Cathepsin B Inhibition Limits Bone Metastasis in Breast Cancer

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Introduction
Early detection of breast cancer has increased the 5-year survival rate to more than 85%. However, progression to metastatic disease in tissues such as lung and bone reduces the survival rate to 23% due to limited curative treatments available (1). Hence, it is important to determine the mechanisms involved in primary tumor cell invasion and spread to distant sites such as bone, to allow identification of molecular targets for new therapies.

Proteases contribute to tumor cell invasion and angiogenesis and are commonly associated with metastasis. It is now recognized that cysteine proteases play pivotal roles in cancer progression. There are 11 members in the human cysteine cathepsin family (cathepsins B, C, H, F, K, L, O, S, V, W, X/Z) and 19 in mouse (2). In normal cells, cysteine cathepsins are expressed primarily in lysosomes and have roles in antigen presentation, apoptosis, autophagy, and cellular homeostasis (3–6). Secreted cathepsin K is well documented to contribute to bone resorption and remodeling (7). In cancer, the cellular localization of lysosomal cysteine cathepsins is often altered. Intracellular, cell surface, and secreted cysteine cathepsins are involved in distinct tumorigenic processes in vivo, such as angiogenesis, invasion through extracellular matrices, and metastasis (8–10).

Of the cysteine cathepsins, B and L have been implicated most in tumorigenesis (9, 11, 12). Cathepsins B and L are prognostic markers in several types of cancer, including breast, with increased primary tumor expression associated with poor outcome (13–15). In the PyMT-induced transgenic mammary carcinoma model, ablation of cathepsin B reduces and delays lung metastasis (16), while increased expression of cathepsin B enhances metastasis in the same model (17). The prometastatic role of cysteine cathepsins has also been shown in a multistage transgenic model of pancreatic cancer. Treatment of mice with a broad spectrum cysteine cathepsin inhibitor decreases tumor vascularization and invasion (18), an effect that is enhanced when cysteine cathepsin inhibitors are combined with chemotherapy (19). Taken together, these data indicate that cysteine cathepsins have important roles in metastasis. Because bone is the most common site of distant metastases, it is a major cause of morbidity in breast cancer patients, emphasizing the importance of identifying molecular drivers of bone metastasis for new therapeutic targets. The endogenous cysteine cathepsin inhibitor stefin A is a suppressor of breast cancer metastasis to bone that is coexpressed with cathepsin B in bone metastases. In this study, we used the immunocompetent 4T1.2 model of breast cancer which exhibits spontaneous bone metastasis to evaluate the function and therapeutic targeting potential of cathepsin B in this setting of advanced disease. Cathepsin B abundance in the model mimicked human disease, both at the level of primary tumors and matched spinal metastases. RNA interference–mediated knockdown of cathepsin B in tumor cells reduced collagen I degradation in vitro and bone metastasis in vivo. Similarly, intraperitoneal administration of the highly selective cathepsin B inhibitor CA-074 reduced metastasis in tumor-bearing animals, a reduction that was not reproduced by the broad spectrum cysteine cathepsin inhibitor JPM-OEt. Notably, metastasis suppression by CA-074 was maintained in a late treatment setting, pointing to a role in metastatic outgrowth. Together, our findings established a prometastatic role for cathepsin B in distant metastasis and illustrated the therapeutic benefits of its selective inhibition in vivo.

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metastasis in breast cancer patients, determining the roles of cysteine cathepsins in bone metastasis is crucial.

Our previous studies support a role for cysteine cathepsins in bone metastasis (20). Using our unique 4T1.2 syngeneic model of spontaneous bone metastasis (21), we identified the endogenous cysteine cathepsin inhibitor stefin A as a metastasis suppressor. Tumor cell stefin A significantly reduced pulmonary and bone metastasis in the murine model and was an independent predictor of good prognosis in a cohort of 142 breast cancer patients (20). Of the cysteine cathepsins, cathepsin B was coexpressed with stefin A in primary tumors and metastases, suggesting that stefin A suppressed metastasis via inhibition of cathepsin B. This was supportive of previous studies that show that a shift in equilibrium between cathepsin B and its endogenous inhibitor stefin A predicts poor survival outcomes (22, 23).

In this study, we used our 4T1.2 model to show a critical function of cathepsin B in breast cancer metastasis to bone. Molecular suppression and selective therapeutic inhibition of cathepsin B significantly reduces pulmonary and bone metastasis. This study provides evidence that cathepsin B is a potential therapeutic target for treatment of breast cancer patients with metastatic disease.

