not inhibit significantly CA I and II, offtarget, highly abundant CA isoforms.[9] Thus, considering such derivatives as lead molecules and continuing our interest in investigating coumarins as CAIs, we report here the synthesis and inhibition studies of a series of derivatives that incorporate sugar moieties into their molecules.[16]

Glycosidic sulfonamide CAIs were explored previously by our group and Poulsen's group,[17,18] and the presence of sugar moieties in the molecules of such compounds was associated with effective inhibition of physiologically relevant isoforms, among which were also CA IX and XII. High selectivity for the inhibition of the tumor-associated over the cytsolic isoforms has not been, however, observed for most of the sulfonamide glycoconjugates.[17,18] However, interesting features of the sugar-containing CAIs are related to the fact that they show good water solubility, and because of the chemical diversity of sugars, a wide range of different chemotypes could be generated easily.[17,18]

Thus, we applied this "sugar approach" to obtain glycosyl conjugates of coumarins. The synthesis of the glycosyl coumarins investigated here is exemplified in Scheme 2 for the mannose derivative [all other derivatives investigated here were obtained in a similar manner, starting from the corresponding protected sugars (see Experimental Procedures for details)]. We used 4-methyl-7-hydroxy coumarin (4-methylumbelliferone) to prepare the sugar coumarin derivatives because its phenol moiety can be easily derivatized,[9,16] leading to novel chemotypes that have not been investigated previously as CAIs.[1,2] Thus, pentaacetylated mannose **1** was treated with morpholine for the selective deprotection at the 1-OH moiety, and the key intermediate **2** transformed into the trichloroacetimidate **3** by treatment with trichloroacetonitrile. Coupling of intermediate **3** with 4-methylumbelliferone **4** led to the tetraacetylated glycosylumbelliferone **5**, which was deprotected with sodium methoxide in methanol leading to the mannosylumbelliferone **6**. Because of the participation of the acetyl at C-2, the major compounds isolated were assigned as α anomers for mannose and rhamnose and as β anomers for the other sugar derivatives. This is supported by the coupling constants of the anomeric protons in the ^{1}H NMR spectra (see Experimental Procedures for details). The coupling constants reported here were also consistent with literature reports on

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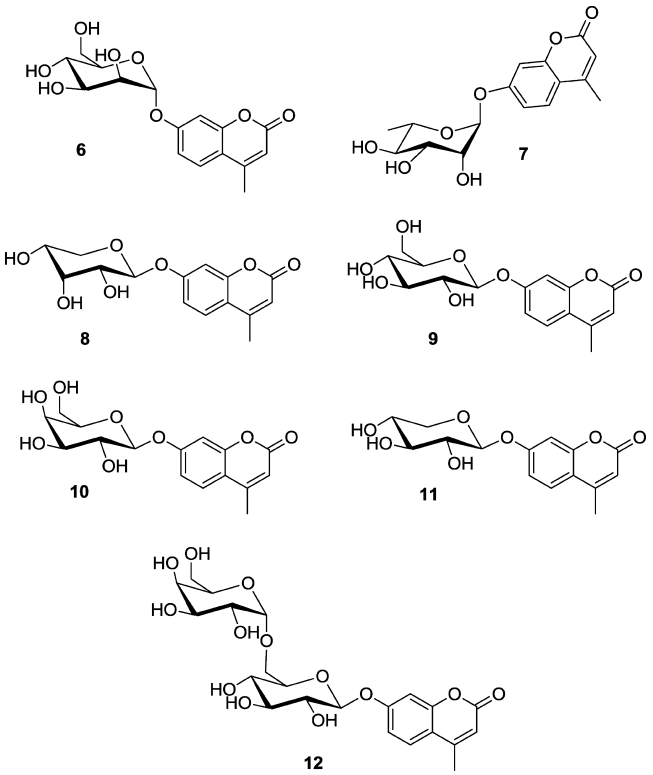
analogous compounds.[16] Both mono- and disaccharides were derivatized by this procedure, leading to the glycosylumbelliferones **6**–**12** (Table 1).

