Differential cathepsin responses to inhibitor-induced feedback: E-64 and cystatin C elevate active cathepsin S and suppress active cathepsin L in breast cancer cells

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Differential cathepsin responses to inhibitor-induced feedback: E-64 and cystatin C elevate active cathepsin S and suppress active cathepsin L in breast cancer cells


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Abstract

Cathepsins are powerful proteases, once referred to as the lysosomal cysteine proteases, that have been implicated in breast cancer invasion and metastasis, but pharmaceutical inhibitors have suffered failures in clinical trials due to adverse side effects. Scientific advancement from lysosomotropic to cell impermeable cathepsin inhibitors have improved efficacy in treating disease, but off-target effects have still been problematic, motivating a need to better understand cellular feedback and responses to treatment with cathepsin inhibitors. To address this need, we investigated effects of E-64 and cystatin C, two broad spectrum cathepsin inhibitors, on cathepsin levels intra- and extracellularly in MDA-MB-231 breast cancer cells. Cathepsins S and L had opposing responses to both E-64 and cystatin C inhibitor treatments with paradoxically elevated amounts of active cathepsin S, but decreased amounts of active cathepsin L, as determined by multiplex cathepsin zymography. This indicated cellular feedback to selectively sustain the amounts of active cathepsin S even in the presence of inhibitors with subnanomolar inhibitory constant values. These differences were identified in cellular locations of cathepsins L and S, trafficking for secretion, co-localization with endocytosed inhibitors, and longer protein turnover time for cathepsin S compared to cathepsin L. Together, this work demonstrates that previously underappreciated cellular compensation and compartmentalization mechanisms may sustain elevated amounts of some active cathepsins while diminishing others after inhibitor treatment. This can confound predictions based solely on inhibitor kinetics, and must be better understood to
effectively deploy therapies and dosing strategies that target cathepsins to prevent cancer progression.

**Graphical Abstract**

![Graphical Abstract Image]

**Keywords**
Proteases; breast cancer; metastasis; pharmaceuticals; feedback; E-64; cystatin C1

1. Introduction

During cancer progression, tumor and tumor-associated cells overexpress cysteine cathepsins (Chauhan et al., 1991; Chen and Platt, 2011; Kopitz et al., 2005; Li et al., 2005; Mohamed and Sloane, 2006; Podgorski et al., 2009; Zhang et al., 2004), which are proteases belonging to the papain family of peptidases (Berti and Storer, 1995). Breast cancer cells have been reported to acidify the extracellular milieu (Montcourrier et al., 1997) which provides an optimal environment for cathepsin activity (Chapman et al., 1997). We previously showed that not only the expression, but also the amount of the active form of the cathepsins is greater in human breast tumors compared to the patient matched normal breast tissue (Chen and Platt, 2011). This has motivated the development of pharmaceutical inhibitor therapies targeting active cathepsins. Many have been efficacious in inhibiting cathepsins and slowing disease progression, but most of the clinical trials have been discontinued due to side effects (Palermo and Joyce, 2008). Only odanacatib, the selective cathepsin K inhibitor, has passed into phase III clinical trials for treatment of osteoporosis (Bone et al., 2015; Jensen et al., 2010; Palermo and Joyce, 2008). Lysosomotropic inhibitors were recognized for being problematic (Desmarais et al., 2007; Falgueyret et al., 2005; Gerbaux et al., 1996), but other consequences of cathepsin inhibitors as treatment strategies still need to be elucidated for proper deployment of these small molecule drugs.

Cathepsins not only have been implicated in degradation of extracellular matrix (ECM) proteins after their secretion into their immediate environment (Chauhan et al., 1991; Stonelake et al., 1997; Szpaderska and Frankfater, 2001), but also play important intracellular roles necessary for homeostasis and proteostasis (Mohamed and Sloane, 2006; Premzl et al., 2003; Szpaderska and Frankfater, 2001). Blocking these, non-specifically, with
inhibitors may cause the side effects observed. This was the case with lysosomotropic
cathepsin inhibitors and the reason their trials were ended (Falguery et al., 2005; Duong,
2012). Intracellular cathepsins activate other procathepsins into the mature, active form by
proteolytic cleavage of the propeptide (Dahl et al., 2001), shed surface bound proteins for
cell signaling (Sobotic et al., 2015), are involved in apoptosis when released into the
cytoplasm (Guicciardi et al., 2000; Kirkegaard and Jaätelä, 2009; Stoka et al., 2001), and
can even influence transcription by their actions on signaling proteins and transcription
factors (Goulet et al., 2007; Qian et al., 2011; Wilkinson et al., 2015).