Methods

Immunohistochemistry

All tissues were fixed in 10% neutral-buffered formalin for 24 hours, bones decalcified in 20% EDTA, pH 8.0, and embedded in paraffin. Archived human material was used in which previous informed consent was obtained from all patients. Citrate buffer (10 mmol/L, pH 6.0) high temperature and pressure antigen retrieval was required for human tissues, and immunohistochemistry (IHC) was then carried out on tissue sections as described previously (20).

Cell maintenance

The 4T1.2 lines (21) were derived in our laboratory from the parental mouse 4T1 mammary tumor cell line. The 4T1 cell line was derived by Fred Miller that, along with a series of other models, was originally derived from a spontaneous mammary tumor in Balb/c mice. Cells were maintained in alpha-modified eagle medium (α-MEM) supplemented with 5% FBS (JRH Biosciences) and cultured at 37°C in an atmosphere of 5% CO2 for no more than 4 weeks.

Generation of stable cathepsin B knockdown and base vector control clones

Short hairpin RNA (shRNA) for cathepsin B (5'-GGATGACGCTGGATACCTA-3') along with control shRNA (5'-AGTACTGCTTACGATACCG-3') lacking a murine gene target were inserted into pRetroSuper retroviral expression plasmids (Oligoengine) before transfection into PFT67 retroviral packaging cells with Lipofectamine 2000 (Invitrogen) and infection of 4T1.2 target cells. Stably transduced cells were selected with puromycin (8 μg/mL). Single cells were sorted (FACSVantage-Diva, BD Biosciences), expanded in culture, and analyzed for gene expression by real-time reverse transcription (RT)-PCR and immunoblotting. Four clones of tumor cells containing control base vector (4T1.2 BV) or cathepsin B–specific hairpins (4T1.2 CTSB kd) were pooled for subsequent analysis.

Immunocytchemistry

4T1.2, 4T1.2 BV, and CTSB kd cells were grown on uncoated glass coverslips for 24 hours, fixed in 3.7% cold paraformaldehyde, and then blocked in PBS containing 2 mg/mL BSA. Cells were then incubated overnight at 4°C with primary antibody, rabbit anti-human cathepsin B (24). Cathepsin B expression was detected with FITC Alexa Fluor 488 donkey anti-rabbit secondary antibody (Invitrogen) and imaged on a Zeiss LSM 510 META confocal microscope.

Live cell proteolysis assay

Proteolysis was measured according to previously published procedures (26, 27). Briefly, glass coverslips were coated with 50 μL of 25 mg/mL quenched fluorescent substrate DQ-collagen IV (Invitrogen) mixed with reconstituted basement membrane (rBM) Cultrex (Trevigen). For collagen I, plastic coverslips were coated with 100 μL of 25 mg/mL DQ-collagen I (Invitrogen) mixed with collagen I (Advanced BioMatrix). The ratio of quenched fluorescent components to nonfluorescent components was 1:40 for all experiments. Cells were seeded at a density of 5,000 cells per coverslip and cultured in serum-containing medium for 48 hours. Activity based probe (ABP) GB123 (1 μmol/L) was added to the media 16 to 18 hours before imaging. Fluorescence was then observed by confocal microscopy on a Zeiss LSM 510 with a 40× water immersion lens. Cell lysates labeled with GB123 were separated by SDS-PAGE and visualized by scanning with a typhoon flatbed laser scanner (excitation/emission 633/680 nm). For inhibitor studies, the highly selective cathepsin B inhibitor CA-074 was synthesized and purified in the Bogyo laboratory, CA and used at 20 μmol/L [dimethyl sulfoxide (DMSO) vehicle] and replenished after 24 hours. The fluorescent GB123 probe was produced as described previously (28).

