Original Article

Dynamic Model of Protease State and Inhibitor Trafficking to Predict Protease Activity in Breast Cancer Cells

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Abstract

Introduction—Cysteine cathepsins are implicated in breast cancer progression, produced by both transformed epithelial cells and infiltrated stromal cells in tumors, but to date, no cathepsin inhibitor has been approved for clinical use due to unexpected side effects. This study explores cellular feedback to cathepsin inhibitors that might yield non-intuitive responses, and uses computational models to determine underlying cathepsin-inhibitor dynamics.

Methods—MDA-MB-231 cells treated with E64 were tested by multiplex cathepsin zymography and immunoblotting to quantify total, active, and inactive cathepsins S and L. This data was used to parameterize mathematical models of intracellular free and inhibited cathepsins, and then applied to a dynamic model predicting cathepsin responses to other classes of cathepsin inhibitors that have also failed clinical trials.

Results—E64 treated cells exhibited increased amounts of active cathepsin S and reduced amount of active cathepsin L, although E64 binds tightly to both. This inhibitor response was not unique to cancer cells or any one cell type, suggesting an underlying fundamental mechanism of E64 preserving activity of cathepsin S, but not cathepsin L. Computational models were able to predict and differentiate between inhibitor-bound, active, and inactive cathepsin species and demonstrate how different classes of cathepsin inhibitors can have drastically divergent effects on active cathepsins located in different intracellular compartments.

Conclusions—Together, this work has important implications for the development of mathematical model systems for protease inhibition in tissue destructive diseases, and consideration of preservation mechanisms by inhibitors that could alter perceived benefits of these treatment modalities.

Keywords—Cathepsins, Mathematical, Cancer, Clinical trials, Zymography.

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ABBREVIATIONS

Pro	Procathepsin
Mat	Active mature cathepsin
Mat _{inact}	Inactive mature cathepsin
MatE64	Cathepsin bound to E64
E64	Cathepsin inhibitor E64
k_{prod}	Rate of cathepsin production
k_{act}	Rate of cathepsin activation
k_{inact}	Rate of cathepsin inactivation
k_{E64}	Rate of cathepsin inhibition by E64
k_{deg}	Rate of degradation for active mature cathepsin
k_{degI}	Rate of degradation for inactive mature cathepsin
k_{degE64}	Rate of degradation for inactive mature cathepsin: E64 complex
$k_{\text{in,E64}}$	Rate of E64 influx
$k_{\text{out,E64}}$	Rate of E64 outflux

INTRODUCTION

Recent advances in breast cancer treatment have drastically increased survival rates for early stage cancer patients, but metastatic stage 4 breast cancer remains extremely difficult to treat with a 5-year survival rate of only 22 percent.⁸ Cysteine cathepsins, including cathepsins K, L, S and V, are proteases that have been implicated in cancer, with recent research revealing integral roles of cysteine cathepsins in promoting tumor growth and metastasis primarily through the cleavage of extracellular matrix substrates including collagen, elastin and laminin.^{26,28} Cysteine cathepsins are attractive pharmaceutical targets for the

prevention of tumor metastasis since they not only are produced by the cancer cells themselves, but also participate in the accessory stromal cells of the tumors and the secondary metastatic sites, as with bone metastases. While some cathepsin inhibitors have shown efficacy in treating bone metastases, to-date, no cathepsin inhibitors have been approved for clinical use due to side effects, particularly skin lesions.^{6,22} The mechanisms responsible for these side effects are unknown, but could include inhibition of off-target proteases, inhibition in off-target tissues, or disruption of proteolytic networks necessary for normal cathepsin regulation.

Cathepsin expression and activity is regulated by a complex proteolytic network consisting of a wide variety of proteases, specific endogenous inhibitors and biological substrates.¹⁶ Cathepsins are translated as inactive procathepsins, which must be cleaved, either autocatalytically or by another protease, before they can become active mature enzymes capable of degrading substrates.^{19,37} Changes to the localization, expression or activity of the proteases responsible for cleaving procathepsins can prevent or stimulate cathepsin activity. Cathepsins and other proteases are also capable of degrading mature enzymes, preventing them from degrading substrates. We have previously reported that cathepsin S is capable of degrading cathepsin K, even in the presence of extracellular matrix substrates, resulting in an overall decrease in collagen proteolysis.² Given the contrary ability of proteases to both increase and decrease the activity of other proteases, it is unsurprising that inhibiting a single protease can cause unexpected side effects in clinical trials and in laboratory settings.