Together, these findings have motivated this investigation of cathepsin regulation during
broad spectrum cathepsin inhibition to identify cellular responses and feedback mechanisms
that could reduce inhibitor efficacy while inducing cellular programs that could lead to
unexpected side effects. The goal was to study active cathepsin amounts and trafficking
mechanisms in cancer cells in the presence or absence of broad spectrum cathepsin
inhibitors. MDA-MB-231 breast cancer cells were selected as they are an aggressive triple
negative breast cancer cell line (Gillet et al., 2009; Sevenich et al., 2014; Sudhan and
Siemann, 2013). We treated them with cystatin C, the 13kDa protein inhibitor of cysteine
cathepsins that is ubiquitously expressed and secreted by numerous cell types, or with its
small molecule analog E-64. Cystatin C inhibits cysteine cathepsins by physically blocking
the three domains of the active site, forming a tight, nearly irreversible bond with
subnanomolar K<sub>i</sub>. E-64 interacts with the thiol group on the cysteine at the active site,
forming an irreversible bond (Abrahamson, 1994; Katunuma and Kominami, 1995). E-64 is
not used clinically since more selective cathepsin inhibitors have been developed to target
specific cathepsins (Fonović and Turk, 2014; Palermo and Joyce, 2008; Wilkinson Richard
et al., 2015), but it is useful in studying cysteine cathepsins in vitro because of its potency
and specificity to cysteine cathepsins among other proteolytic families. It also can be used as
a model broad spectrum inhibitor that cross-reacts with multiple cathepsins, an unfortunate
consequence that has been found with other cathepsin inhibitors. Here, we show that
treatment with broad spectrum cathepsin inhibitors upregulate the vesicular active cathepsin
S, but not cathepsin L, which was primarily located in the cytoplasm. This is meaningful
because cathepsin S has been implicated in cancer as well as other diseases such as
atherosclerosis (Chapman et al., 1997; Lafarge et al.; Samokhin et al.; Samouillan et al.,
2014; Sukhova et al., 2003), emphysema (Chapman et al., 1997), abdominal aortic
aneurysms (Chapman et al., 1997; Sho et al., 2004; Sukhova et al., 2005), arthritis (Hou et
al., 2002), and cystic fibrosis (Lecaille et al., 2013) and more effective treatment options are
needed.

2. Materials and Methods

2.1 Materials

Red fluorescent protein (RFP)-labeled and non-labeled MDA-MB-231 breast cancer cells
were obtained from Cell Biolabs, Inc (San Diego, CA, USA) or American Type Culture
Collection (ATCC) (Manassas, VA, USA), respectively. Anti-human cathepsin S and L
antibodies (R&D Biosystems), anti-actin (Santa Cruz Biotechnology), and secondary
donkey anti-goat antibodies tagged with an infrared fluorophore (Li-Cor) were used to detect protein with a Li-Cor Odyssey scanner.

2.2 Cell Culture

RFP tagged MDA-MB-231 breast cancer cells (Cell Biolabs, Inc.) were transfected with one of the plasmids containing full-length expression sequences of either cystatin C or an empty vector control under control by the CMV promoters (Origene) using Lipofectamine 2000 (Invitrogen). The cells were then in DMEM (Lonza) medium with 10% FBS, 1% L-glutamine, and 1% non-essential amino acids and incubated for 24 hours at 37°C. Cells were incubated with either the cysteine cathepsin broad-spectrum small molecule inhibitor E-64 (Calbiochem), the intracellular cysteine cathepsin inhibitor E-64d (Calbiochem), or the protein inhibitor of cysteine cathepsins recombinant cystatin C (BBI Solutions).