In vivo metastasis assay

Cells (1 × 105) in PBS were mixed 1:1 with Matrigel (BD Biosciences) and injected orthotopically into the 4th mammary gland of 6-week-old female Balb/c mice (Walter and Eliza Hall Institute), 20 mice per group. Tumor volume was calculated as: volume = length (mm) × width (mm)2/2. Mice were injected (intra peritoneal, 200 μL/20 g mouse) daily with 50 mg/kg JPM-OEt (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute, MD), CA074, or vehicle (saline/5% DMSO), 3 days after tumor inoculation. After 30 days, all mice were culled upon any signs of distress due to metastasis by inhalation overdose and the organs resected. Tumor burden was measured by quantitative RT-PCR (qRT-PCR) as previously validated and published (Eckhardt and colleagues, 2005) and 3 mice out of 20 were randomly selected for histology. For qPCR, multiplex amplification of hygromycin/vimentin was used to measure tumor cell DNA signal (hygromycin) relative to a marker present in all cells (vimentin). Reactions were conducted on an ABI Prism 7000 thermocycler. Relative tumor burden (RTB) in an organ was...
calculated by: \( RTB = \frac{10,000}{25} \), in which \( \Delta C_t = C_t (\text{hygromycin}) - C_t (\text{vimentin}) \). Late treatment studies were conducted as above but with resection of primary tumors at day 15 (~0.3 g) and treatment of mice with CA-074 or vehicle daily beginning at day 20 and until signs of metastasis were evident in either group, at which point all mice were culled and assessed for metastatic burden. Any mice with primary tumor regrowth were excluded from the study. Mouse studies were conducted only after approval by the Peter MacCallum Ethics Review Board.

**Noninvasive imaging of 4T1.2 tumor-bearing burden mice**

4T1.2 cells were injected into the 4th mammary fat pad of 6-week old Balb/c mice followed 3 days later by daily intraperitoneal injections of CA-074 (50 mg/kg) or vehicle (5% DMSO/saline.) Probes (25 nmol GB123 or 2 nmol osteosense750, VisEn Imaging, Inc.) were administered through the tail vain in a solution of 67% DMSO, 33% PBS in 100 \( \mu \)L final volume, 24 hours before imaging. Mice were then anesthetized with 3% isoflurane, and tomographic images taken with the fluorescence tomography (FMT)2500 imaging system, using the 680 or 750 nm channels. All animal experiments were approved by the Stanford Administrative Panel on Animal Care.

**Statistical analysis**

Statistics were conducted with the data analysis package within GraphPad Prism 5.0 for Windows (GraphPad Software). Unless otherwise stated, tests comparing 2 means are a Student t test, with equal variance assumed. Error bars indicate SEM unless otherwise stated.

**Results**

**Expression of cathepsin B in 4T1.2 spontaneous bone metastasis mimics that of the human disease**

To support a role for cathepsin B in bone metastasis, we evaluated protein levels in a cohort of human primary tumors (\( n = 10 \)) and bone metastases (\( n = 5 \)). In agreement with previous reports (15, 29), cathepsin B was detected in primary tumor and stromal cells (Fig. 1A). Importantly, cathepsin B was also present in bone metastases. In all tumors, cathepsin B was expressed in tumor-associated stromal cells, including the local vasculature (Fig. 1A) and more than 60% of tumors expressed the protease in the tumor cells specifically. This
Figure 2. Cathepsin B knockdown in 4T1.2 tumor cells reduces the degradation of DQ-collagen. A, Western blot detection of cathepsin B protein expression in 4T1.2 parental cells, pooled 4T1.2 BV, and CTSB kd clones. B, cathepsin B activity. C, cathepsin B expression (green) in 4T1.2 cells (blue). Frame captures of 3D reconstructions of parental 4T1.2 and CTSB kd tumor cells (cyan nuclei staining) grown on rBM containing 25 μg/mL DQ-collagen IV (D; green) or collagen I containing 25 μg/mL DQ-collagen I (E; green), in the presence of 1 μmol/L GB123 (red), along with 4T1.2 cells treated with 20 μmol/L CA-074. F, quantification of GB123 cathepsin activity and DQ-collagen IV degradation products from panel D (G). H, quantification of GB123 cathepsin activity and DQ-collagen I degradation products from panel E (I). ∗∗, P < 0.05; ∗∗∗, P < 0.005; ∗∗∗∗, P < 0.0001.
staining pattern was consistent in all primary breast tumors and bone metastases examined. To ensure that the cathepsin B expression in human tumors was similar in our 4T1.2 murine model (30), expression was assessed in primary tumors and matched metastases in bone. Cathepsin B was most intense at the periphery of the primary tumors, suggestive of a role in tumor invasion through the extracellular space (ECM). In spine metastases, levels were highest in tumor cells adjacent to bone and other stromal components (Fig. 1B). This distribution of cathepsin B confirmed the value of the 4T1.2 model to dissect the function of cathepsin B in bone metastasis. Cathepsin B expression was also apparent in human and murine pulmonary metastases (Supplementary Fig. S1).