Table 1. Inhibition of hCA I, II, IX, and XII with Coumarins 4, 6–12, and A–C (as standard inhibitors) As Determined by a Stopped-Flow, CO_2 Hydration Assay Method (6 h incubation of enzyme with coumarin)[19]

	K_I[a]			
compd	hCA I[b]	hCA II[b]	hCA IX[c]	hCA XII[c]
4	>100 μM	>100 μM	560 nM	8100 nM
6	>100 μM	>100 μM	9.2 nM	43 nM
7	>100 μM	>100 μM	201 nM	184 nM
8	>100 μM	>100 μM	3200 nM	53 nM
9	1.0 μM	>100 μM	370 nM	54 nM
10	0.59 μM	77 μM	93 nM	8.5 nM
11	3.4 μM	>100 μM	350 nM	105 nM
12	0.88 μM	0.59 μM	820 nM	101 nM
A	0.078 μM	0.059 μM	54500 nM	48600 nM
B	3.1 μM	9.2 μM	>500000 nM	>500000 nM
C	58.4 μM	>100 μM	478 nM	754 nM

[a]Errors in the range of ±5–10% of the reported value, from three different determinations. [b]Full-length, cytosolic isoform. [c]Catalytic domain, recombinant enzyme.



CA Inhibition. Inhibition data for compounds **6**–**12** reported here and **A**–**C** as standards against four CA isozymes, i.e., hCA I, II, IX, and XII, are listed in Table 1.[19] The following structure–activity relationship (SAR) observations can be drawn from the data in Table 1 for these glycosyl coumarin derivatives.

(i) The cytosolic isoform hCA I (h, human enzyme) was weakly inhibited by the parent coumarin **4** and umbelliferone **C**, as well as by the mannose, rhamnose, and ribose coumarin derivatives (**6**–**8**, respectively), with inhibition constants in the range of 58.4–100 μM. The glucose, galactose, xylose, and melobiose coumarin derivatives (**9**–**12**, respectively) as well as the unsubstituted coumarin **B** were medium-potency hCA I inhibitors, with K_I values in the range of 0.59–3.4 μM. Only the natural product coumarin **A** showed potent hCA I inhibition, as reported previously (K_I = 78 nM).[1]

(ii) The ubiquitous, offtarget isoform hCA II was weakly inhibited by most of the coumarins investigated here (i.e., **4**–**11** and **C**), which showed K_I values in the range of 77–100 μM. Among the glycosyl coumarins reported here, only disaccharide derivative **12** showed some level of hCA II inhibition (K_I = 0.59 μM). The unsubstituted coumarin **B** is a weak hCA II inhibitor, but the natural product **A** with a K_I of 59 nM is an effective inhibitor of this isoform.[1]

(iii) The tumor-associated isoform hCA IX was weakly or not at all inhibited by coumarins **A** and **B** (K_I values of 54.5 μM for natural product **A** and >500 μM for **B**), whereas the simple hydroxylated compounds **4** and **C** were medium-potency inhibitors, with K_I values in the range of 478–560 nM. The glycosylated coumarins investigated here also showed very interesting inhibitory properties against this isoform. Thus, the ribose derivative **8** was a weak hCA IX inhibitor (K_I = 3200 nM); rhamnose **7**, glucose **9**, xylose **11**, and melibiose **12** coumarins were medium-potency inhibitors (K_I values in the range of 201–820 nM), whereas mannose **6** and galactose **10** derivatives were potent hCA IX inhibitors, with K_I values of 9.2 nM in the case of **6** and 93 nM in the case of **10**. These inhibition data illustrate very well one of the most salient features of coumarin derivatives as CAIs: for a congeneric series of derivatives like those investigated here, a very large range of activities are observed, from highly potent, low nanomolar inhibitors to ineffective, micromolar (or millimolar) inhibitors. As we have shown previously,[1,2,9] this is primarily due to the mechanism of inhibition with this class of compounds, because the hydroxycinnamic acids formed by the active site-mediated hydrolysis of the coumarin prodrug bind in an active site region that is different in all CA isoforms.[1,2] Furthermore, very minor variations in the original coumarin structure strongly influence the inhibitory power of the compound, because the hydroxycinnamic acids formed after hydrolysis may adopt cis or trans conformations and interact with various amino acid residues at the entrance of the enzyme active site cavity. This type of behavior is rarely or never seen for the sulfonamide CAIs.[3]

(iv) CA XII is also present in many tumor types, being like CA IX a transmembrane isoform, with an extracellular active site, and involved in many physiologic and pathologic processes.[3] It may be observed that coumarins **A** and **B** are ineffective as hCA XII inhibitors, whereas the hydroxylated simple derivatives **4** and **C** are weak inhibitors (K_I values of 754–8100 nM). However, all glycosylated coumarins prepared here showed a considerable inhibition of this isoform, with inhibition constants in the range of 8.5–184 nM. CA XII was thus the isoform most prone to inhibition with glycosyl coumarins among the four CAs investigated here. The best inhibitor was the galactose derivative **10**, but effective inhibition (K_I < 60 nM) was also observed for the mannose **6**, ribose **8**, and glucose **9** derivatives.