Cathepsin inhibitors have a history of provoking unexpected results *in vivo* and *in vitro*.^{4,38} E64 (*trans*-Epoxy succinyl-L-leucylamido(4-guanidino)butane) is a cell impermeable, small molecule, broad inhibitor of the cysteine cathepsin family,¹⁷ and there is an endogenous protein inhibitor of all cysteine cathepsins, cystatin C.¹ Our group has previously shown that treatment of MDA-MB-231 cells with the small-molecule cathepsin inhibitor E64 can increase the amount of active cathepsin S, while decreasing the amount of active cathepsin L in breast cancer cells.⁴⁰ In MDA-MB-231, cathepsin S is located in endolysosomal vesicles where membrane impermeable E64 is transported through endocytosis before escaping into the cytoplasm where it can bind cathepsin L.⁴⁰ The underlying mechanism responsible for this unexpected reaction to inhibitor treatment has not been previously established.

While pharmaceutical cathepsin inhibitors display strong, nanomolar enzyme specificity *in vitro*, inhibitor specificity can decrease substantially in intracellular environments. Many early cathepsin inhibitors were

lysosomotropic, weakly basic, lipophilic molecules capable of passing through lipid membranes until they are protonated in acidic compartments such as the lysosome.³ Lysosomotropic inhibitors, such as bafilomycin, have shown high intracellular potency, but poor specificity, preventing them from passing clinical trials.^{7,34} Emerging non-basic, non-lysosomotropic cathepsin inhibitors have shown improved *in vivo* selectivity and efficacy in clinical trials for osteoporosis and metabolic bone disease, but even these more specific inhibitors have resulted in unexplained side effects in patients.⁹

Unravelling the specific interactions of this network is difficult with only experimental methods due to the number of molecules involved and limitations of tracking specific molecules intracellularly. Mathematical modeling of cathepsin kinetics has been instrumental in characterizing the unique responses of recombinant proteins to substrates and inhibitors *in vitro*.^{24,25}

This study seeks to uncover the post translational regulatory mechanisms responsible for the previously observed increase in active cathepsin S following cathepsin inhibitor treatment in breast cancer cells. Additionally, this work expands the focus from the MDA-MB-231 triple-negative breast cancer cell line, to other breast cancer and macrophage cell lines, as well as primary human tumor and patient-matched normal tissue. This work utilizes a combination of experimental and mathematical modeling approaches to explore the mechanism responsible for previously observed increase in active cathepsins following cathepsin inhibitor treatment. The development of accurate cathepsin-inhibitor mathematical models will be instrumental to designing cathepsin inhibitor regimens capable of suppressing tumor growth and metastasis in breast cancer patients.

MATERIALS AND METHODS

Cell Culture

MDA-MB-231, MCF-7, and MCF-10A cells were incubated in DMEM (Lonza) medium with 10% FBS, 1% L-glutamine, and 1% non-essential amino acids and at 37 °C with 5% CO₂. Thp-1 macrophages were derived from Thp-1 monocytes cultured in RPMI-1640 (Lonza) medium with 10% FBS, 1% L-glutamine and 1% Penicillin–Streptomycin. Thp-1 monocytes were differentiated into macrophages by treatment with 0.1 μM phorbol 12-myristate 13-acetate (PMA) for 24 h, followed by 24 h in fresh medium without PMA before E64 (*trans*-Epoxy succinyl-L-leucylamido(4-guanidino)butane) treatment.

Human Tissue

Human cancer and normal breast tissue specimens were from National Disease Research Interchange (Philadelphia, PA).

Multiplex Cathepsin Zymography

Total protein concentration of cell lysate and homogenized tissue was measured using the Pierce Micro BCA Protein Assay (Thermo Scientific). Equal quantities of protein were prepared in nonreducing SDS-PAGE loading buffer and cathepsin zymography was performed as previously described.^{14,39} Briefly, samples were loaded into a gelatin-embedded polyacrylamide, lysate proteins were separated by molecular weight during electrophoresis. Following electrophoresis, cathepsins in gels were renatured before being incubated overnight in cathepsin-specific assay buffer at 37 °C. The gels were then stained with Coomassie Blue, then destained. White bands indicate the presence of active cathepsins capable of degrading gelatin embedded in the polyacrylamide gel. Lysate from RAW 264.7 murine macrophages was loaded and used as a positive control, as these cells are known to produce large amounts of active cathepsins.