Reduced cathepsin B in tumor layers lowers spontaneous metastasis to bone

To determine the functional consequences of lowering cathepsin B levels specifically in tumor cells, we stably transduced 4T1.2 cells with cathepsin B–specific shRNA (CTSB kd) or control hairpins (4T1.2 BV). Stable knockdown of cathepsin B was verified by real-time RT-PCR (Supplementary Fig. S2) and immunoblotting (Fig. 2A). Inhibition of cathepsin B activity was also confirmed by a fluorometric assay that utilizes the selective cathepsin B substrate, Z-Arg-Arg-NHMec (25). Consistent with decreased levels, cathepsin B activity in cell lysates was significantly lower in cathepsin B knockdown cells (Fig. 2B and Supplementary Fig. S3). In addition, intracellular and membrane-associated cathepsin B staining was almost undetectable in the knockdown cell lines compared with the parental and base vector lines (Fig. 2C). Importantly for proteolysis and metastasis assays, cathepsin B knockdown did not alter cell proliferation (Supplementary Fig. S4).

To assess proteolytic activity in the 4T1.2 clonal pool, we used a recently established three-dimensional (3D) in vitro confocal assay that mimics the architecture of the tumor microenvironment and enables imaging and quantification of proteolysis by living cells (26, 27). The degradation of collagen IV or collagen I by 4T1.2 parental cells, CTSB kd, cells, and cells treated with the cathepsin B selective inhibitor CA-074 (31) was assessed in the presence of the cathepsin B selective ABP GB123. Cells were grown in rBM containing DQ-collagen IV or in collagen I containing DQ-collagen I. Images of the cells grown on rBM revealed tight 3D spheroids (Fig. 2D). In contrast, cells embedded in collagen I grew as monolayers (Fig. 2E). 3D images of cell induced proteolysis are available online (Supplementary Materials, movie M1–M6). Knockdown or inhibition of cathepsin B did not affect cellular morphology on either matrix. Importantly, GB123 staining confirmed a significant reduction in cysteine cathepsin activity in 3D cultures by CTSB knockdown or inhibition by CA-074 compared with parental 4T1.2 cells grown on either rBM or collagen I (Fig. 2F and H respectively, P < 0.005). Degradation of DQ-collagen IV was reduced by cathepsin B knockdown (Fig. 2G) and to a greater extent by the CA-074 inhibitor. In addition, cathepsin B knockdown or inhibition in 4T1.2 cells led to a significant decrease in DQ-collagen I degradation (Fig. 2F, P < 0.05). Because collagen I is the predominant bone matrix protein, this is suggestive of a role for cathepsin B in bone degradation. These studies also support the use of small-molecule cathepsin B inhibitors to reduce proteolysis.

We next injected parental 4T1.2, pooled 4T1.2 BV controls or CTSB kd cells into the mammary gland of Balb/c mice to test the impact of cathepsin B knockdown on metastasis. There was no difference in orthotopic tumor growth between the parental and CTSB kd groups (Fig. 3A) even though knockdown was maintained (Fig. 3B). Base vector control tumors expressed cathepsin B at the tumor edge as seen previously with the 4T1.2 tumors, whereas expression in CTSB kd tumor cells was negligible. Although cathepsin B knockdown did not reduce orthotopic mammary tumor growth, lung metastasis was significantly decreased in mice-bearing CTSB kd tumors compared with 4T1.2 parental or 4T1.2 BV tumors (Fig. 3C, p < 0.0001). Although control 4T1.2 BV tumors grew at a slower rate than 4T1.2 parental tumors (Fig. 3A), the metastatic burden at endpoint was not altered, hence 4T1.2 BV cells were appropriate controls for assessing the effect of cathepsin B knockdown on metastatic burden. The decrease in lung metastatic burden was confirmed by histology (Fig. 3D). Of particular interest to this study, bone (spine) metastasis was also reduced dramatically in the CTSB kd group compared with 4T1.2 (Fig. 3E, P < 0.005) and base vector control (Fig. 3E, P < 0.05). Histologic analysis of spine from 4T1.2 BV and CTSB kd tumor-bearing mice revealed tumor deposits in the control tissue that were not detectable in the spines of mice-bearing CTSB kd tumors (Fig. 3F).

