Targeting Tumor Hypoxia: Suppression of Breast Tumor Growth and Metastasis by Novel Carbonic Anhydrase IX Inhibitors

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Abstract

Carbonic anhydrase IX (CAIX) is a hypoxia and HIF-1–inducible protein that regulates intra- and extracellular pH under hypoxic conditions and promotes tumor cell survival and invasion in hypoxic microenvironments. Interrogation of 3,630 human breast cancers provided definitive evidence of CAIX as an independent poor prognostic biomarker for distant metastases and survival. shRNA-mediated depletion of CAIX expression in 4T1 mouse metastatic breast cancer cells capable of inducing CAIX in hypoxia resulted in regression of orthotopic mammary tumors and inhibition of spontaneous lung metastasis formation. Stable depletion of CAIX in MDA-MB-231 human breast cancer xenografts also resulted in attenuation of primary tumor growth. CAIX depletion in the 4T1 cells led to caspase-independent cell death and reversal of extracellular acidosis under hypoxic conditions *in vitro*. Treatment of mice harboring CAIX-positive 4T1 mammary tumors with novel CAIX-specific small molecule inhibitors that mimicked the effects of CAIX depletion *in vitro* resulted in significant inhibition of tumor growth and metastasis formation in both spontaneous and experimental models of metastasis, without inhibitory effects on CAIX-negative tumors. Similar inhibitory effects on primary tumor growth were observed in mice harboring orthotopic tumors comprised of lung metatstatic MDA-MB-231 LM2-4^{Luc+} cells. Our findings show that CAIX is vital for growth and metastasis of hypoxic breast tumors and is a specific, targetable biomarker for breast cancer metastasis. *Cancer Res; 71(9); 3364–76. ©2011 AACR.*

Introduction

Cancer metastasis is a complex process that results in establishment of secondary tumors in distant organs (1). There is increasing recognition that hypoxia plays an important role in cancer progression and metastasis (2, 3), including breast cancer metastasis (4, 5). Furthermore, there is now growing evidence that altered tumor metabolism and hypoxia-inducible factor 1α (HIF-1α)-regulated enzymes such as carbonic anhydrase IX (CAIX) and CAXII may be vital to the process of tumor progression to metastasis (2, 6).

CAIX is a dimeric membrane-bound enzyme that efficiently catalyzes the reversible hydration of CO_2 (7, 8). CAIX is selectively expressed in hypoxic tumors, including breast malignancies (9, 10), and its presence is a poor prognostic marker for patients with breast cancer (11, 12). The tumor-specific expression of CAIX and its association with cancer progression and poor treatment outcome has led to interest in targeting this enzyme for cancer therapy (8). Studies have focused on the utilization of CAIX as a biomarker of hypoxic tumors, spurring the development of specific antibodies and sulfonamide-based small molecules for imaging CAIX *in vivo* (13–17). However, the relevance of CAIX function to the biology of tumors has only recently come into focus. Evidence suggests that together with the activity of proteins, such as the Na^+/H^+ exchanger NHE1, Na^+-HCO_3^- cotransporters, and monocarboxylate transporters MCT-1 and MCT-4 (18, 19), the activity of CAIX plays an important role in the survival of tumor cells in hypoxic regions of tumors (18, 20) through the regulation of tumor pH (18, 20). The HCO_3^- produced at the

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extracellular surface by CAIX is transported into the cytosol to control an intracellular pH (pHi) that is challenged by the abnormally acidic extracellular pH (pHe) produced in hypoxia (18, 20–22). The protons derived from CAIX activity further contribute to the decrease in pHe, thereby potentiating extracellular matrix breakdown and cell invasion (6, 19). Therefore, CAIX may increase metastatic potential by allowing aggressive tumor cells to survive the hostile environment imposed by hypoxia, and may further function to potentiate extracellular acidosis, facilitating growth and invasion of surviving cells (18, 20–22).

Although targeting CAIX for the treatment of cancer has garnered much scientific and clinical interest, appropriate carbonic anhydrase-relevant cell and animal models of tumor hypoxia for testing novel, CAIX-active compounds have only recently become available (23). To date, studies have focused on the role of CAIX in the regulation of primary tumor growth (22, 24). Importantly, neither the functional requirement of CAIX in breast tumor growth and metastasis *in vivo*, nor the benefit of therapeutic targeting this enzyme in aggressive breast cancer has been addressed.

In this study, we provide definitive evidence, using a large (>3,600) cohort of human breast cancer samples, that CAIX is a poor prognostic marker for distant metastasis and survival. Furthermore, using a combination of gene depletion strategies and pharmacologic inhibition with novel small molecule inhibitors, we show a functional requirement of CAIX in the growth and metastasis of mouse and human breast tumors in several preclinical models. Our findings establish CAIX as a therapeutic target for the treatment of CAIX-positive breast cancer.

Materials and Methods

Cell culture and hypoxic exposure

The acquisition, generation, and culture of the luciferase expressing mouse breast cancer cell lines 4T1, 66cl4, and 67NR have been described previously (25). The MDA-MB-231 human breast cancer cell line was obtained from the American Type Culture Collection and was maintained as described previously (26). The MDA-MB-231 LM2-4^{Luc+} cell line was provided by Dr. Robert Kerbel (University of Toronto, Canada) in July, 2010 and cells were cultured as described previously (27). For *in vitro* studies, cell lines were passaged for a maximum of 3 months, after which fresh seed stocks were thawed for experimental use. All cells were incubated at 37°C with 5% CO_2 in a humidified incubator (normoxia). For culture in hypoxia, cells were maintained in 1% O_2 and 5% CO_2 balanced with N_2 at 37°C in a humidified incubator in a sealed anaerobic workstation. Cell lines were evaluated routinely for morphology, hypoxia-induced CAIX expression, and *in vivo* tumor growth.

Generation of transfected and transduced cells

shRNAmir vectors targeting mouse CAIX and a nonsilencing sequence (Open Biosystems) were transfected into 4T1 cells by LipofectAMINE-PLUS (Invitrogen Life Technologies) according to the manufacturer's instructions. Transfected cells were selected by using hygromycin. Stable shCAIX clones were derived by limited dilution cloning. For (re)introduction of CAIX, human CAIX (gift from Dr. Jacques Pouysségur, University of Nice, Nice, France) was transfected into 4T1 cells stably expressing mouse shCAIX, and Zeocin was used for selection.

For stable depletion of human CAIX in the MDA-MB-231 cells, 2 different shRNAmir constructs (Open Biosystems) were transduced into cells by using lentivirus as per the manufacturer's instructions. Transduced cells were selected by using puromycin.

All transfected and transduced cell lines were selected, propagated, and frozen as seed stocks at early passage. For *in vivo* studies, cells were thawed from frozen stocks, passaged 1–2 times to expand the culture, and implanted in mice. Cell lines were tested for mycoplasma contamination by a commercial testing facility prior to implantation in mice.

Measurement of extracellular pH

Changes in pHe were assessed by using procedures published previously (28–30). In brief, cells were plated and allowed to recover overnight. A standard volume of 3 mL of fresh media/dish was then added and cells were incubated in normoxia or hypoxia for 72 hours. Care was taken to ensure that cultures grown in normoxia and hypoxia were subconfluent and contained similar cell numbers. Media was collected and pH was measured immediately by a digital pH meter.

Pharmacological inhibitors

The chemical properties of the sulfonamide, CAI17, have been described previously (8, 30). For *in vitro* studies, CAI17 was dissolved in dimethylsulfoxide, stored at −80°C and diluted into culture medium prior to application. Subconfluent cells were incubated with CAI17 for 72 hours, washed 3 times in PBS, and imaged by a Zeiss Axioplan epifluorescence microscope. For *in vivo* studies, CAI17, ureido-sulfonamide U-104, and glycosyl coumarins GC-204 and GC-205 were solubilized in 37.5% PEG400/12.5% ethanol/50% saline prior to injection. Drug aliquots were made fresh daily or were prepared, frozen at −80°C in single-use aliquots, and thawed prior to administration. Drugs were administered by i.p. injection, except for CAI17, in which the first 2 doses were administered by i.v. injection, followed by i.p. injection of the remaining doses. Specific dosing schedules are described in the appropriate figures.