Systems Modeling of Cathepsin Dynamics

Previous studies of cathepsins in the MDA-MB-231 cell line have shown that cathepsin S and L are localized in distinct compartments.⁴⁰ Accordingly, we have assumed that cathepsin S and L dynamics are driven by independent systems, and the network diagram (Fig. 1) shows the assumed structure, variables and parameters of both systems. The schematic shows relationships between the four cathepsin species, as well as, the presence of the inhibitor, E64, which has been reported to irreversibly bind to mature active cathepsin S or L.

Based on the diagram shown in Fig. 1, we have derived a system of ordinary differential equations (ODE) that describe cathepsin dynamics using simple mass action (Eqs. (S1)–(S5)). This system of equations contains five dynamic variables corresponding to the four cathepsin species and E64, as well as, nine kinetic parameters describing the fluxes within the system. Based on our zymography and Western blot data we aimed to identify values of the kinetic parameters that are in agreement with our experimental findings. Western blot images are not included here as they were previously published and can be viewed there.⁴⁰

Baseline levels of total and active cathepsins in breast cancer cells were approximated from standards of recombinant cathepsins used in loading previous

Pro = Procatepsin

Mat = Active mature cathepsin

Mat_{inact} = Inactive mature cathepsin

MatE64 = Cathepsin bound to E64

E64 = Cathepsin inhibitor E64

k_{prod} = Rate of cathepsin production

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k_{deg} = Rate of degradation for active mature cathepsin

k_{degI} = Rate of degradation for inactive mature cathepsin

k_{degE64} = Rate of degradation for inactive mature cathepsin:E64 complex

$k_{in,E64}$ = Rate of E64 influx

$k_{out,E64}$ = Rate of E64 outflux

FIGURE 1. Model reaction diagram of cathepsin state and inhibition in cancer cells. Cathepsins are translated as inactive procatepsins that are activated to mature cathepsins. Mature cathepsins can bind to E64, be degraded or inactivate through non-E64 mediated mechanisms. Abbreviations for model variables and parameters are shown.

Western blots and zymograms. We compared our measurements with published literature values and we were within an order of magnitude with reasonable assumptions based on cell type and cathepsin levels.⁴² These baseline values were then used to scale the E64 induced fold change in total and active cathepsins.

Parameterization of the dynamic model was performed using a steady state approach. Endocytosis of membrane impermeable E64 increases the E64 concentration in the lysosomal compartment, which is where cathepsin S is localized. We assumed the lysosomal E64 concentration reaches a steady state concentration equal to the specified media concentration after 24 h based on previous experimental observations, which found E64 induced feedback on cathepsins was dose dependent, and the appeared to reach a steady state by 24 h.⁴⁰ Steady state concentrations of cathepsins and E64 in the cytosol, which is where cathepsin L is localized, were scaled based on an approximation that the cytosol volume is 35 times larger than the lysosomal volume. Based on these assumptions, and the proposed system of ODEs (SEqs. (1)–(5)), steady state equations were defined for the three tested concentrations of E64: 0, 10, and 50 μ M. This system of equations includes seven unknown steady state kinetic parameters, as well as, steady state

concentrations of cathepsin species that are not directly measurable based on available experimental data. Detailed equations (SEqs. (6)–(28)) and parameter estimation steps are included in Supplementary Methods.

The steady state equations were reduced to a system of six equations (SEqs. (29)–(34)) with six cathepsin-related kinetic parameters as unknowns. SEquation (29)–(32) define the relationship between the kinetic parameters and the steady state concentrations, while SEqs. (33) and (34) serve as constraints connecting the steady state concentrations to the experimental data.