2.3 Multiplex Cathepsin Zymography

Cell lysates or conditioned media were collected after a specified incubation duration. Total protein amounts in the cell lysates were determined using the Pierce Micro BCA Protein Assay (Thermo Scientific) and prepared as previously described (Li et al., 2010). The conditioned media were concentrated using VivaSpin 500 concentrators (Sartorius Stedim Biotech GmbH) and the same amount of volume per sample was loaded. The cell lysates and conditioned media were assayed as previously described, but briefly, equal amounts of protein or volume were loaded in gelatin embedded polyacrylamide gels to separate the protein using SDS-PAGE techniques (Wilder et al., 2011). The gel was washed in renaturing buffer and assay buffer followed by staining with a Coomassie blue stain and destain. The gel was then imaged using an ImageQuant LAS 4000 (GE Healthcare Life Sciences). The bands were then quantified using ImageJ.

2.4 Western Blots

Cell lysates or conditioned media were collected after a specified incubation duration. Total protein amounts in the cell lysates were determined using the Pierce Micro BCA Protein Assay (Thermo Scientific). The conditioned media were concentrated using VivaSpin 500 concentrators (GE Healthcare) and the same amount of volume per sample was loaded. Equal amounts of protein or volume was loaded in polyacrylamide gels to separate the protein using SDS-PAGE techniques. Protein was transferred to a nitrocellulose membrane (Bio-Rad) and proteins were then probed with primary antibodies overnight at 4°C followed by an hour secondary antibody incubation.

2.5 Immunocytochemistry

Non-tagged MDA-MB-231 breast cancer cells were incubated with or without 50 μM of the cathepsin broad-spectrum small molecule inhibitor E-64 (Calbiochem). For the gelatin degradation assay, cells were also incubated with 0.05 mg/ml DQ-gelatin from bovine skin, fluorescein conjugate (Invitrogen) at 37°C for 24 hours. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X. After which cells were incubated overnight with a primary antibody against cathepsin S, cathepsin L (R&D Systems), cystatin C (Millipore), or cystatin B (Santa Cruz Biotechnology) at 4°C. The cells were then rinsed.
2.6 Quantitative RT-PCR

MDA-MB-231 breast cancer cells were incubated with and without E-64 for 24 hours at 37°C. After, the cells were lysed and RNA isolated using the RNeasy Mini Kit (Qiagen). The RNA was reversed transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen) and cDNA was amplified using SYBR® Green PCR Master Mix (Applied Biosystems) and primer sequences for cathepsin S, cathepsin L, and GAPDH.

2.7 Statistical Analysis

To calculate the weighted co-localization coefficients, the pixels with intensities from both red and green fluorescent channels were summed and divided by the sum of total red pixels. The pixel’s value is equal to its intensity value in order to take into account the brightness of the pixel. The pixel values range from zero to one. Two-tailed student paired t-test with two-sample equal variance was performed on all statistical analysis.

3. Results

3.1 E-64 treatment of MDA-MB-231s increased intracellular amounts of active cathepsin S

To determine the effects of cathepsin inhibition on levels of active cathepsins in breast cancer cells, we treated confluent MDA-MB-231 cells with a range of E-64 concentrations from 0 to 50 μM for 24 hours. This inhibitor range was chosen to include concentrations high enough to ensure uptake of the cell-impermeable inhibitor by general pinocytosis (Wilcox and Mason, 1992). The cells were then lysed and processed for multiplex cathepsin zymography to detect changes in the amount of active cathepsins. Incubation with as low as 10 μM E-64 caused a significant increase (38%) in the amount of active cathepsin S in cell lysates (n=4, *p<0.05), while the amount of cathepsin L detected was significantly decreased by 34% with as low as 5 μM E-64 (n=5, *p<0.05) (Fig. 1 A). Since cathepsin L yields a more intense zymography signal when incubated at pH 4, which also provides selectivity over other cathepsins (Wilder et al., 2011), zymography was also completed under these conditions with the same samples to confirm the loss of active cathepsin L after E-64 treatment. Even at pH 4 the 20 kDa band was not present with 50 μM E64 (Fig 1A).

Zymography only detects the active form, so to quantify the total amount of cathepsin S or L protein, cell lysates were also prepared for Western blotting. There was no significant difference in the amounts of pro- and mature- cathepsin S at any of the concentrations of E-64 by Western blot (n=6) (Fig. 1A). The amount of pro cathepsin L and short chain cathepsin L was increased with 50 μM E-64 (n=8, *p<0.05). The active and total amounts of cathepsins was quantified using ImageJ analysis (Supplemental Fig. S1).