Analysis of protein expression

Cells or flash-frozen tumor tissues were lysed as described previously (26). Equal amounts of protein were loaded on SDS-PAGE gels. Western blots were carried out as described previously (26) by using mouse CAIX (1:500), human CAIX (1:1,000; R&D Systems), caspase 3 (1:1,000; Cell Signaling), PARP-1 (1:1,000; Cell Signaling), and β-actin (1:10,000, Sigma) antibodies.

Mouse tumor models

All animal studies and procedures were done in accordance with protocols approved by the Institution Animal Care

Committee at the BC Cancer research Centre and the University of British Columbia (Vancouver, British Columbia, Canada).

Syngeneic orthotopic tumors and spontaneous metastasis

4T1 cells (1×10^6) or 67NR cells (2×10^6) were orthotopically implanted into the fourth mammary fat pad of 7- to 9-week-old female BALB/c mice as described previously (25). Primary tumor growth rates were calculated from caliper measurements by using the modified ellipsoid formula, $(L \times W^2)/2$ (L, length; W, width). Tumor formation and metastasis progression was monitored and quantified by bioluminescent imaging as previously described (25, 27).

Experimental metastasis assays

For studies involving genetic depletion of CAIX, 4T1 or 67NR cells (5×10^5) were injected directly into the tail vein of 7- to 9-week-old female BALB/c mice. Mice were imaged once per week to follow the growth of metastases. Mice were euthanized 20 days postinjection and lungs were resected for further analysis. Tumor burden in the lung was quantified by manually counting nodules visible on the lung surface. For studies by U-104, 4T1 cells (1×10^5) were injected as described earlier in the text, whereas 2×10^5 cells were used for studies with GC-204 and GC-205.

Human xenograft tumors

For studies involving CAIX depletion, 1×10^7 MDA-MB-231 cells suspended in a 50% Matrigel/PBS solution were implanted subcutaneously in 6- to 8-week-old female NOD. CB17-prkdc^{scid}/J mice. For primary breast tumor xenografts by the MDA-MB-231 LM2-4^{Luc+} variant, cells were implanted orthotopically in mice as described earlier in the text. Therapy was initiated when the tumor volumes reached 200 mm^3. For both models, tumor growth was monitored by caliper measurement.

Immunohistochemistry

Two hours before tumor excision, mice were injected i.p. with a saline solution containing 1,500 mg/kg bromodeoxyuridine (Sigma) and 60 mg/kg Pimonidazole (Chemicon), and i.v. 5 minutes before with $DiOC_7(3)$ (70 μL, 0.6 mg/mL; Molecular Probes). Tumors were then harvested and analyzed for vasculature, perfusion, hypoxia, apoptosis, proliferation, and necrosis as described previously (31, 32). Paraffin-embedded tumor sections were also stained for CAIX (1:50 for lung metastases; Santa Cruz Biotechnology) as previously described (25).

Apoptosis assay

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL; Roche Applied Science) was employed for analysis of apoptosis according to the manufacturer's instructions. Briefly, subconfluent cells grown on coverslips were incubated for 48 hours in normoxia or hypoxia in 1% serum, fixed, and analyzed for TUNEL-positive cells. Quantification was achieved by counting the number of TUNEL-positive cells in 5 random fields/cell line at ×20 magnification.

Clinical analysis

The methods used to create the tumor tissue microarrays (TMA) have been described (33). A total of 3,630 cases had adequate tumor and staining results for assessment of all biomarkers. Immunohistochemistry for ER, PR, HER2, CK 5/6, EGFR, and Ki67 was carried out concurrently on serial sections and scored as described previously (33). CAIX expression was assessed by using a murine monoclonal antibody (M75; 1:50; ref. 34). Scoring of CAIX expression was either 0 (no staining) or 1 (any staining) and done independently and blindly by 2 pathologists. This scoring system has been developed and validated for this very large TMA, and has been used previously to show prognostic significance for breast cancer biomarkers such as HER-2 and Ki67 (33, 35). The stained images of the complete tissue core set are available at the publicly accessible website http://bliss.gpec.ubc.ca. Prior approval of the study was obtained from the Ethics Committee of the University of British Columbia.

Statistical analysis

Results were subjected to statistical analysis by the Data Analysis ToolPack in Excel software. Two-tailed P values were calculated by Student's t-test. Data were considered significant for $P < 0.05$. Statistical analysis for the clinical outcomes was carried out by SPSS 13.0, S-Plus 6.2, and R 2.1.1 (http://www.r-project.org). In univariate analysis, BCSS (date of diagnosis of primary breast cancer to date of death with breast cancer as the primary or underlying cause), RFS (date of diagnosis of primary breast cancer to the date of a local, regional, or distant recurrence), and distant RFS (date of diagnosis of primary breast cancer to the date of a distant recurrence) were estimated by Kaplan–Meier curves. Log-rank test was used to estimate the survival differences. For multivariate analysis, a Cox proportional hazards model was used to estimate the adjusted hazard ratios and significance. To assess the violations of proportional hazard models, smoothed plots of weighted Schoenfeld residuals were used.

Results and Discussion

CAIX is a poor prognostic marker in a large cohort of breast cancer patients

Although previous studies have reported that CAIX expression in several types of cancer, including breast cancer, correlates with poor patient prognosis (11, 34, 36, 37), the sample sizes have been relatively small and adjuvant treatments not uniform. To provide definitive evidence for CAIX as an important breast cancer prognostic marker, we analyzed the expression of CAIX in a primary breast TMA containing more than 3,600 patient samples subjected to standardized treatment with a median follow-up of 10.5 years (Supplementary Table S1). Previously, we showed prognostic significance of CAIX in a cohort of 103 breast cancers in which CAIX expression was examined both as a continuous and a categorical variable (11). We found that even 1% staining was

significantly prognostic. For the large cohort of patients examined here, a simplified scoring system (present vs. absent) allows for less analytical variability, an issue which plagues immunohistochemical testing for hormone receptors and the HER-2 receptor in breast cancer. CAIX expression was seen in 15.6% of assessable tumors and CAIX was differentially expressed among the biological subtypes, with the highest correlation in the basal breast cancers (51%) and the lowest proportion in the luminal A subtype (8%; Supplementary Table S2).

In Kaplan–Meier analyses, CAIX expression was significantly associated with worse relapse-free survival (Fig. 1A), distant relapse-free survival (Fig. 1B), and breast cancer–specific survival (Fig. 1C), achieving very high levels of statistical significance. The 10-year distant relapse-free survival and breast cancer specific survival rates in the CAIX-positive versus CAIX-negative groups were 57% compared with 73%, and 62% compared with 78%, respectively. In multivariate analyses, including all standard prognostic variables and biological subtypes, CAIX expression remained a strong independent poor prognostic factor with a hazard ratio of 1.4 (Supplementary Table S3). These data confirm and extend the results of previous studies, and show a clear link between CAIX expression and a higher rate of distant metastasis for breast cancer. Our data substantiate previous studies that have also shown CAIX as a poor prognostic marker of breast cancer (11, 12). In addition, other studies have shown a clear correlation between the expression of CAIX, hypoxia-induced HIF-1α, and altered metabolic proteins such as GLUT-1 (4, 9, 38), although HIF-1α is a more labile protein, and thus more susceptible to preanalytical variables.