Selective inhibition of cathepsin B suppresses distant metastasis

To test the therapeutic efficacy of cysteine protease inhibitors in vivo, we treated 4T1.2 tumor–bearing mice with JPM-OEt (a broad spectrum cysteine protease inhibitor; ref. 32) and the selective CA-074 inhibitor (31). Treatment of 4T1.2 tumor–bearing mice with either inhibitor had no impact on primary tumor growth (Fig. 4A and D). However, analysis of metastasis revealed a significant difference between these compounds. Treatment with the broad spectrum JPM-OEt inhibitor did not significantly reduce metastasis to lung or bone when compared with vehicle (Fig. 4B and C). In contrast, CA-074 treatment significantly decreased metastasis to lung (Fig. 4E, P < 0.05) and bone (Fig. 4F, P < 0.05). The effect of the inhibitors in vivo was further confirmed by histopathologic analysis. Visible lung and spine metastatic nodules were observed in vehicle and JPM-OEt treatment groups, whereas tumors were undetectable in the CA-074 group (Fig. 4H and I). Importantly, measurement of cysteine cathepsin B and L activity in primary tumor tissue lysates derived from mice treated with JPM-OEt and CA-074 revealed that both inhibitors reduced tumor cathepsin B activity significantly (Fig. 4G, P < 0.005), yet CA-074 was more effective. In contrast, only JPM-OEt inhibited cathepsin L activity (Fig. 4G, P < 0.05). Hence, the reduced efficacy of JPM-OEt could be due to the inhibition of other cysteine cathepsins that may have antitumorigenic functions (33) and/or due to a slightly reduced ability to suppress cathepsin B activity.

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The CA-074 treatment studies were conducted from day 3 until metastasis was evident. This did not specifically allow assessment of the role of cathepsin B in the later steps of metastasis, preventing metastatic outgrowth once tumor cells had already lodged in distant tissues. We therefore carried out a repeat CA-074 treatment experiment aimed at recapitulating a late-treatment setting. Primary tumors were removed and treatment began at day 20, a time when micrometastases can be detected in lung and bone. Analysis of metastatic burden revealed that there was a significant decrease in lung and overall bone metastases in this setting (Fig. 5), revealing a role for cathepsin B in late stages of metastatic spread.

### Noninvasive in vivo imaging of cathepsin B activity in bone metastases

We used the ABP GB123 to label active cysteine cathepsins in vivo in the presence and absence of cathepsin B.

| Figure 3. | Cathepsin B knockdown reduces spontaneous metastasis to lung and bone. A, primary tumor volumes. Error bar, SEM. B, IHC detection of cathepsin B in primary tumors from 4T1.2 BV and CTSB kd tumor-bearing mice. C, real time qPCR detection of tumor burden in lung (with hygromycin-tagged 4T1.2 cells). Relative tumor burden compares tumor cell hygromycin DNA signal to that of vimentin expression in all murine cells. D, hematoxylin and eosin (H&E)-stained lung sections of 4T1.2 BV and CTSB kd tumor-bearing mice. E, real-time qPCR detection of tumor burden in spine (with hygromycin-tagged 4T1.2 cells). F, H&E-stained spine sections of 4T1.2 BV and CTSB kd tumor-bearing mice. T, tumor region. Scale bar, 50 μm. *P < 0.05; **P < 0.005; ***P < 0.0001. |
Figure 4. Treatment of tumor-bearing mice with cathepsin inhibitors. Mice-bearing 4T1.2 tumors were treated with 50 mg/kg JPM-OEt, CA-074, or vehicle (5% DMSO/saline), 3 days after tumor inoculation. Primary tumor volumes more than 28 days in, JPM-OEt (A) and CA-074 (D) treatment groups. Error bar, SEM (n = 20). Real-time qRT-PCR of genomic DNA containing the tumor-specific hygromycin reporter gene was used for detection of tumor burden in lung (B and E) and spine (C and F) of JPM-OEt- and CA-074–treated mice, respectively. Relative tumor burden is a measure of the hygromycin signal relative to vimentin signal that is present in all cells. G, cathepsin B and L activity in vehicle-, JPM-OEt-, and CA-074–treated primary tumors. H&E–stained lung (H) and spine (I) sections of vehicle-, JPM-OEt–, and CA-074–treated 4T1.2 tumor–bearing mice. T, tumor region. Scale bar, 50 µm. *, P < 0.05; **, P < 0.005; ***, P < 0.0005.
inhibitors. Mice-bearing 4T1.2 tumors were treated with CA-074 as described previously. To visualize cysteine cathepsin activity, GB123 was injected before in vivo imaging with (FMT2500; VisEn Medical). FMT whole body imaging allowed visualization of cysteine cathepsin activity at the site of orthotopic mammary tumors (Fig. 6A). Cysteine cathepsin activity was reduced in the CA-074-treated animals even though the tumors were similar in size, suggesting that the loss in GB123 fluorescence was due to decreased cathepsin B activity (Fig. 6A). This loss was confirmed by ex vivo imaging of the tumors (Fig. 6B), however was not significant and likely due to detection of other active cysteine cathepsins such as cathepsin L in the primary tumor by GB123. The cellular localization of cysteine cathepsin activity was additionally resolved by confocal microscopy (Fig. 6C). Strongest activity was detected at the boundaries of the 4T1.2 tumors and this was lost in CA-074–treated mice. We next completed whole body scans of mice injected with both GB123 and Osteosense750 probes. Osteosense750 is a fluorescent diphosphonate imaging agent that detects active bone remodeling and hence allows bone visualization. Noninvasive whole body images of the spine region revealed GB123 signal in the spine of mice-bearing 4T1.2 tumors that aligned with Osteosense750 signal (Fig. 6D), and hence bone remodeling suggestive of an osteolytic tumor region. The GB123 signal was decreased in mice treated with CA-074 (Fig. 6D). Ex vivo images of the spines confirmed this (Fig. 6E and F). Quantitation of total GB123 fluorescence in spine, revealed a significant reduction in fluorescence in CA-074–treated compared with control-treated mice (Fig. 6G, P < 0.05).