To determine if this result of E-64 causing differential effects between cathepsins L and S was temporal, a time course of E-64 treatment was completed using the highest dose of 50 μM compared to 0 μM E64. Equal amounts of protein were loaded for multiplex cathepsin zymography with aliquots assayed at pH 6 and at pH 4, to specifically intensify the signal from cathepsin L. As early as 8 hours, E-64 stimulated a 94% increase in the amount of
active cathepsin S (Fig 1B) with a maximum of 160% by 24 hours compared to controls (n=5, ** p < 0.01) (Fig 1C). Cathepsin L, however, was reduced by 57% after 8 hours and by 77% after 24 hours (n=3, ** p < 0.01) (Fig 1D). Western blots of cathepsin L and S protein over this time course and quantification are included in Supplemental figure 2.

This differential effect of E-64 on cathepsin L vs. cathepsin S led us to question which of these proteases would be interacting with substrates internalized for degradation. As a membrane impermeable inhibitor, E-64 must be uptaken by endocytosis from the extracellular environment. To confirm the location of the cathepsins within the cells and their access to substrate, MDA-MB-231 cells were incubated with DQ-gelatin, a fluorogenically quenched gelatin that produces fluorescence when cleaved, and treated with 50 μM E-64 or vehicle for 24 hours. After which, the cells were fixed, immunostained for cathepsins S or L, and imaged using confocal microscopy. With E-64 treatment, cathepsin S co-localized with the fluorogenic substrate in vesicular compartments (Fig 2A), but cathepsin L did not (Fig 2B). This co-localization of cathepsin S with substrate was increased with E-64 treatment. This was also the condition in which more active cathepsin S was detected by zymography (Fig 1A). This suggested that the inhibitor treatment elevated the amount of active cathepsin S which co-localized with substrate in a different way than it does for cathepsin L.

3.2 Inhibition with cystatin C also upregulates the amount of active cathepsin S

E-64 is a small molecule that uses the same mode of inhibition as cystatin C, the endogenous cysteine cathepsin protein inhibitor encoded in the genome and constitutively secreted by most enucleated cells and present at high concentrations in all extracellular fluids (Abrahamson et al., 1986). To test if the protein inhibitor would induce the same response as the small molecule pharmaceutical inhibitor, we incubated MDA-MB-231 cells with cystatin C and monitored if it would upregulate the amount of active cathepsin S protein, similar to E-64. After 24 hours of incubation with increasing concentrations of cystatin C, cells were lysed and assayed by zymography and by Western blotting for cystatin C protein (Fig 3A). As with E-64, there was a dose dependent increase in the amount of active cathepsin S in response to cystatin C treatment with a 58% increase at 500 nM and 1 μM of cystatin C (n=6, * p < 0.05 and ***p < 0.005, respectively) (Fig. 3B). Similar to E-64 treatment, procathepsin L protein amounts were upregulated with 1 μM cystatin C incubation (Supplemental Fig. S3). Intracellular cystatin C levels were also elevated suggesting that the exogenously added cystatin C protein was being endocytosed by the cells during treatment.

We then asked if overexpressing cystatin C would also lead to increased amounts of cathepsin S, analogous to an autocrine feedback loop. To test this, MDA-MB-231 cells were either transfected to overexpress cystatin C, an empty plasmid, or mock transfected (Fig 3C). Compared to the mock and empty plasmid transfected group, cystatin C overexpression increased the amount of active cathepsin S (n=4, * p < 0.05), but did not decrease active cathepsin L (n=3, p=0.2) (Fig. 3D). These studies were repeated with E-64 treatment to test if the combination with cystatin C overexpression would cause a synergistic effect on the amount of active cathepsin S. There was no significant difference in active cathepsin S with combined E-64/CysC treatment than just overexpressing cystatin C alone as confirmed by
the quantification (Fig. 3D). Active cathepsin L reduction still occurred with the combination of E-64 treatment and cystatin C overexpression (n=3, *p< 0.01), but the amount of procathepsin L increased under these conditions. Together, this suggests treatment with cathepsin inhibitors, whether small molecule or native protein inhibitor cystatin C, caused elevated amount of active cathepsin S and reduced active amount of cathepsin L.