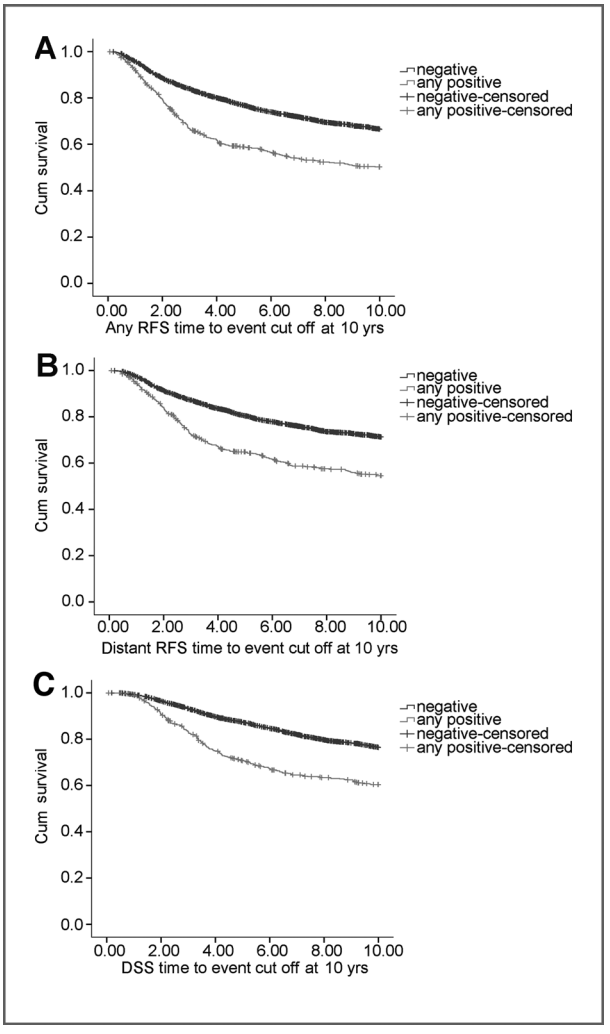

Figure 1. CAIX expression is an independent prognostic factor in a large cohort of breast cancer patients. The Kaplan–Meier plots show cumulative survival (Cum survival) as a function of time to event cut off at 10 years postdiagnosis. CAIX expression was significantly associated with poor relapse-free survival (RFS; A), distant RFS (B), and disease specific (breast cancer) survival (C). DSS, distant site survival. $P < 10^{-17}$, $P < 10^{-16}$, and $P < 10^{-13}$ for A, B, and C, respectively. CAIX expression on the TMA was binarized as 0 and 1 for analysis.

Preclinical models for interrogating the requirement of hypoxia-induced CAIX expression in breast cancer

Tumor hypoxia is linked both to the expression of CAIX and to the selection of tumor cells that are better able to metastasize. However, although a few studies have investigated the role of CAIX in the regulation of primary tumor growth (22, 24), the relationship between CAIX expression and metastatic potential has not been investigated. Therefore, to interrogate the functional role of CAIX in metastatic breast cancer, we were interested in selecting tumor models that exhibit both hypoxic microenvironments and the ability to metastasize. We have shown previously that the highly metastatic 4T1 mouse mammary tumor (39) overexpresses CAIX (25). Further characterization of this model revealed that it is poorly vascularized and contains large regions of hypoxia and necrosis (Fig. 2A). Indeed, tumors formed by 4T1 cells have significantly less blood vessels, and significantly higher amounts of hypoxia, necrosis, and apoptosis compared with tumors formed by isogenic, nonmetastatic (25) 67NR cells (Fig. 2A and B). Furthermore, bioinformatic analysis of differential gene expression data (25) identified several hypoxia-regulated genes, including CAIX, that are expressed at higher levels in the 4T1 tumors relative to the 67NR tumors (Fig. 2C). These attributes make the 4T1 mouse mammary tumor a robust model for examining the effects of manipulating CAIX expression and activity on the progression of breast cancer.

In addition to this syngeneic mouse model, we selected the MDA-MB-231 human breast tumor cell line, as previous studies have shown this model to be hypoxic and to have hypoxia-inducible levels of CAIX (40, 41). We confirmed the effect of hypoxia on CAIX expression in these cell lines to validate them as appropriate models for subsequent *in vivo* studies. In keeping with previously published findings (25, 41), we found that both cell lines induced CAIX expression in hypoxia (Fig. 2D; Supplementary Fig. S1A), in contrast to the absence of hypoxia-induced CAIX in the 67NR cells (Fig. 2D; Supplementary Fig. S1A). These models reflect the clinical findings shown in Figure 1 and in other studies (11, 12) of hypoxia-induced upregulation of CAIX expression.