Parameter estimation was initially performed for cathepsin S and L, where the squared residuals for SEqs. (29)–(34) were minimized with respect to the six kinetic parameters. These equations define a nonlinear root finding problem composed of six equations and six unknowns, where an exact solution cannot be determined analytically. Therefore, a numerical approximate solution was obtained using a newly developed optimization algorithm for black-box problems by identifying kinetic parameter values that minimized the sum of the squared residuals for SEqs. (29)–(34).¹¹ The applied algorithm is surrogate-based, and utilizes a special class of polynomials to approximate the relationship between the optimization variables and the objective function. For this problem the optimization variables were the parameter values and the sum of the squared-residuals of the steady-state equations was the objective function that was being minimized. The algorithm starts with an initial set of samples that fall on a Smolyak sparse-grid, and then iteratively samples the parameter space by using the polynomial approximation to predict where the best samples lie and then updates the approximation following the collection of new samples. Local optimization of the polynomial approximation is also used in identifying which samples to collect next (i.e. the parameter combinations to evaluate), and criteria based on the improvement of the polynomial approximation are used for determining convergence. Based on this model, parameter estimation for cathepsin L proved successful with the identification of a solution with low residuals, but for the case of cathepsin S no reasonable solution was found (Table S1, Fig. S1).

During parameter estimation, we investigated two different definitions for the relationship between our experimental measurements and the species of the ODE system. The first definition assumes that all mature cathepsin species are detectable by Western blot (WB), while only active mature cathepsin [Mat] is detectable through zymography (*Zymo*), not E64-bound cathepsin [MatE64], as defined by Eqs. (1) and (2).

$$WB = [\text{Mat}] + [\text{Mat}_{\text{inact}}] + [\text{MatE64}] \quad (1)$$

$$\text{Zymo} = [\text{Mat}] \quad (2)$$

The second definition still assumes all cathepsin species are detectable by Western blot, but differs from definition 1 for *Zymo*, in that active mature cathepsin and E64-bound cathepsin are both assumed to be detectable through zymography, with the added assumption that what was E64-bound in the intact cells has come unbound in the zymo preparation, allowing the cathepsin to be active (Eqs. (3) and (4))

$$WB = [\text{Mat}] + [\text{Mat}_{\text{inact}}] + [\text{MatE64}] \quad (3)$$

$$\text{Zymo} = [\text{Mat}] + [\text{MatE64}] \quad (4)$$

Dynamic simulations were performed based on the model diagram (Fig. S3) steady state kinetic parameters (Table S3) and the system of ODEs defined by SEqs. (37)–(47). The rate of inhibitor influx, outflux and transport from the endolysosomal to cytoplasmic compartments were approximated to ensure that the assumed steady state E64 concentrations were reached within 24 h. Additionally, the kinetic parameters were scaled to ensure the system reached a steady state within 24 h. Numerical simulations were performed using the deSolve package in the R statistical language.³⁵

Statistical Analysis

Zymography band intensities were quantified by densitometry in ImageJ and data were normalized by dividing each band intensity on a gel by the intensity measured from cells or tissue treated with 0 μM E64 (setting this group to 1 across all gels). Densitometry graphs display the mean and standard deviation of biological replicates of these normalized values. Two-tailed Student's t-test with two-sample equal variance was performed on all statistical analysis.

RESULTS

Incubation with E64 Reduces Amount of Active Cathepsin L in MDA-MB-231 Cells, But Increases the Amount of Active Cathepsin S

Whether the response of increased active catS and decreased catL after incubation with the broad cathepsin inhibitor E64 was specific to MDA-MB-231 cells or could occur in other cell types was yet to be established. To test this, the invasive estrogen receptor positive MCF-7,¹³ non-transformed, non-invasive breast epithelial MCF-10A,³² and invasive, triple neg-

active MDA-MB-231 cell lines,¹⁰ were incubated with increasing concentrations of E64 for 24 h then lysed and prepared for cathepsin zymography to determine amounts of active cathepsins. E-64 incubation increased active cathepsin S but led to a decreased amount of active cathepsin L in MDA-MB-231 cells (Fig. 2a). E64 appeared to slightly increase active cathepsin S in MCF-7 and MCF10A cells, but this increase was not statistically significant when averaged across three biological replicates. Neither MCF-7 nor MCF10A cells expressed active cathepsin L detectable by zymography, regardless of E64 treatment (Figs. 2b and 2c).