3.3 Cathepsin S protein turnover is slower than that of cathepsin L

The next goal was to delineate if the results above were due to differences in cathepsin S and L protein turnover or feedback regulation at the transcriptional level. Cycloheximide was used to halt protein synthesis in MDA-MB-231 cells that were treated with or without exogenous cystatin C for 24 hours. There was a significant reduction in cathepsin L signal when cells were treated with cycloheximide, but no significant difference for cathepsin S (Fig 4; n=3, *p<.001) confirming that cathepsin S protein turnover is slower than cathepsin L. Of note, intracellular cystatin C levels were significantly elevated only when cystatin C was added exogenously, in the presence or absence of cycloheximide, which confirmed that it was being endocytosed by the MDA-MB-231 cells and not being produced by them (n=3, p < 0.001). Quantitative real-time PCR for cathepsins L and S after treatment with E-64 did not show increased mRNA levels; there was a significant decrease for cathepsin S mRNA and no change for cathepsin L mRNA (Supplemental Fig S4). This suggests that protein turnover and post-translational control is more critical to yielding these cathepsin responses to inhibitors than transcriptional regulation.

3.4 Cystatin C co-localized with cathepsin S, but not cathepsin L, when MDA-MB-231 cells were treated with exogenous cystatin C

With the different responses between active cathepsins S and L to either the E64 treatment or cystatin C incubation, it became important to determine if cystatin C co-localized with either cathepsin S or L. MDA-MB-231 cells were incubated in the presence or absence of 500 nM cystatin C for 24 hours, followed by immunostaining for cystatin C, cathepsin S, and cathepsin L. Co-localization was quantified using the Zeiss co-localization analysis to calculate the weighted co-localization coefficient by summing the weighted pixels with intensities from both red and green fluorescent channels and dividing by the sum of total red pixels. Cathepsin S and cystatin C co-localized (Fig. 5A) after exogenous addition and endocytosis of cystatin C, but cathepsin L did not (Fig 5B). This suggested that cathepsins S and L were in separate intracellular compartments. Co-immunostaining for cathepsins S and L, in the presence or absence of exogenously added cystatin C, did not yield overlapping signal, which confirmed that these two proteases were in separate cellular locations (Fig. 5C). Quantification of the weighted co-localization coefficient was used to confirm these findings (n = 3, *p < 0.005) (Fig. 5D).

3.5 Cathepsin L is in the cytoplasm of MDA-MB-231 cells, but cathepsin S is intravesicular

Cathepsin L staining patterns in the MDA-MB-231 cells were not the canonical punctate lysosomal pattern that display intravesicular patterns, and it has been reported previously that cathepsin L was found in the cytoplasm of cancer cells and podocytes (Goulet et al., 2007; Sever et al., 2007). To test if cathepsin L was located in the cytoplasm, MDA-MB-231 cells were co-immunostained for either cathepsin L or cathepsin S with cystatin B, also
known as stefin B, a cytoplasmic cysteine protease inhibitor (Calkins et al., 1998). There was dominant co-localization between cathepsin L and cystatin B as indicated by the overlapping yellow fluorescence, which was confirmed by the weighted co-localization coefficient (n=6, *p < 0.05) (Fig 6). Almost all the immunopositive cathepsin L was in vesicular compartments immunopositive for cystatin B (Fig. 6A), and no co-localization was observed between cathepsin S and cystatin B (Fig. 6B).

As confirmation, E-64d, which is membrane permeable but converts to the membrane-impermeable E-64c derivative upon cleavage of the ethyl ester in the cytoplasm (Katunuma and Kominami, 1995; McGowan et al., 1989), was incubated with MDA-MB-231 cells at increasing concentrations for 4 hours to specifically inhibit cytoplasmic cathepsins. This earlier time point was chosen to minimize any inhibitor-induced transcription or translational cathepsin feedback that could occur over the 24-hour period, and to inhibit the cathepsins present in the cytoplasm during this short window. Cathepsin L inhibition was evident by as low as 1 μM E-64d (n=4, * p < 0.05), but the amount of active cathepsin S was unchanged (Fig. 6D) (Supplemental Fig. S5), indicating that cathepsin L, but not cathepsin S, was susceptible to inhibition by the cytoplasmic cysteine protease inhibitor.