Effects of Glycosyl Coumarin CA IX Inhibitors on Primary Breast Tumor Growth. CA IX is selectively expressed in hypoxic tumors, including breast malignancies,[14,15,20–22] and its presence is an independent poor prognostic marker for patients with breast cancer.[15,22,23,24]

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We have shown recently that 4T1 syngeneic mouse mammary tumors are hypoxic and overexpress CA IX and that 4T1 cells induce biologically active CA IX in hypoxia in vitro.[15] Furthermore, we have demonstrated that treatment of 4T1 tumors with certain novel ureido-substituted benzenesulfonamides[14] results in attenuation of primary tumor growth and metastasis in spontaneous[15] and experimental metastatic disease.[14,15] Novel CA IX-selective glycosyl coumarin inhibitors were also found to limit the metastatic burden in a model of experimental metastasis.[15]

To evaluate the effect of pharmacologic inhibition of CA IX activity on primary tumor growth in vivo using the novel glycosyl coumarin compounds described here, we treated mice harboring established 4T1 tumors with the glycosyl coumarin **6**, a compound that demonstrates excellent CA IX and XII inhibitory activities in vitro, and a very good selectivity ratio for inhibiting the tumor-associated CA isoforms compared to the cytosolic CA isoforms, as discussed above. We implanted 4T1 cells into the mammary fat pad of mice and allowed the tumors to establish. Subsequent to the establishment of tumor growth, at a time when the tumors became hypoxic and expressed CA IX,[15] mice were treated with the inhibitor or equal amounts of vehicle by intravenous administration. We observed significant inhibition of tumor growth in mice treated with glycosyl coumarin **6**, compared to vehicle controls (Figure 1). The inhibitor concentrations were well-tolerated, and no significant weight reduction was noted in any of the treated mice for the duration of treatment. It is noteworthy that the inhibition of primary tumor growth observed in this study was somewhat more modest than that seen with the sulfonamide-based inhibitors described previously.[14,15] There are at least two potential explanations for this observation. First, previous studies used daily administration by intraperitoneal injection, while the current set of experiments employed intravenous injection, a clinically relevant but technically challenging mode of administration in mice that resulted in the delivery of substantively fewer doses of the inhibitor. Nonetheless, statistically significant inhibition of tumor growth was achieved in these mice, similar to previous data showing that novel CA IX-selective glycosyl coumarin inhibitors effectively limit the metastatic burden in a model of experimental metastasis.[15] Second, glycosyl coumarin 6 is not cytotoxic and is targeted to CA IX, the expression of which may be variable among tumors and depends on local levels of hypoxia, thereby exerting its effect on a subset of the tumor cell population. Thus, selective CA IX inhibitors such as glycosyl coumarin **6** may be best applied in combination with conventional chemotherapy or radiation to target hypoxic cells typically resistant to these conventional therapies. Taken together, these data demonstrate that one of the glycosyl coumarin inhibitors of CA IX reported here is effective in attenuating primary tumor growth in a model of aggressive, CA IX-positive breast cancer and provide proof of principle for the use of CA IX-selective glycosyl coumarins as anticancer/antimetastatic agents for the treatment of hypoxic tumors.