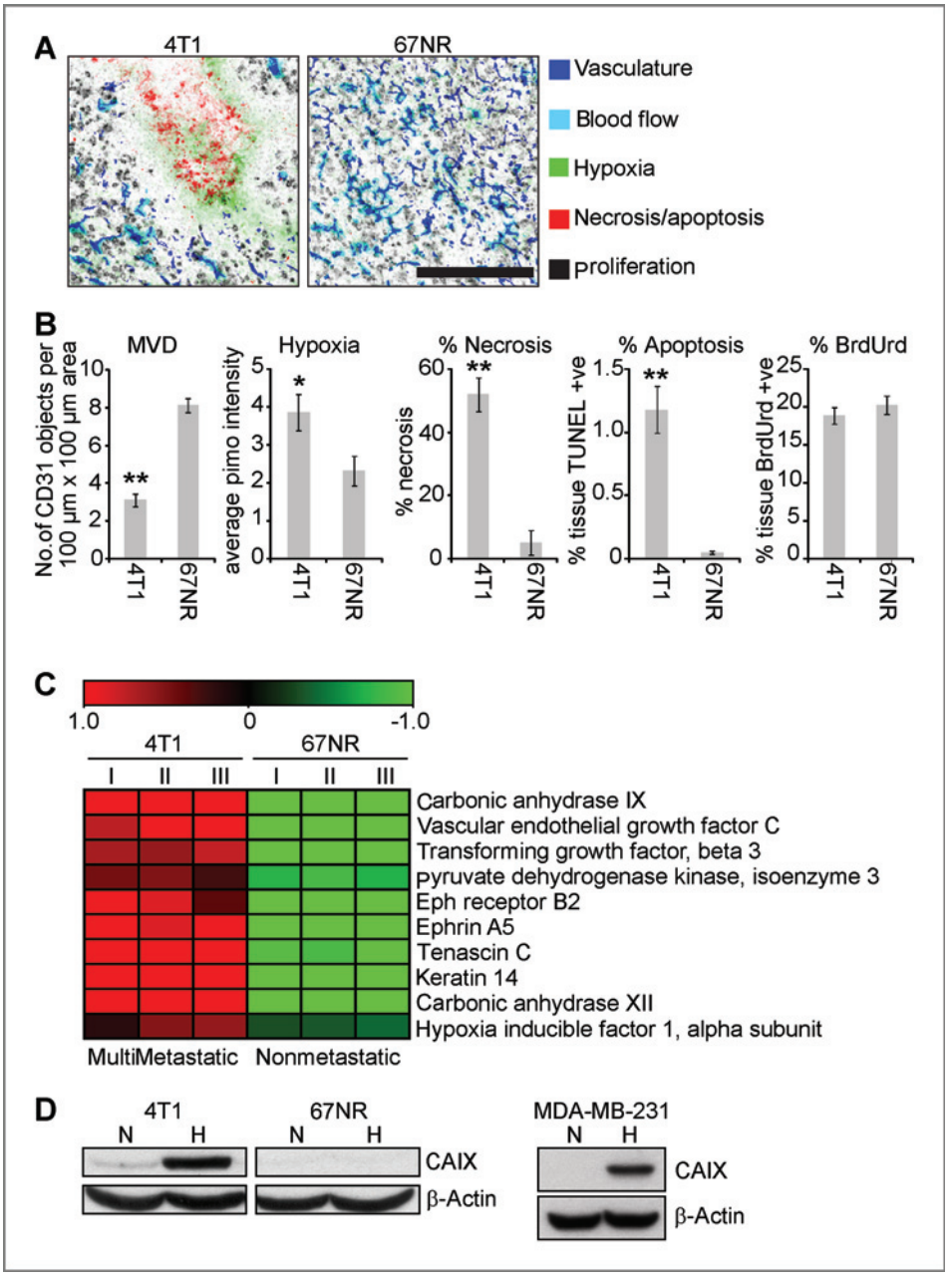

Figure 2. The metastatic 4T1 primary tumor is a valid preclinical model of hypoxia-induced CAIX expression. A, representative composite, pseudocolored images of 4T1 and 67NR tumor tissue sections showing the distribution of the indicated parameters. Scale bar, 150 µm. B, quantification of the parameters outlined in (A) by using whole tumor tissue sections. $n = 10$ animals/group. *, $P \leq 0.02$; **, $P < 10^{-5}$. C, differential gene expression data derived from tumor tissue from each cell model (25) was analyzed bioinformatically for expression of hypoxia-induced genes (high expression, red; low expression, green). $n = 3$ animals/group. D, the indicated cell lines were cultured in normoxia or hypoxia and levels of CAIX expression were analyzed by Western blot. β-Actin served as a loading control.

Depletion of CAIX in 4T1 cells inhibits cell survival and alters pHe

Next, we silenced CAIX gene expression in the 4T1 cells (Fig. 3A) and the MDA-MB-231 cells (Fig. 3B) by stably expressing CAIX short hairpin RNA (shRNA) constructs. 4T1 cells expressing a nonsilencing control shRNA (shNS) upregulated expression of CAIX in hypoxia as expected, whereas hypoxia-induced CAIX expression was markedly attenuated in 2 independent clones expressing a single, identical shRNA targeting mouse CAIX (shCAIX; Fig. 3A; Supplementary Fig. S1B). Similarly, for the MDA-MB-231 cell line, 2 distinct shRNA sequences targeting human CAIX were transduced and hypoxia-induced CAIX expression was analyzed. Only one of the transduced shRNA constructs was found to effectively deplete CAIX expression (Fig. 3B; Supplementary Fig. S1C). The cell line expressing the "nonsilencing" CAIX shRNA sequence was used as a control in subsequent *in vivo* experiments.

Recent data suggest that the regulation of pH by CAIX may be important for cell survival in conditions of hypoxic stress (22, 42) and previous studies have shown a reduction in clonogenic survival in hypoxia of MDA-MB-231 cells treated with siRNA to CAIX (41). To examine whether depletion of CAIX may be influencing cell survival in the 4T1 system, we

cultured control and CAIX-depleted 4T1 cells in hypoxia and assessed the amount of cell death by using a TUNEL assay. 4T1 shCAIX cells showed a significant increase in cell death compared shNS cells (Fig. 3C). To determine whether the increase in TUNEL-positive cells was because of an increase in apoptosis, we analyzed the levels of active caspase-3 and PARP. No clear evidence of increased caspase-3 cleavage (Supplementary Fig. S2A) or PARP cleavage (Supplementary Fig. S2B) was observed, suggesting that cell death in the CAIX-depleted cells may be occurring by a caspase-independent mechanism, possibly related to depletion of the intracellular ATP concentration (Supplementary Fig. S2C). Although the decrease in ATP concentration was modest, it was statistically significant and similar in magnitude to data reported previously (43).

CAIX is functionally linked to the control of tumor pH (18, 22, 42), and hypoxia-induced, extracellular acidosis is a measure of the biological activity of CAIX in cultured cells (30). Therefore, we examined the effect of CAIX depletion on pHe in hypoxia. Acidification of the extracellular medium in hypoxia was inhibited in the shCAIX-expressing 4T1 clones relative to the parental and shNS-expressing 4T1 cells (Fig. 3D), suggesting that silencing CAIX gene expression induces functional inhibition of pHe regulation in this cell line.

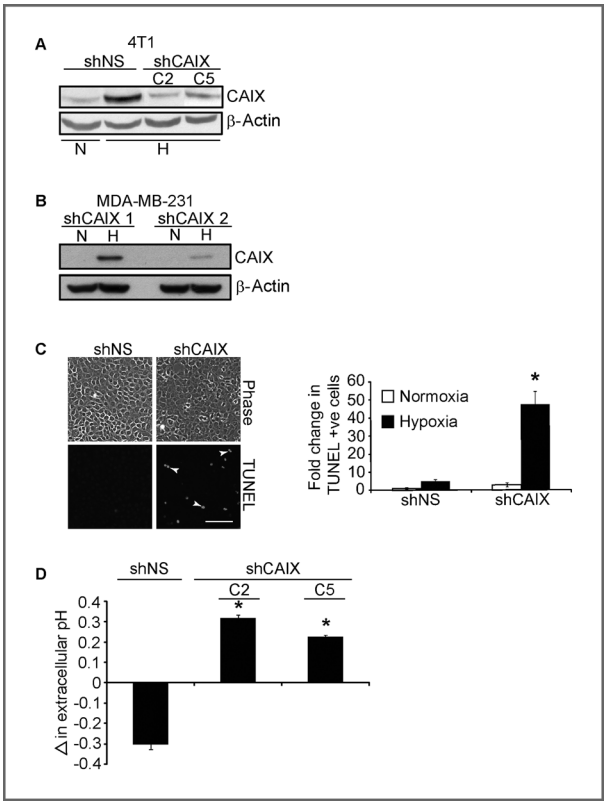

Figure 3. Silencing CAIX expression in metastatic 4T1 cells inhibits cell survival and alters pHe in hypoxia. A, cells expressing nonsilencing shRNA (shNS) or shRNA targeting mouse CAIX (shCAIX) were cultured in normoxia (N) or hypoxia (H) and analyzed for CAIX expression. Two independent clones (C2, C5) expressing a single, identical shCAIX construct were tested. B, MDA-MB-231 cells expressing either of 2 different shRNA constructs targeting human CAIX (shCAIX 1 and shCAIX 2) were cultured as above and analyzed for CAIX expression. C, 4T1 cells expressing shNS or shCAIX were cultured in hypoxia and the amount of cell death was analyzed. Left, representative images of TUNEL-positive cells (arrows). Scale bar, 100 μm. Right, quantification of the TUNEL-positive cells. Data are expressed as fold change in TUNEL-positive cells, compared with control cells cultured in normoxia. $n = 5$. *, $P < 0.01$, compared with the controls. D, cells were cultured in normoxia or hypoxia and pHe was measured. $n = 3$. Data show the mean change in pH ± SEM. Changes in the pHe in hypoxia are assessed relative to the baseline pHe values in normoxia. *, $P < 0.01$, compared with the 4T1 cells expressing shNS in hypoxia.

Depletion of CAIX expression results in regression or growth inhibition of mouse and human breast tumors

Having evaluated the biological response of 4T1 cells to CAIX depletion *in vitro*, we next tested the impact of silencing CAIX expression on the growth of these tumors *in vivo*. We observed that whereas control 4T1 cells formed tumors that grew steadily over 30 days, tumors established from CAIX-depleted cells regressed significantly after initial tumor growth (Fig. 4A). The regression of the tumors seemed to be stable, as there were only 2 mice with primary tumor recurrence appearing toward the end of the study (Supplementary Table S4). Examination of the primary tumors confirmed downregulation of CAIX expression in the tumors (Supplementary Fig. S3). The reduction of CAIX expression had a dramatic effect on the overall survival of the mice (Fig. 4B). Although the animals bearing tumors that express CAIX did not survive, the survival rate of animals inoculated with CAIX-depleted 4T1 cells remained at 100% over the course of the study.