E64 Treatment of Human Normal and Cancerous Breast Tissue Yields Variable Responses of Active Cathepsins

After observing increased active cathepsins following E64 treatment in one breast cancer cell line, the next step was to determine if this same response would occur in primary human tissue. Breast tissue contains

multiple cell types, but we hypothesized that human breast tumors would exhibit similar increases in active cathepsin S following E64 treatment. Patient-matched tumor and normal breast tissue were acquired, cut into small pieces, and incubated in increasing concentrations of E64 for 24 h. Normal and cancer tissues were homogenized in lysis buffer prior to being assayed by cathepsin zymography (Fig. 3a); zymography results were quantified by densitometry (Fig. 3b).

In the absence of E64, active cathepsins S and L were detectable in cancer and normal tissue (Fig. 3a), consistent with previous publications indicating cathepsin involvement in breast cancer.²³ Amount of active cathepsin was highly variable between patient samples, with four of the samples showing active cathepsin V signal in addition to previously detected active cathepsins L and S. For the most part, E64 increased cathepsin S in normal tissue and decreased cathepsin L. However, in cancerous tissue, E64 caused a significant decrease in cathepsins L, S, and V, with some amount of patient to patient variability.

FIGURE 2. Incubation with E64 reduces amount of active cathepsin L in MDA-MB-231 cells, but increases the amount of active cathepsin S. MDA-MB-231 (a), MCF-7 (b), and MCF-10A (c) cells were grown to confluence prior to incubation in the presence of 0, 1, 5, 10 and 50 μ M E64 for 24 h. Cells were lysed and equal amounts of protein were loaded for multiplex cathepsin zymography. Densitometry was used to quantify the intensity of the active cathepsin S and L signals and shown in the graphs on the right for each cell type. There was an almost 50% increase in active cathepsin S, but a 75% loss of active cathepsin L in the MDA-MB-231 cells ($n = 3$, $*p < 0.05$, $\%p < 0.001$). Neither MCF-7 nor MCF-10A had detectable levels of active cathepsin L.

FIGURE 3. Treatment with E64 stimulates higher amounts of active cathepsin S in some normal, but not tumor tissue. Excised, patient-matched normal and tumor breast tissue was collected from seven human patients, and equal size thin pieces were incubated in media containing 0, 5, or 50 μ M of E-64 for 24 h, then lysed, and equal amounts of proteins were loaded for multiplex cathepsin zymography (a). Active cathepsins L and S were detectable in all patients, and active cathepsin V was detectable in four patients. Active cathepsins and response to inhibitor treatment was highly variable between patients, but active cathepsins L, S and V were significantly decreased by over 50% in the cancer, but not the normal tissues ($n = 4-7$, * $p < 0.05$, # $p < 0.001$) (b).

Macrophages Respond to E64 Incubation with Higher Amounts of Active Cathepsins S and V and Reduced Amounts of Active Cathepsin L

Macrophages have been shown to express cathepsins L, S and V⁴³ and tumor associated macrophages can comprise up to 50% of breast tumor mass.³⁶ Macrophages represent a non-epithelial cell lineage that could be responsible for the active cathepsin V detected in the patient matched samples. Thp-1 macrophages were treated with increasing doses of E64 for 24 h, lysed and assayed for active cathepsins. Macrophages showed increased active cathepsin S and decreased active cathepsin L when treated with E64 in a dose dependent manner (Fig. 4a), similar to the breast cancer cells. Macrophages also showed significantly increased active amounts of cathepsins V (Fig. 4b), showing that E64 was capable of increasing active cathepsin S in multiple cell lineages and of

increasing amount of active enzyme among multiple cathepsin family members.

Mass Action Model of Cathepsin Inhibition by E64: Assumptions and Corrections

A mathematical model of cathepsin L and S interactions with E64 in MDA-MB-231 cells was constructed to explain the counterintuitive responses to E64. Reactions in the model include: synthesis/production of procatepsins, activation from procatepsin to the mature, active cathepsin, degradation, inactivation, and inhibition by E64 (Fig. 1).