Discussion

Bone metastases occur in a high proportion of breast cancer patients with metastatic disease and are associated with severe morbidity and eventual mortality when visceral organs become involved. Dissecting the molecular mechanisms involved in tumor cell survival and outgrowth in bone is essential for the development of targeted therapies to reduce patient mortality. Here, we report that tumor-derived cathepsin B is a key contributor to bone metastasis. Our data show for the first time that selective inhibition of cathepsin B with small-molecule inhibitors significantly reduces metastasis and has therapeutic potential.

There are a number of critical steps required for growth of tumors in bone. This includes collaboration between the tumor cells themselves and the bone stroma for stimulating angiogenesis, invasion through the bone matrix and growth beyond the micrometastatic stage. Cathepsin B upregulation has been documented in several human cancers, including breast, prostate, and melanoma (15, 34, 35). Our data show that cathepsin B has critical tumor-specific functions in breast cancer metastasis that are completely independent from primary tumor growth. We show that tumor cells themselves can directly degrade the major bone matrix protein collagen I and that cathepsin B is important, if not essential, in this process. Previous studies have implicated tumor cell cathepsin B in
degradation of ECM proteins including fibronectin, laminin, and collagen IV (36, 37). In addition, and in support of our studies, inhibition of cathepsin B has been shown to reduce collagen I degradation by prostate carcinoma cell lines (38), again suggestive of a tumor cell–specific role in bone lysis. Consistent with a role in invasion, we showed a critical function of tumor-derived cathepsin B in metastasis in the 4T1.2 model. Although orthotopic tumor growth was not altered by cathepsin B knockdown, pulmonary and bone (spine) metastases fluorescence in spines excised 24 hours after probe injection and imaged ex vivo. This indicates that cathepsin B inhibition is likely to reduce metastatic burden.