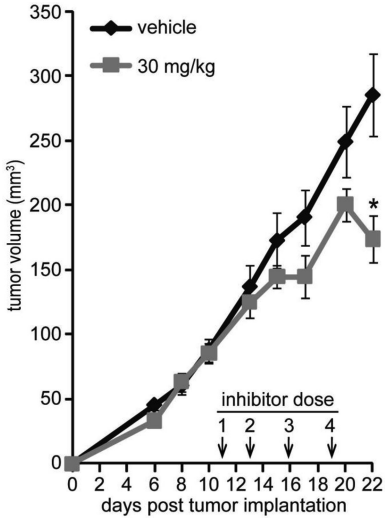

Figure 1. Treatment with the CA IX-selective glycosyl coumarin **6** attenuates the growth of mouse breast tumors. 4T1 murine mammary tumor cells were implanted orthotopically into BALB/c mice, and tumors were established. Treatment was initiated 11 days post-tumor cell injection. Glycosyl coumarin **6** was administered by intravenous injection via a lateral tail vein at a dose of 30 mg/kg. The dosing schedule is given in the graph and involved injections every other day for doses 1 and 2, followed by injections every third day for the remaining doses. Tumor growth was monitored by caliper measurement. The initiation and termination of treatment are indicated. Vehicle-treated animals served as controls. $n = 7$ for each group. Results are expressed as mean tumor volumes ± the standard error of the mean for each group. *$P = 0.01$, compared to vehicle controls.

CONCLUSION

A small series of 7-glycosylated 4-methyl coumarins was prepared and investigated for the inhibition of four physiologically relevant CA isoforms, CA I and II (cytosolic, offtarget isoforms) and CA IX and XII (transmembrane, tumor-associated enzymes). These compounds were generally ineffective or weak inhibitors of CA I and II (activities in the micro- to millimolar range), but many of them were effective, nanomolar CA IX and XII inhibitors. One of these compounds, 7-mannosyl-4-methylumbelliferone, significantly inhibited the growth of primary tumors by the highly aggressive 4T1 syngeneic mouse mammary tumor cells at a concentration similar to that used previously in a setting of experimental metastasis.[15] Such compounds thus constitute interesting candidates for the development of conceptually novel anticancer drugs. In conjunction with the previous demonstration[15] of the antimetastatic effects of some of these coumarins, we claim that members of this class of compounds possess important anticancer activity through a novel mechanism of action that takes advantage of the fact that CA IX and XII are overexpressed in hypoxic tumors (which do not respond to classical chemo- and radiotherapy) and are present in limited amounts in normal tissues. Thus, tumor CA IX and XII inhibition may lead to significantly fewer side effects compared to classical anticancer agents in clinical use or the monoclonal antibodies (mAbs) targeting CA IX, which are in advanced (Phase III) clinical trials.[3a] Indeed, this mAb (Girentuximab and its radio-iodinated variant) seems to be highly effective in treating patients with metastatic renal carcinomas as well as other solid tumors.[3a]

EXPERIMENTAL PROCEDURES

Chemistry. Peracetylated sugar derivatives, umbelliferone, solvents, and other reagents were of the highest available purity (Sigma Aldrich). CA isoforms were recombinant ones prepared in house as reported previously.[1,2] All compounds reported here were >98% pure,

as determined by high-performance liquid chromatography and elemental analyses.

Synthesis of 2,3,4,6-Tetra-O-acetyl-D-mannopyranose (2). D-Mannose pentaacetate (**1**) (10.25×10^{-3} mol) was dissolved in dry CH_2Cl_2 (40 mL). Morpholine (41×10^{-3} mol) was then added, and the mixture was stirred under an N_2 atmosphere at room temperature overnight. The mixture was then washed twice with 40 mL of 1 N HCl and 3 × 20 mL of water, dried ($MgSO_4$), and concentrated under vacuum to give the 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose (**2**).

Synthesis of 2,3,4,6-Tetra-O-acetyl-D-mannopyranosyl Trichloroacetimidate (3). The 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose (**2**) (4.31×10^{-3} mol) was dissolved in dry CH_2Cl_2 (38 mL). Trichloroacetonitrile (43.1×10^{-3} mol) was added, and the mixture was stirred under an N_2 atmosphere at 0 °C for 1 h. Then diazabicyclo[5.4.0]undec-7-ene (DBU) (0.86×10^{-3} mol) was added, and the mixture was stirred under an N_2 atmosphere at 0 °C for 30 min and concentrated under vacuum. The crude 2,3,4,6-tetra-*O*-acetyl-D-mannopyranosyl trichloroacetimidate (**3**) was used without further purification in the next step.