The model was fit to previously published Western blot and cathepsin zymography data collected from MDA-MB-231 cells treated with 0, 10, or 50 μ M E64 for 24 h with the assumption that zymography detected mature, but not inactive, cathepsins (Mat), since en-

FIGURE 4. Macrophages have reduced amounts of active cathepsin L after incubation with E64, but higher amounts of active cathepsins S and V. Thp-1 monocytes were stimulated to differentiate into macrophages with PMA, and confirmed by the upregulated amounts of active cathepsins L, S, and V. These differentiated macrophages were then incubated with 0, 5, or 50 μ M E64 for 24 h, lysed, and equal amounts of protein were loaded for multiplex cathepsin zymography (a). E-64 incubation caused an 80% loss of active cathepsin L in the macrophages at 50 μ M dose, but two-fold amounts of active cathepsin S and cathepsin V (n = 7–9, *p < 0.05, #p < 0.001), as quantified and shown in the densitometry (b).

zymes must be active to generate a signal.⁴⁰ The pro-cathepsins, mature cathepsin, inactivated and inhibited cathepsin forms would all be detected by Western blots. Assumptions of the model also were that cathepsin L was in the cytoplasm and cathepsin S was located in endolysosomal vesicles as shown previously.⁴⁰ Membrane impermeable E64 was modeled as an endocytosed inhibitor taken up by the cell into endolysosomal compartments that fuse with lysosomes, where E64 is then able to bind to cathepsin S. The model assumes the small molecule E64 is able to leak out of the endolysosomal compartment into the cytosol in a similar manner to other lysosomal contents that have been shown to move into the cytoplasm in cancer cells.¹² Once these minute amounts of E64 are in the cytoplasm, they are able to bind to cathepsin L present in the cytoplasm, as demonstrated by immunocytochemistry previously.⁴⁰

The model was able to simulate the behavior of E64 reducing active cathepsin L, but not the unexpected result of E64 treatment increasing active cathepsin S. A number of alternative reaction scenarios were considered, and preliminarily modeled to determine if they could fit the data and better describe the system in action, but none of these models were able to recapitulate the results of increased active cathepsin S as seen in the zymography results. Transcriptional regulation of cathepsin S was not considered because E64 treatment did not affect cathepsin S mRNA or protein in MDA-MB-231 cells.⁴⁰ After exhausting these alter-

native explanations, it was necessary to reassess the initial assumption that cathepsin S bound by E64 was not detectable by cathepsin zymography.

E64 Does Not Block Cathepsin S Zymography Signal Under Cell Lysate Conditions

E64 is considered an irreversible cathepsin inhibitor, due to the formation of a covalent bond between the C2 atom of the E-64 epoxy ring and the active site cysteine of the cathepsin, as well as hydrogen bonding between E-64 and polar residues near the cathepsin active site.¹⁸ However, under the partial denaturing conditions of zymography followed by refolding after the electrophoresis, the cathepsin-E64 complex could potentially be disassociated or shifted into a non-inhibitory conformation. To test the hypothesis that cathepsin S and E64 were uncoupling during the denaturation/renaturation of zymography, MDA-MB-231 cells were cultured as before, with 0 or 50 μ M E64 for 24 h, then collected in zymography lysis buffer. An additional 10 μ M of fresh E64 was added to the cell lysate, assuming additional E64 would bind to free cathepsin L and S in the lysate. MDA-MB-231 cells treated with 50 μ M E64 showed decreased active cathepsin L and increased active cathepsin S, consistent with previous results (Fig. 5a). There was no change in the zymography signal for those samples that had an additional 10 μ M E64 added to the lysate

FIGURE 5. E64 does not block cathepsin S zymography signal under cell lysate conditions. (a) MDA-MB-231 cells were cultured with 0 or 50 μ M E64 for 24 h then the cells were lysed and lysate collected from each of the incubation conditions. Aliquots of each lysate were then incubated with 0 or 10 μ M E64 for 10 min to allow binding of E64 to active cathepsins. Then equal protein amounts from these samples were prepared for cathepsin zymography as usual (a), or 10 μ M fresh E64 was added to the overnight incubation step during cathepsin degradation of the embedded gelatin (b). There was no observed difference in active cathepsin zymography signal between lysates treated with additional, fresh E64 or not, but incubation with E64 during the degradation completely blocked all active cathepsin zymograms signal. Densitometry was used to quantify the zymography signal ($n = 3$, $*p < 0.05$). ImageJ was used to quantify band intensity in each lane, then the value for each band was divided by the highest intensity band in its row (either CatL or CatS). Then the means and standard deviations of these normalized values were plotted in the graphs (c).

in lysis buffer compared to the control samples for neither cathepsin L nor S (Fig. 5a).