To validate the observed *in vivo* effects of CAIX depletion on primary human breast tumor growth, we conducted similar experiments by using the MDA-MB-231 cells expressing human shCAIX (Fig. 3B). Depletion of CAIX significantly attenuated tumor growth of MDA-MB-231 xenografts (Fig. 4C). Importantly, both the parental cells and the cells expressing the nonsilencing construct (shCAIX 1 NS) formed tumors at a similar rate (Fig. 4C), showing the specificity of the effect of CAIX depletion on tumor growth. These data provide strong evidence for an important functional role of CAIX in the growth of hypoxic primary breast tumors.

To show that the tumor regression seen with the 4T1 shCAIX cells was dependent specifically on CAIX, we attempted to rescue the tumor growth by introducing human CAIX (resistant to mouse CAIX shRNA) into 4T1 cells expressing mouse-specific shCAIX. Human CAIX expression was readily detectable on the cell surface, together with stable expression of mouse shCAIX (Fig. 4D). Cell cultures expressing human CAIX in tandem with shRNA to mouse CAIX showed low numbers of TUNEL-positive cells (Supplementary Fig. S4), similar to control 4T1 cells (Fig. 3C). Furthermore, the mammary tumors established from these cells grew at rates similar to those of the parental 4T1 tumors (Fig. 4D), confirming the specificity of CAIX-targeting in the regression of the 4T1 tumors.

Previous work in a xenograft model of colon cancer reported a requirement of hypoxia-induced CAIX and CAXII

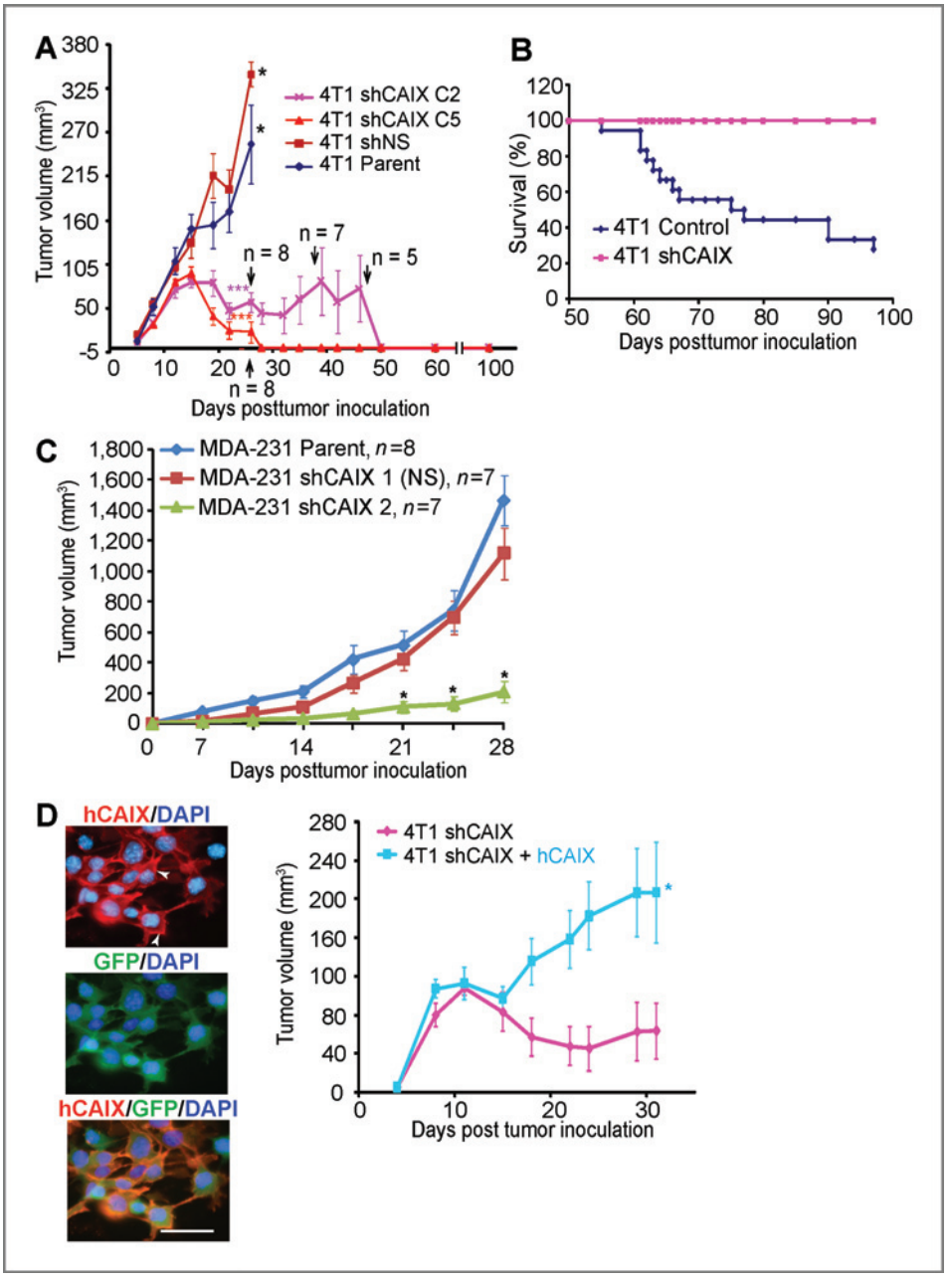

Figure 4. Growth of primary breast tumors characterized by hypoxia requires the expression of CAIX. A, parental 4T1 cells, or 4T1 cells expressing shNS or shCAIX were implanted orthotopically into BALB/c mice and tumor volume was monitored over time. $n = 10$ for each group. Arrows, changes in n due to surgery and revised values are indicated. *, completion of primary tumor excision from the control groups. Results are expressed as mean ± SEM for each group. ***, $P < 10^{-11}$, compared with the shNS group. B, the groups of mice described in (A) were monitored for the percentage of mice surviving (pooled control and shCAIX groups) over time. $n = 18$/group. C, parental MDA-MB-231 cells or cells expressing shCAIX 1 or shCAIX 2 were implanted subcutaneously into the flank of NOD/SCID mice and tumor growth was monitored. Note that shCAIX 1 does not silence CAIX expression (see Fig. 3B) and is used as a nonsilencing (NS) control. $n = 7$ for each group. *, $P < 0.01$, compared with NS control tumors. D, 4T1 cells expressing mouse shCAIX were transfected with human CAIX (hCAIX) and immunocytochemistry was done to assess levels of human CAIX expression (red, arrowheads) in these cells (shCAIX + hCAIX). Cells are simultaneously positive for green fluorescent protein (GFP; mouse shCAIX) and are counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Cells were implanted orthotopically in BALB/c mice and monitored for tumor growth. $n = 9$ for each group. *, $P < 0.04$, compared with the shCAIX group.

in the regulation of tumor growth (22). In this model, shRNA-mediated depletion of CAIX expression resulted in a partial reduction in tumor growth and compensatory upregulation of CAXII, whereas depletion of expression of both proteins resulted in greater inhibition of tumor growth (22). Our current findings suggest that, in breast cancer, CAIX alone is important for the growth of breast tumors. Our data showing that overriding CAIX depletion by constitutive expression of human CAIX can rescue tumor growth strongly implicates the dependency of these metastatic tumors on CAIX function. In addition, CAIX depletion in MDA-MB-231 human breast cancer cells, a cell line which is deficient in CAXII expression (44), also significantly reduced tumor growth. The reasons for the lack of CAXII dependence in these models are not known, but it is possible that the relative expression of CAIX versus CAXII in different cell types may be an important factor.