Synthesis of 4-Methylumbellifer-7-yl-2,3,4,6-tetra-O-acetyl-α-D-mannopyranose (5). The crude 2,3,4,6-tetra-*O*-acetyl-D-mannopyranosyl trichloroacetimidate (**3**) (4.31×10^{-3} mol) was dissolved in dry CH_2Cl_2 (38 mL). 7-Hydroxy-4-methyl coumarin (**4**) (4.31×10^{-3} mol) and boron trifluoride metherate ($BF_3{\cdot}Me_2O$) (0.86×10^{-3} mol) were then added, and the mixture was stirred under an N_2 atmosphere at room temperature overnight. Twenty milliliters of CH_2Cl_2 was further added, and the solution was washed with water, dried over anhydrous $MgSO_4$, and concentrated under vacuum. The crude product (**5**) was then purified by crystallization from MeOH or by silica gel column chromatography [5/5 (v/v) EP/AcOEt] to give the expected compound in 60% yield.

Synthesis of 4-Methylumbellifer-7-yl-α-D-mannopyranoside (6). The 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl coumarin (**5**) (0.59×10^{-3} mol) was added to a solution of MeONa (0.88×10^{-3} mol) in dry MeOH (5 mL). The mixture was stirred at room temperature for 30 min. The product (**6**) was then purified by crystallization or by silica gel column chromatography [5/5 (v/v) EP/AcOEt]. The reaction is quantitative.

4-Methylumbellifer-7-yl-α-D-mannopyranoside (6): 51% overall yield; R_f = 0.24 (9/1 CH_2Cl_2/MeOH); mp 132−134 °C; ^{1}H NMR (400.13 MHz, DMSO-d_6) δ 2.4 (d, 3H, J = 0.8 Hz), 3.33 (m, 1H), 3.47 (m, 1H), 3.51 (t, 1H, J = 9.4 Hz), 3.57 (m, 1H), 3.69 (dd, 1H, J = 9.2 Hz), 3.86 (d, 1H, J = 1.2 Hz), 5.53 (d, 1H, J = 1.6 Hz), 6.24 (d, 1H, J = 1.2 Hz), 7.09 (d, 1H, J = 2.4 Hz), 7.11 (dd, 1H, J = 8.8, 2.4 Hz), 7.70 (d, 1H, J = 8.8 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 18.82, 61, 66.95, 70.43, 71, 76.06, 99.48, 104.31, 112.38, 114.38, 114.79, 127.14, 160.80, 159.83, 155.02, 154.05; MS (ESI$^+$) m/z 339.24 [M + H]$^+$, 361.29 [M + Na]$^+$, 699.37 [2M + Na]$^+$. Anal. Calcd for $C_{16}H_{18}O_8$: C, 56.80; H, 5.36. Found: C, 56.84; H, 5.33.

4-Methylumbellifer-7-yl-α-L-rhamnopyranoside (7): 58% overall yield; R_f = 0.4 (9/1 CH_2Cl_2/MeOH); mp 207−209 °C; ^{1}H NMR (400.13 MHz, $CDCl_3$) δ 1.14 (d, 3H, J = 6.4 Hz), 2.35 (d, 1H, J = 1.2 Hz), 3.86 (q, 1H, J = 5.3 Hz), 5.10 (t, 1H, J = 10 Hz), 5.42 (d, 1H, J = 3.6 Hz), 5.44 (t, 1H, J = 2.3 Hz, H_2), 5.45 (t, 1H, J = 2.2 Hz), 6.13 (d, 1H, J = 0.8 Hz), 7.02 (d, 1H, J = 2.4 Hz), 7.06 (dd, 1H, J = 8.8, 2.4 Hz), 7.47 (d, 1H, J = 8.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.05, 21.11, 69, 69.27, 69.51, 70.1, 95, 104.26, 113.23, 113.61, 125, 152.52, 155.10, 158.61, 170.15, 170.31; MS (ESI$^+$) m/z 345.31 [M + Na]$^+$, 667.39 [2M + Na]$^+$. Anal. Calcd for $C_{16}H_{18}O_7$: C, 59.62; H, 5.63. Found: C, 59.58; H, 5.65.