3.6 Cathepsin L is secreted by MDA-MB-231 cells, but cathepsin S is not

Since cathepsins S and L were not in the same compartments, we hypothesized that they were being differentially trafficked intracellularly. To test if these different compartmental locations were related to selective cathepsin secretion, amounts of secreted cathepsin S and L were investigated. Serum free conditioned media from MDA-MB-231 cells incubated in the presence or absence of 50 μM E-64 were collected after 24 hours, centrifugally concentrated, and equal volumes of the media were loaded and examined by Western blot. Minimal amounts of extracellular cathepsin S was detected, but cathepsin L was abundant in the conditioned media (Fig. 7A) indicating that cathepsin L may not only be in the cytoplasm, but also in secretory vesicles being shuttled out. Similar results were seen from the conditioned media collected from cells incubated with 500nM cystatin C for 24 hours (n=3) (Fig. 7B). There was no significant difference of the cathepsin protein amounts in the conditioned medium between untreated and inhibitor-treated groups.

4. Discussion

This study has shown that treatment of breast cancer cells with broad spectrum cathepsin inhibitors induced compensatory mechanisms resulting in elevated amounts of active cathepsin S while inhibiting cathepsin L. This inhibitor-induced feedback occurred with either a small molecule inhibitor, E-64, or protein inhibitor, cystatin C, indicating that the inhibition, and not the molecule directly, was causal for inducing this type of unexpected feedback response. Although both cathepsins S and L are members of the lysosomal cysteine cathepsin family belonging to the papain family of peptidases, these findings demonstrate that they have opposing feedback responses to inhibition. Cathepsin L’s susceptibility to inhibition by E-64 impaired processing of the cathepsin L protein resulting in accumulation of procathepsin L, and was largely immunopositive in the cytoplasm. These differences in cellular locations could also be for their alternative trafficking since cathepsin
L was predominantly located in the cytoplasm, but moderate intravesicular staining, distinct from cathepsin S, could be for vesicles targeted for secretion since cathepsin L was detected in the conditioned medium but cathepsin S was not. Cathepsin S was being targeted to intravesicular endosomes/lysosomes, confirmed by its co-localization with endocytosed fluorogenic substrate, and lack of secreted cathepsin S being detected. The slower protein turnover of cathepsin S determined after halting protein synthesis with cycloheximide confirmed a separate mechanism by which the active amounts of cathepsins S and L could differ and how the presence of stabilizing inhibitors might be contributory.

Upregulation of one or more active cathepsin proteins in response to broader, non-selective inhibition could be a mechanism behind unexpected side effects observed with the testing of other cathepsin inhibitors. A phase I clinical trial demonstrated similar findings as ours with the cathepsin S inhibitor LY3000328, which led to increased cathepsin S activity and overall protein mass in the plasma of healthy subjects after treatment (Payne et al., 2014). Cellular compensatory mechanisms, such as the sustained elevated levels of active cathepsin S could be utilized to ensure degradation of lysosomal cargo during inhibitor treatments to maintain proteolytic homoeostasis. This inhibitor-induced elevation in the amount of active cathepsin S was not mediated at the transcriptional level since inhibitor treatment did not increase cathepsin S mRNA (Supplemental Fig. S4). Instead, the level of regulation was likely post-translational, and due to increased stabilization or activation of the active cathepsin S protein by the inhibitors or other cell factors. Even after new protein synthesis was halted for 24 hours with cycloheximide, cathepsin S protein was detected in the cells, but the cathepsin L protein signal was significantly diminished (Fig 4).

The concentrations of cystatin C used in this study (0–1 μM) are in the physiological range reportedly ranging from 0.1 to 3 μM in human body fluids (Abrahamson et al., 1986). Endocytosis of cystatin C does occur and can accumulate in lysosomes at high concentrations (Ekström et al., 2008; Merz et al., 1997), but it also dimerizes in the acidic environment of the lysosomes reducing its inhibitory capacity (Merz et al., 1997) and allowing cathepsin-mediated substrate degradation. Therefore, the upregulated cathepsin S detected in vesicles with cystatin C endocytosis could lead to elevated intracellular cleavage of substrate (Fig. 4A and 2A), while preventing extracellular substrate cleavage in this microenvironment.