Inhibition of CAIX inhibits metastasis of 4T1 breast tumors

Although a reduction in colon tumor growth in response to CAIX depletion has been shown previously (22) and CAIX has been shown to influence cell migration *in vitro* (45), CAIX has not been previously linked to metastasis

in vivo. Given the metastatic potential of the 4T1 cells, in particular, we were interested in determining whether inhibition of CAIX expression or activity also inhibited breast tumor metastasis. To investigate the effects of CAIX depletion on spontaneous metastasis of primary breast tumors *in vivo*, we employed bioluminescent imaging to detect metastases arising from control (parental and shNS) and CAIX-depleted 4T1 tumors (Fig. 5A). Whereas the mice implanted with control cells showed clear evidence of metastasis to several organs, (In Vivo Imaging System) IVIS-detectable metastases were not observed in mice that had been implanted with the CAIX-depleted cells.

To show that the observed effects on the formation of metastases were not simply because of primary tumor regression, we injected the various 4T1 tumor cell lines intravenously and assessed the ability of the cells to form lung metastases (Fig. 5B). We found that, whereas the shNS cells formed robust lung metastases, cells depleted of CAIX showed almost no visible metastasis to the lungs. 67NR cells also showed little evidence of experimental metastasis (Fig. 5B). Quantification of bioluminescent signal at 2 and 3 weeks postinjection showed a significant increase in signal in the shNS group, but not in the shCAIX and 67NR groups (Fig. 5B). Examination of the gross lungs revealed that

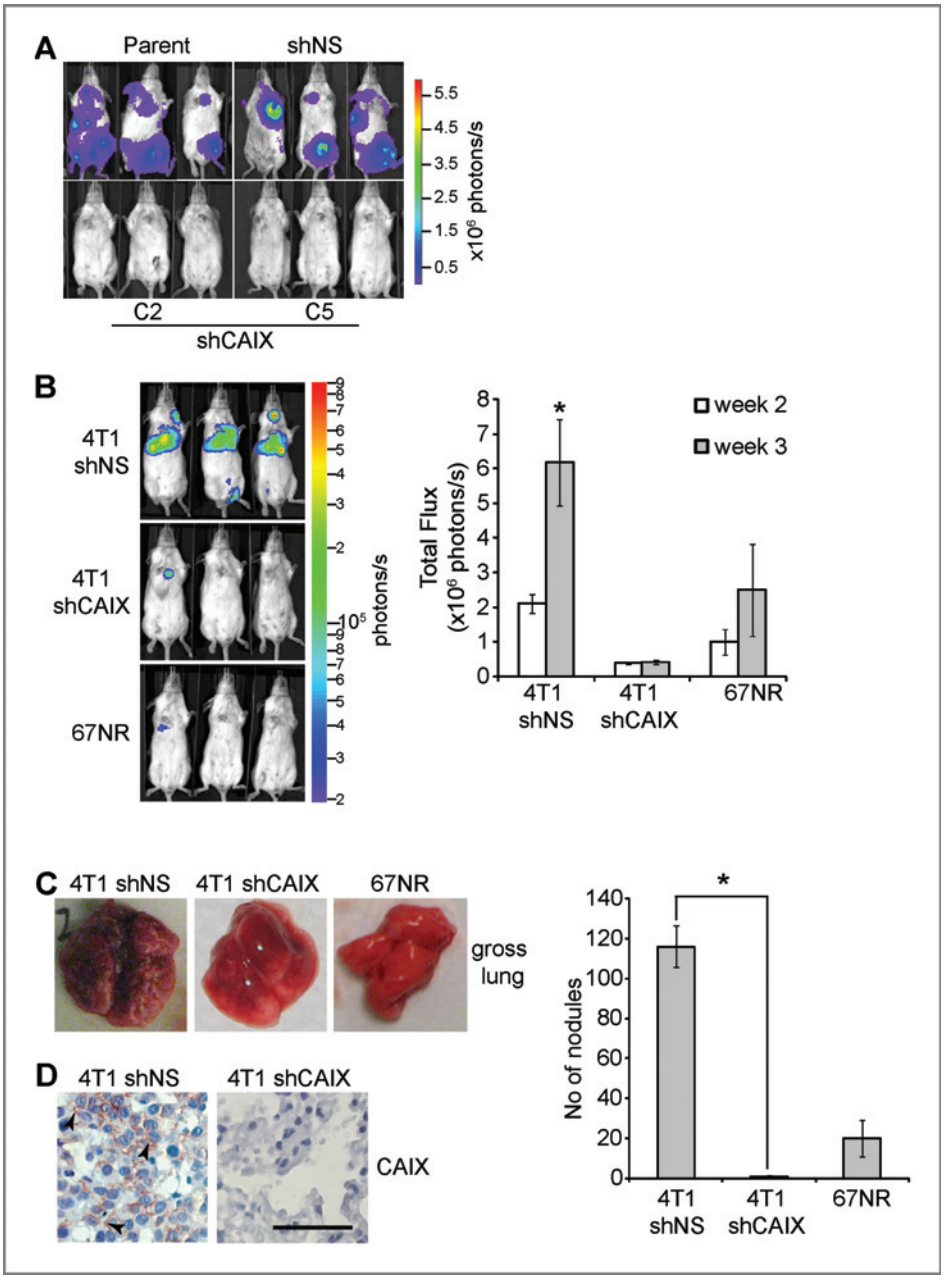

Figure 5. Inhibition of CAIX expression or activity attenuates metastasis of 4T1 mouse mammary tumors. A, using the experimental design detailed in Figure 4A, mice were examined for evidence of spontaneous metastasis. Primary tumors in the control arm were removed by day 30 postimplantation, whereas primary tumors in the CAIX-depleted groups either regressed or were removed in a similar fashion to control tumors. Representative bioluminescent images are shown as heat maps (blue, least intense; red, most intense;) overlaid on gray-scale body images. B, 4T1 cells expressing shNS or shCAIX, or 67NR cells were injected directly into the tail vein of BALB/c mice. Representative images of tumor cell bioluminescence at 20 days postinoculation are shown. The graph shows quantification of bioluminescence at week 2 and week 3 postinjection of tumor cells. $n = 8$/group. *, $P < 0.02$. C, comparison of surface nodules on gross lungs resected from animals at 3 weeks postinjection. The graph shows quantification of the number of metastatic foci (nodules) on the lung surface. $n = 8$. *, $P = 0.0001$. D, representative images of lung metastases stained immunohistochemically for CAIX (arrowheads). Scale bar = 50 μm.

animals injected with shNS cells exhibited large number of lung surface nodules, whereas nodules were largely absent in mice injected with shCAIX cells or 67NR cells (Fig. 5C). Quantification of surface nodules showed that mice harboring shNS cells had significantly higher counts than mice harboring shCAIX or 67NR cells (Fig. 5C). Membrane-localized CAIX expression was also evident in some of the metatstatic foci in histologic sections taken from control animals, but not from mice inoculated with CAIX-depleted cells (Fig. 5D). Taken together, these data suggest that CAIX may be required for colonization and growth of metastatic cells at secondary sites.