4-Methylumbellifer-7-yl-β-D-ribopyranoside (8): 60% overall yield; R_f = 0.45 (8/2 AcOEt/MeOH); ^{1}H NMR (400.13 MHz, DMSO-d_6) δ 2.38 (d, 3H, J = 1.2 Hz), 3.91 (m, 1H), 4.03 (m, 1H), 4.70 (t, 1H, J = 5.4 Hz), 5.07 (d, 1H, J = 6 Hz), 5.61 (d, 1H, J = 2 Hz), 6.23 (s, 1H), 6.77 (d, 1H, J = 2 Hz), 6.96 (dd, 1H, J = 8.4, 2 Hz), 7.68 (d, 1H, J = 8.4 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 18.09, 62.518, 70.40, 74.46, 84.81, 103.27, 105.05, 111.55, 113.36, 113.84, 126.46, 153.32, 155.3, 159.32, 160.05; MS (ESI$^+$) m/z 331.26 [M + Na]$^+$, 639.25 [2M + Na]$^+$. Anal. Calcd for $C_{15}H_{16}O_7$: C, 58.44; H, 5.23. Found: C, 58.40; H, 5.25.

4-Methylumbellifer-7-yl-β-D-glucopyranoside (9): 55% overall yield; R_f = 0.39 (8/2 CH_2Cl_2/MeOH); mp 210−212 °C; ^{1}H NMR (400.13 MHz, DMSO-d_6) δ 2.41 (s, 3H), 3.17 (dd, 1H, J = 14.2, 8.8 Hz), 3.29 (dd, 2H, J = 11.9, 7.4 Hz), 3.40−3.53 (m, 2H), 5.08 (d, 1H, J = 5.3 Hz), 6.25 (s, 1H), 7.03 (d, 1H, J = 2.4 Hz), 7.05 (dd, 1H, J = 9.2, 2.4 Hz), 7.71 (d, 1H, J = 9.2 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 18.35, 60.86, 69.85, 73.35, 76.70, 77.36, 100.21, 103.42, 111.92, 113.60, 114.29, 126.63, 153.56, 154.61, 160.33, 160.37; MS (ESI$^+$) m/z 361.38 [M + Na]$^+$. Anal. Calcd for $C_{16}H_{18}O_8$: C, 56.80; H, 5.36. Found: C, 56.85; H, 5.41.

4-Methylumbellifer-7-yl-β-D-galactopyranoside (10): 64% overall yield; R_f = 0.35 (8/2 CH_2Cl_2/MeOH); mp 248 °C; ^{1}H NMR (400.13 MHz, DMSO-d_6) δ 2.41 (s, 3H), 3.44 (ddd, 1H, J = 9.2, 5.5, 3.3 Hz), 3.48−3.65 (m, 3H), 3.68 (t, 1H, J = 6.3 Hz), 3.72 (t, 1H, J = 3.8 Hz), 4.99 (d, 1H, J = 7.7 Hz), 6.25 (s, 1H), 7.02 (d, 1H, J = 2.4 Hz), 7.05 (dd, 1H, J = 9.1, 2.4 Hz), 7.70 (d, 1H, J = 9.1 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 18.15, 60.39, 68.13, 69.87, 73.22, 75.71, 100.60, 103.15, 112.24, 112.85, 114.79, 126.17, 153.89, 154.75, 160.19, 160.19; MS (ESI$^+$) m/z 361.35 [M + Na]$^+$. Anal. Calcd for $C_{16}H_{18}O_8$: C, 56.80; H, 5.36. Found: C, 56.75; H, 5.31.

4-Methylumbellifer-7-yl-β-D-xylopyranoside (11): 45% overall yield; R_f = 0.58 (8/2 CH_2Cl_2/MeOH); mp 223 °C; ^{1}H NMR (400.13 MHz, DMSO-d_6) δ 2.40 (s, 3H), 3.27 (d, 2H, J = 2.3 Hz), 3.40 (m, 2H), 3.76 (m, 1H), 5.12 (d, 1H, J = 3.9 Hz), 6.25 (s, 1H), 7.01 (d, 1H, J = 2.4 Hz), 7.03 (dd, 1H, J = 9.2, 2.4 Hz), 7.70 (d, J = 9.2 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 18.13, 62.73, 69.27, 72.95, 76.32, 100.32, 102.74, 112.74, 113.36, 114.13, 126.47, 153.32, 155.3, 159.32, 160.05; MS (ESI$^+$) m/z 331.32 [M + Na]$^+$. Anal. Calcd for $C_{15}H_{16}O_7$: C, 58.44; H, 5.23. Found: C, 58.49; H, 5.20.