Even though it was suggested that the stabilization of bound cathepsin proteins to inhibitors could be contributing to the elevated protease activities (Desmarais et al., 2007), our results indicate that the inhibitor-induced feedback occurring in breast cancer cells is also dependent on localization of the cathepsin. Although cathepsin L was initially deemed a lysosomal cysteine protease, immunostaining largely placed it in cytoplasm of MDA-MB-231 breast cancer cells (Fig. 5). This finding is supported by cytoplasmic cathepsin L in podocytes, where it was involved in cleavage of dynamin, which resulted in cytoskeleton reorganization (Sever et al., 2007). Nuclear cathepsin L has also been reported to cleave the CCAAT-displacement protein/cut homeobox transcription factor (Goulet et al., 2007; Goulet et al., 2006) and cleave and modify histones (Adams-Cioaba et al., 2011; Bulynko et al., 2006). These studies indicate functional roles for cathepsin L in other cellular locations, and
downregulation of cathepsin L during inhibitor treatment could disrupt these cellular responses as a consequence.

Previous reports have investigated various cathepsin inhibitors in vitro and in vivo and indicate differences in cathepsin specific responses in rodent models. The less potent cysteine cathepsin inhibitor leupeptin was shown to increase cathepsin B activity in rat fibroblasts as well as in mouse calvaria, while E-64 still maintained inhibitory effects on the cathepsin B activity (Montenez et al., 1994). In addition, the more potent inhibitor EP-475, also known as E-64c (Barrett et al., 1982), was found to increase the amount of and extend the half-lives of cathepsins B, H, and L protein in the liver of rats (Kominami et al., 1987), which may be occurring here for cathepsin S in human breast cancer cells.

In conclusion, we demonstrated that a feedback mechanism within the cathepsin proteolytic network occurs upon broad-spectrum inhibition of cysteine cathepsins in breast cancer cells. Understanding these cell-mediated feedback mechanisms will be critical for developing effective therapeutics and dosing strategies targeting cysteine cathepsins for treating cancer invasion and metastasis. Breast cancer therapies have been successful with in vitro or in vivo animal models (Bell-McGuinn et al., 2007; Jensen et al., 2010), but have not yet been translated to the clinic due in part to side effects elicited by small molecule therapeutics (Bromme and Lecaille, 2009; Le Gall et al., 2008; Palermo and Joyce, 2008). Even though these cathepsin inhibitors were designed to be highly selective, off target inhibition are increased when inhibitors enter into the intracellular environment (Bromme and Lecaille, 2009; Desmarais et al., 2007; Falgueyret et al., 2005). These mechanisms must be further elucidated to successfully deploy these well-designed inhibitors to prevent pathological cathepsin activity in breast cancer and other tissue destructive diseases such as cardiovascular disease, arthritis, tendinopathy, endometriosis, and cystic fibrosis, which have had cathepsin upregulation implicated (Chapman et al., 1997; Hou et al., 2002; Lafarge et al.; Porter et al., 2015; Samokhin et al.; Seto et al., 2015; Sukhova et al., 2003; Voynow et al., 2008).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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Figure 1. Inhibitor-induced increase of active cathepsin S and reduction of cathepsin L occurs intracellularly in MDA-MB-231 breast cancer
(A) MDA-MB-231 cells incubated with increasing amounts of E-64 for 24 hours were lysed and equal amounts of total protein loaded for multiplex cathepsin zymography with either a pH6 or pH4 assay buffer incubation (n=3 for each treatment, *p<0.05). The same lysates from MDA-MB-231 cells treated with varying E-64 doses for 24 hours were run with Western blot analysis and probed for intracellular pro- and mature- forms of cathepsin S and cathepsin L. Increased amounts of active cathepsin S were detected by zymography, but E-64 treatment decreased amounts of active cathepsin L. There were no changes in cathepsins S and L protein in the Western blots (n=6). (B) MDA-MB-231 cells were incubated with 50 μM E-64 or a vehicle control for 0, 2, 4, 8, 12, and 24 hours and lysates were collected at each time point. Equal amounts of total protein were loaded for multiplex cathepsin zymography with either a pH6 or pH4 assay buffer incubation. From the time course, the amounts of active cathepsin S (C) and cathepsin L (D) were quantified using ImageJ analysis (n=3, *p<0.05, ** p<0.01).
Figure 2. E-64 increased cathepsin S localization with intracellular gelatin substrate degradation, but not for cathepsin L.