Pharmacologic inhibition of CAIX reduces the growth and metastasis of mouse and human tumors

Our data showing the requirement of CAIX expression for survival and eventual metastasis of primary breast tumor cells suggested that targeting the activity of CAIX with specific pharmacologic inhibitors may be useful for inhibiting disease progression. In previous studies, treatment of

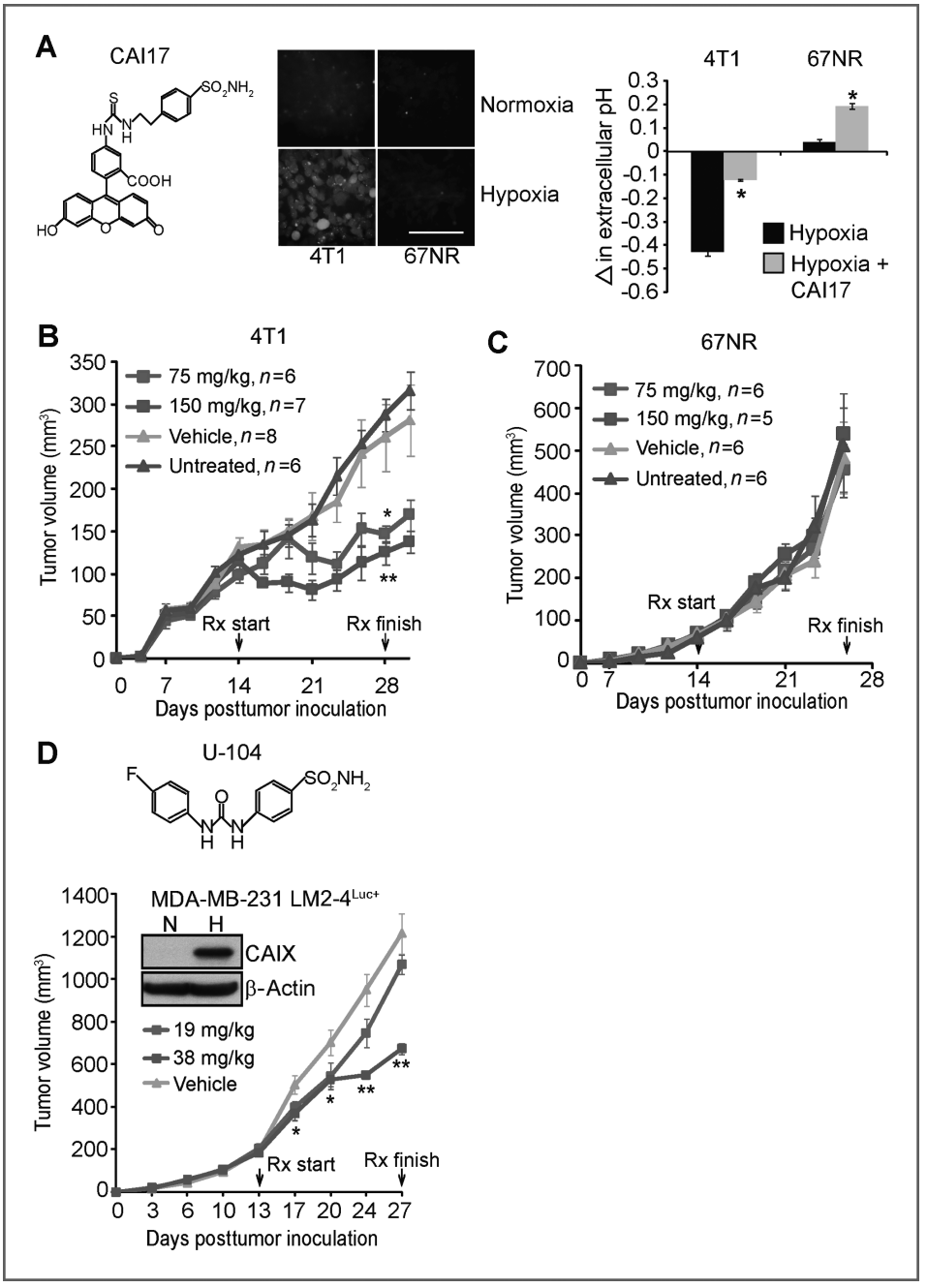

Figure 6. Targeting CAIX activity with selective small molecule inhibitors of CAIX attenuates the growth of mouse and human breast tumors. A, left, chemical structure of sulfonamide-based CAIX inhibitor CAI17 (8). Middle, cells were cultured with 10 μM CAI17. Shown are representative images of the FITC-tagged inhibitor (green) bound to cells in normoxia and hypoxia. Right, cells were cultured with or without 400 μmol/L CAI17. The mean changes in pHe ± SEM are shown. Changes in pHe in hypoxia were assessed relative to the baseline pHe values measured in parallel cultures grown in normoxia. $n = 3$. *, $P < 0.001$. B, BALB/c mice were inoculated orthotopically with 4T1 cells and tumors were grown for 14 days. Animals then received CAI17 3 times per week for 2 weeks and tumor growth was monitored. Treatment initiation and termination are indicated. Vehicle-treated and untreated animals served as controls. $n = 6$ to 8. *, $P < 0.02$, **, $P < 0.01$, compared with vehicle controls. C, animals were implanted with 67NR cells, treated as described in (B), and monitored for tumor growth. $n = 5$ to 6. D, top, chemical structure of U-104; bottom, MDA-MB-231 LM2-4^{Luc+} cells were implanted orthotopically into NOD/SCID mice. When tumors reached an average of 200 mm^3, animals received the indicated doses of U-104 daily and tumor growth was monitored. $n = 8$/group. *, $P < 0.03$, **, $P < 0.001$. Inset, hypoxia-induced CAIX expression by LM2-4^{Luc+} cells.

Table 1. K_i values for ureido-sulfonamide and glycosyl coumarin inhibitors of CAIX

Compound	K_i (nmol/L)*			
	CAI	CAII	CAIX	CAXII
U-104	5,080	9,640	45.1	4.5
GC-204	>100,000	>100,000	9.2	43
GC-205	>100,000	>100,000	201	184

*K_i values were derived by using *in vitro* assays for CA activity as described in ref. 47.

renal clear cell carcinoma xenografts with fluorescein- and albumin-based membrane-impermeant derivatives of acetazolamide, a general carbonic anhydrase inhibitor, resulted in inhibition of tumor growth compared with vehicle-treated controls (24). These data have provided initial proof of principle for sulfonamide-based antitumor effects, but acetazolamide lacks specificity for CAIX. First, we investigated the ability of the metastatic and nonmetastatic cells to bind to a previously described, highly selective sulfonamide-based inhibitor of CAIX (CAI17) with a K_i*in vitro* of 24 nmol/L (8). This fluorescent inhibitor (Fig. 6A) interacts only with active CAIX in hypoxic conditions (8) and has been used successfully to image hypoxic xenografts (15). Similar to findings in MDCK cells expressing CAIX (30), we observed that the inhibitor was not able to bind appreciably 4T1 or 67NR cells in normoxia (Fig. 6A). In contrast, CAI17 bound to the cell surfaces of metastatic, CAIX-expressing 4T1 cells cultured in hypoxia, but not the nonmetastatic, CAIX-negative 67NR cells cultured in similar conditions (Fig. 6A). We next tested the effect of CAI17 on hypoxia-induced changes in pHe in these 2 cell types. In the absence of the inhibitor, the pHe of cultured 4T1 cells decreased significantly in hypoxia, but remained unchanged in the 67NR cultures (Fig. 6A). Treatment of the 4T1 cells with CAI17 reversed the hypoxia-induced decline of pHe, indicating functional inhibition of CAIX activity (Fig. 6A).

To evaluate the effect of pharmacologic inhibition of CAIX activity *in vivo*, we treated mice harboring established 4T1 tumors with CAI17. We observed significant inhibition of tumor growth in mice treated with the inhibitor, compared with vehicle controls (Fig. 6B). To test for the possibility of nonspecific cytotoxic activity of CAI17, we took advantage of the 67NR tumor model as a negative control. We treated 67NR cell-derived tumors by using a dosing schedule and concentrations of CAI17 identical to those used for the 4T1 model (Fig. 6C). There was no effect of the CAI17 compound relative to the vehicle control, even at the highest concentration of the inhibitor (Fig. 6C). The inhibitor concentrations and the dosing schedule were well tolerated, and no significant weight reduction was noted in any of the treated mice (Supplementary Fig. S5A and B). In addition to CAI17, we tested the effect of a novel ureido-sulfonamide inhibitor of CAIX, U-104 (Fig. 6D; Table 1), on primary breast tumor growth by using a highly metastatic variant of the MDA-MB-231 cell line (27). These cells were observed to robustly induce CAIX in hypoxia (Fig. 6D, inset). Tumor volume measurements showed significant inhibition of primary tumor growth in the mice treated with the U-104 compound compared with vehicle controls (Fig. 6D). Taken together, these data suggest the ability of sulfonamide-based CAIX inhibitors to specifically target CAIX-expressing tumors.