4-Methylumbellifer-7-yl-β-D-melibiopyranoside (12): 47% overall yield; R_f = 0.1 (8/2 AcOEt/MeOH); mp 103−105 °C; ^{1}H NMR (400.13 MHz, DMSO-d_6) δ 2.41 (s, 3H), 3.18 (dd, 1H, J = 25.6, 13.2 Hz), 3.32 (m, 3H), 3.40 (dd, 2H, J = 10.7, 6.3 Hz), 3.55 (m, 6H), 4.65 (d, 1H, J = 3.4 Hz), 5.00 (d, 1H, J = 7.3 Hz), 6.26 (s, 1H), 7.04 (d, 1H, J = 2.4 Hz), 7.10 (dd, 1H, J = 8.8, 2.4 Hz), 7.71 (d, 1H, J = 8.8 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 20.66, 59.99, 60.08, 68.25, 68.35, 69.88, 70.09, 71.14, 74.32, 75.05, 77.26, 98.89, 100.02, 104.67, 111.38, 112.53, 114.23, 126.55, 154.17, 160.28, 166.57, 173.79; MS (ESI$^+$) m/z 523.16 [M + Na]$^+$. Anal. Calcd for $C_{22}H_{28}O_{13}$: C, 52.80; H, 5.64. Found: C, 52.75; H, 5.61.

CA Inhibition. An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. 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 a buffer and 20 mM Na_2SO_4 (for maintaining a 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 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 inhibitor solutions (0.1 mM) were prepared in distilled−deionized water, and dilutions of up to 0.01 nM were made thereafter with distilled−deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min to 72 h at room temperature (15 min) or 4 °C (all other incubation times) prior to the assay, to allow the formation of the E−I complex or the eventual active site-mediated hydrolysis of the inhibitor. Data reported in Table 1 show the inhibition after incubation for 6 h, which led to the completion of the in situ hydrolysis of the coumarin and formation of the 2-hydroxycinnamic acids.[1,2] The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, as reported previously,[1,2] and represent the mean from at least three different determinations. CA isofoms were recombinant ones obtained in house as reported previously.[1,2]

Pharmacological Inhibitors. For in vivo studies, the glycosyl coumarin **6** was dissolved in a 37.5% PEG400/12.5% ethanol/50% saline mixture prior to injection. Solutions were heated gently (~40 °C) to completely dissolve the compound. Solutions of the inhibitor

were prepared in batches, distributed into single-use aliquots, and stored frozen at −80 °C prior to being used. Fresh aliquots were thawed daily just before being administered. The inhibitor and vehicle were administered by intravenous injection via a lateral tail vein using a volume of 200 μL for a 20 g mouse. Specific dosing schedules are described in Figure 1.

Syngeneic Orthotopic Breast Tumor Model. All animal studies and procedures were performed in accordance with protocols approved by the Institution Animal Care Committee at the BC Cancer Research Centre and the University of British Columbia. 4T1 cells (1×10^6 cells/mouse) were implanted orthotopically into the fourth mammary fat pad of 7−9-week-old female BALB/c mice, and tumor growth rates were calculated from caliper measurements using the modified ellipsoid formula $(LxW^2)/2$ as described previously.[15,20] After initial implantation of cells, tumors were allowed to establish, and then treatment was initiated. Tumors were measured, and animals were mixed just prior to the onset of treatment to ensure a similar average tumor volume between the treatment groups. Glycosyl coumarin **6** was administered by intravenous injection via a lateral tail vein at a dose of 30 mg/kg. The dosing schedule involved injections every other day for doses 1 and 2, followed by injections every third day for the remaining doses. Caliper measurements were taken three times per week. $n = 7$ for each group. Data were subjected to statistical analysis using the Data Analysis ToolPack in Excel. Two-tailed P values were calculated using a Student's t test. Data were considered significant for $P < 0.05$.

AUTHOR INFORMATION

Corresponding Author

*J.-Y.W.: telephone, +33-4 67 14 72 34; fax, +33-4 67 14 43 44; e-mail, jean-yves.winum@univ-montp2.fr. C.T.S.: telephone, +39-055-457 3005; fax, +39-055-4573385; e-mail, claudiu.supuran@unifi.it.

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