Cathepsin B has also been implicated in stromal cell–associated protumorigenic functions. In breast cancer, this has been shown with the MMTV-PyMT model in which host-derived cathepsin B promotes lung metastasis (39). The expression of cathepsin B predominately in tumor-infiltrating macrophages indicated that macrophage-derived cathepsin B contributed to metastasis. The expression of cathepsin B in tumor-associated endothelial cells and macrophages has been associated with tumor progression via promotion of angiogenesis (17, 18). Cathepsin B has also been reported to be expressed in stromal fibroblasts and macrophages in colon and prostate cancers (40, 41). Taken together, cathepsin B has key roles in tumor progression, by invasion through the ECM and stimulation of angiogenesis. Our treatment of 4T1.2 tumor–bearing mice with the selective small-molecule cathepsin B inhibitor CA-074 enabled inhibition of both tumor and stromal cathepsin B and allowed assessment of the therapeutic benefit of inhibiting this protease in vivo. Consistent with cathepsin B transcript knockdown, treatment with CA-074 did not alter primary tumor growth yet suppression of metastasis was impressive. In fact, bone metastases could not be detected histologically in tumor-bearing mice treated with the inhibitor, an impressive result considering the aggressive nature of 4T1.2 tumors. Due to the role of cathepsin B in degradation of collagen IV and I, we hypothesized that metastasis inhibition was caused by decreased invasion at both the primary and metastatic site. Our results in a late treatment setting support a role for cathepsin B in metastasis posttumor cell arrest in distant tissues. Commencement of CA-074 treatment after primary tumor resection significantly decreased metastatic growth in lung and bone. The use of cathepsin inhibitors to decrease spontaneous breast cancer metastasis to bone from the mammary gland has not been reported previously. However, use of cathepsin K (another cysteine cathepsin; ref. 42) and cathepsin G (a serine protease; ref. 43) inhibitors have been documented to suppress the formation and growth.

Figure 6. In vivo imaging of cathepsin B activity in tumor-burdened mice. A, fluorescence images of live mice-bearing 4T1.2 tumors treated with vehicle or CA-074 followed by GB123. B, total tumor fluorescence in tumors excised 24 hours after probe injection and imaged ex vivo with the FMT2500 system. C, histology of vehicle- and CA-074–treated mammary tumor tissues from A. Nuclei were visualized with DAPI. D, fluorescence spinal images of live mice-bearing 4T1.2 tumors treated with vehicle or CA-074 followed by GB123 and osteosense 750. E, total metastases fluorescence in spines excised 24 hours after probe injection and imaged ex vivo. F, histology of vehicle- and CA-074–treated spine tissues from D. Tissues were stained with DAPI, and images were taken with a 60× objective. Red, GB123 fluorescence; blue, DAPI. The colorimetric scale bars indicate nanometers of fluorescence. G, quantitation of total GB123 metastases fluorescence in vehicle- and CA-074–treated spines with the FMT2500 system. Mean fluorescence with SE is shown. *P < 0.05. Scale bar, 50 μm.
of breast cancer osteolytic lesions in experimental models of metastasis.

The therapeutic benefit of CA-074 treatment in the 4T1.2 model was in contrast to the broad spectrum inhibitor JPM-OEt, which did not decrease lung or spine metastases. In agreement with our studies, treatment of polyoma middle T oncogene-induced mammary carcinomas with JPM-OEt did not alter tumor weights and lung metastasis (33). Together, these studies reveal the importance of using specific targeted therapies in vivo. JPM-OEt targets several cysteine cathepsins, some of which could actually have tumor suppressive functions. For example, cathepsin I activity is decreased in highly metastatic 4T1.2 tumors compared with weakly or nonmetastatic tumors (20) suggesting inhibitory effects of this protease on tumor progression, as has been suggested before in skin tumors (20) suggesting inhibitory effects of this protease in tumor progression, as has been suggested before in skin tumors. The importance of using specific protease inhibitors is clear from early clinical trials using matrix metalloproteinase (MMP) inhibitors in which inhibition of certain MMPs actually has deleterious effects in patients (45, 46). These studies highlight the significant effort needed to determine which specific proteases contribute to advanced disease progression before potential drugs can be tested in the clinic.

Current treatments available for patients with bone metastasis are aimed primarily at reducing the morbidity associated with bone lysis, a hallmark of breast cancer bone disease. One target of such treatments is cathepsin K. Cathepsin K is expressed predominantly in osteoclasts in which it is secreted and has a key role in bone proteolysis, including degradation of collagen I, osteonectin, and osteopontin (47). The potential role of cathepsin K in osteolytic bone tumors is evident from its expression in tumor-associated osteoclasts (48), in breast and prostate metastases in bone, and in giant cell tumor of bone (49). Cathepsin K inhibitors block bone resorption (50), reducing the pain associated with osteolytic disease, and in turn slow the growth of bone tumors. However, as with the use of bisphosphonates that also suppress osteolysis, treatment is not curative. Therefore, an ideal treatment (or combination of treatments) is one that targets both the tumor cell and the associated stromal populations that promote osteolysis, angiogenesis, and tumor growth. The fact that cathepsin B has essential roles throughout the metastatic cascade, in tumor and stromal cells, suggests that combining cathepsin B inhibitors with conventional therapies may be of clinical value.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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