MDA-MB-231 cells were incubated with DQ-gelatin along with either 50 μM E-64 or vehicle control for 24 hours. The cells were then fixed and immunostained for (A) cathepsin S (red) or (B) cathepsin L (red). Representative confocal images of degraded DQ-gelatin (green) co-localized with cathepsin S or cathepsin L are depicted. Scale bar, 10 μm.
Figure 3. Inhibitor induced cathepsin S upregulation also occurs with cystatin C, but without reducing active cathepsin L

(A) MDA-MB-231 cells treated with exogenously added recombinant cystatin C protein for 24 hours at increasing concentrations were lysed and equal protein amounts from cell lysates were loaded for multiplex cathepsin zymography and Western blotting for cystatin C. The amount of active cathepsin S was significantly higher with increased cystatin C doses, (B) as quantified (*p<0.05, **p<0.005). (C) MDA-MB-231 cells were either mock transfected, transfected with an empty plasmid, or cystatin C overexpression plasmid followed by incubation with E-64 for 24 hours. After 24 hours, the cells were lysed, equal amounts of protein were loaded for multiplex cathepsin zymography with a pH6 or pH4 assay buffer and quantification shown (D). The amount of active cathepsin S in the cystatin C transfected cells increased compared to the mock and empty vector control (M, mock; E, empty; C; cystatin C) (n=3, *p<0.05; n=4, *p<0.05, **p<0.01).
Figure 4. Cathepsin S protein turnover is slower than that of cathepsin L

(A) MDA-MB-231 cells were treated with or without exogenous cystatin C and 0.75 μg/ml cycloheximide (CHX) or vehicle (veh) for 24 hours. After treatment, equal protein amounts were loaded for Western blot to determine intracellular amounts of cathepsin S, cathepsin L, and cystatin C after halting protein synthesis. (B) Densitometry quantification of the procathepsin bands indicate significant reduction in cathepsin L signal when cells were treated with CHX, but no significant difference for cathepsin S (n=3, *p<.001). (Pro-procathepsin; mat-mature; DC-double chain; cysC-cystatin C).
Figure 5. Cystatin C and cathepsin S co-localize in vesicles with inhibitor treatment, but not with cathepsin L

(A) MDA-MB-231 cells were treated for 24 hours with 500 nM exogenous cystatin C, then immunostained for cathepsin S (green) and cystatin C (red). Representative confocal images of the immunofluorescent images are shown. Note the cathepsin S and cystatin C co-localization in large vesicles after exogenous cystatin C treatment (white arrows). Scale bars, 10 μm. (B) Cathepsin L (green) and cystatin C (red) co-localization was determined. Scale bars, 10 μm. (C) Cathepsin S (red) and cathepsin L (green) co-localization. Scale bars, 5 μm. (D) The co-localization between proteins was quantified using Zeiss co-localization analysis to calculate the weighted co-localization coefficient (n=3, *p<0.005).
Figure 6. Cathepsin L is in the cytoplasm and co-localizes with cystatin B

(A) MDA-MB-231 cells were fixed and immunostained for cathepsin L (green) or cystatin B (red), with nuclear staining (DAPI, blue) or (B) cathepsin S (Alexa Fluor ® 488, green), cystatin B (Alexa Fluor® 568, red), and nuclear (DAPI, blue). Representative confocal images of the immunostained MDA-MB-231 cells shows cathepsin L and cystatin B cytoplasmic co-localization. Scale bars, 20μm and 5μm on the zoomed-in images. (C) The co-localization between cathepsin L or cathepsin S with cystatin B was quantified using Zeiss co-localization analysis to calculate the weighted co-localization coefficient (n=6, *p<0.05). (D) MDA-MB-231 cells treated with E-64d dose curve for 24 hours were lysed and equal amounts of protein lysates were loaded for multiplex cathepsin zymography with pH 6 and pH 4 assay buffers. Intracellular active cathepsin L decreased with as little as 1 μM E-64d (n=4, p<0.001), but there was no difference in the amount of active cathepsin S (n=5).
Figure 7. **Cathepsin L was secreted by MDA-MB-231 cells but cathepsin S was not, regardless of inhibitor treatment**

Serum free conditioned media from MDA-MB-231 cells incubated with A) 50 μM E-64 or B) 500 nM cystatin C for 24 hours was collected, concentrated, and equal volumes were loaded for Western blots (n=3). Cathepsin S had minimal detection by immunoblot but cathepsin L was abundantly detectable.