Having shown that selective sulfonamide-based compounds inhibit the growth of primary breast tumors, we next tested U-104 for its ability to inhibit metastasis formation in the 4T1 experimental metastasis model. Intravenous injection of 4T1 cells into mice and subsequent daily treatment of these animals beginning 24 hours postinjection with U-104 resulted in inhibition of metastases formation (Fig. 7A). Quantification of the bioluminescent signal revealed a statistically significant decrease in the formation of metastases in the treated mice (Fig. 7A), suggesting that CAIX-specific inhibition may be useful in treating metastatic disease in breast cancer. Further structure/function analyses for the ureido-sulfonamide compounds will be described elsewhere (46).

Finally, we also tested the ability of 2 additional selective inhibitors of CAIX, GC-204, and GC-205 (Fig. 7B; Table 1), to inhibit metastasis in the same model. These 2 novel compounds are glycosyl coumarins and are representatives of the coumarin class of CAIX inhibitors. GC-204 and GC-205 were effective in limiting colonization in the lungs (Fig. 7B). Quantification of the bioluminescent signal revealed a statistically significant decrease in the formation of metastases in the treated mice (Fig. 7C), with GC-205 being particularly efficacious at 15 mg/kg (Fig. 7C). Collectively, these pharmacologic studies provide strong "proof of principle" data for the therapeutic inhibition of CAIX activity for breast tumor growth and metastasis formation. Moreover, our results suggest that perturbation of CAIX function reduces metastasis both by inhibiting cell survival in hypoxia and perhaps also by preventing migration and invasion, as inhibition of CAIX reduced the metastatic burden in models of experimental metastasis.

In conclusion, our results not only solidify CAIX as a poor prognostic biomarker for human breast cancer, but also show it to be a promising therapeutic target for breast tumor growth and metastasis. Our data show that CAIX is an essential factor in the survival of tumor cells in hypoxic regions of breast tumors and, in addition, its activity contributes to metastasis in breast cancer. Its use would allow for the identification and selection of patients whose tumors are likely to metastasize, and for treatment with CAIX inhibitors to prevent this deadly process. Although CAIX expression is elevated in approximately 16% of breast cancer patients, this percentage falls in with the frequency of upregulation of Her-2 and of basal breast cancers which have the highest expression of CAIX (Supplementary Table S2). Because these subgroups of breast cancers are the most difficult to treat and are also the most aggressive in terms of metastatic potential, we suggest that CAIX inhibitors, such as those described here, should be used to treat hypoxic breast tumors with elevated CAIX expression. The development of small molecule inhibitors

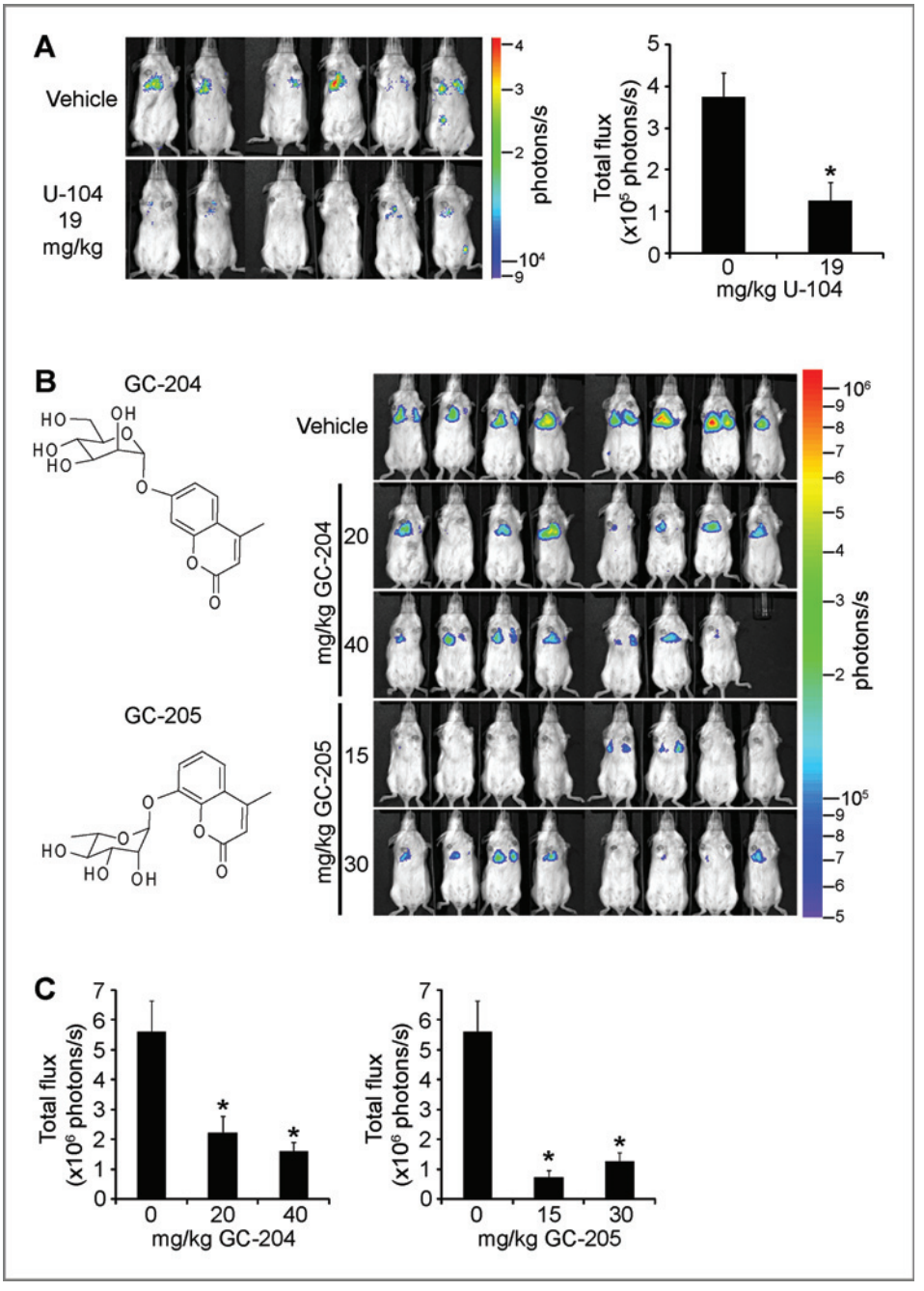

Figure 7. Novel selective small molecule inhibitors of CAIX inhibit metastasis formation by 4T1 mammary tumor cells. A, 4T1 cells were injected directly into the tail vein of BALB/c mice. Daily treatment for 5 days with vehicle or U-104 was initiated 24 hours postinoculation of cells and mice were imaged 24 hours following the final dose. The graph shows quantification of bioluminescence. $n = 6$ per group. *, $P < 0.01$. B, left, chemical structures for the 2 glycosyl coumarins, GC-204 and GC-205; right, using the experimental design outlined in (A), mice were treated daily for 6 days with GC-204 or GC-205. Mice were imaged 24 hours following the final dose. C, quantification of bioluminescence shown in (B). Data are reported as the mean ± SEM. $n = 8$/group. *, $P < 0.02$.

of CAIX activity (8), anti-CAIX neutralizing antibodies, and CAIX imaging agents, should accelerate clinical translation of our findings. Finally, our findings are likely to be applicable to other CAIX-expressing tumors.

Disclosure of Potential Conflicts of Interest

S. Dedhar and C.T. Supuran are founding members of Metasignal Therapeutics Inc.

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