To ensure that 10 μ M E64 was sufficient to bind and inhibit cathepsins, a zymogram was run in parallel, but was treated differently after electrophoresis and cathepsins were renatured; the gel was incubated in cathepsin assay buffer containing 10 μ M E64. Incubation in assay buffer with 10 μ M E64 completely abrogated the cathepsins' zymography signal, even in the RAW264.7 macrophage lysate used as a positive control (Fig. 5b). Densitometry of the active cathepsin zymography signal is shown (Fig. 5c). The ability of E64 to inhibit cathepsins when added to the assay buffer after renaturation of the enzymes into their native conformation, but not when added to lysate in the presence of detergents and other chemicals, suggests the zymography process disrupts the inhibitory properties of E64, possibly through conformational changes to the cathepsin active site brought on by so-

dium dodecyl sulfate (SDS), detergents in the lysis buffer, basic pH of lysis buffer or electrophoresis buffers. This potential unbinding of E64 under non-reducing SDS-PAGE conditions was not expected because E64 has been classified as an irreversible covalently binding inhibitor of the active site cysteine,¹⁸ but this led to reconsideration of the experimental data to which the computational model was being fit.

Correcting for Cathepsin S-E64 Zymography Signal Significantly Improves Model Fit, Parsing Inhibited Cathepsin from Uninhibited Cathepsins, and Active from Inactive Conformations

The mathematical model was revised to incorporate the ability of cathepsin S bound by E64 in living cells to generate a zymography signal, as shown schematically in Fig. 6a. Model fitting with these new assumptions generated parameters with much lower

FIGURE 6. Correcting for cathepsin S-E64 zymography signal significantly improves model fit, and parses bound from unbound states and active from inactive conformations. Revised model architecture assumes cathepsin S is detectable by Western blot when in its pro state, free mature state, inactive state or when bound by E64 and detectable by zymography when in its free mature state or when bound by E64 (a). Model predictions for enzyme species at steady state for cathepsins S and L. The model predicts that E64 treatment increases cathepsin bound E64 complexes and decreases free cathepsins in a dose dependent manner. E64 is also predicted to decrease non-E64 inactivated cathepsin L by over 50% while non-E64 inactivated cathepsin S is less affected (b).

TABLE 1. Parameters for final steady state model.

Parameters	Cathepsin L	Cathepsin S
k_{prod}	1.28×10^3	13.04
k_{act}	8.97×10^2	2.75×10^{-1}
k_{inact}	4.29×10^2	2.58×10^{-2}
k_{deg}	1.30×10^4	3.55×10^{-1}
k_{degl}	1.45×10^4	5.84×10^{-2}
$k_{\text{E64}} (\mu\text{M}^{-1})$	1.77×10^4	6.49×10^{-3}
k_{degE64}	2.35×10^4	1.71×10^{-1}

residuals for the cathepsin S parameters (Table S2), recapitulating the experimental results garnered from Western blots and zymography (Fig. S2). Steady state simulations of the model predicted dose dependent accumulation of cathepsin-E64 complexes for both cathepsins L and S (checkerboard segments of graphs), but only cathepsin S-E64 complexes, not cathepsin L-E64 complexes, were allowed to contribute to zymography signal (Fig. 6b). Taken together, the red signals indicate zymography signal generating species with a direct relationship with E64 concentration for cathepsin S but an inverse relationship for cathepsin L.

Model parameters governing cathepsin production (k_{prod}), activation (k_{act}), inactivation (k_{inact}), and degradation (k_{deg}) were estimated to be several orders of magnitude greater for cathepsin L than cathepsin S (Table 1), indicating cathepsin L production and turnover to be greater than that of cathepsin S in MDA-MB-231 cells.

A Priori Predictions of Dynamic Responses of Cathepsins L and S to Different Classes of Cathepsin Inhibitors

Following the successful construction of the steady state cathepsin L and S model, we were interested in constructing a dynamic model with utility to describe the kinetics of different classes of cathepsin inhibitors binding to and inhibiting cathepsins over time. The model includes extracellular, lysosomal (cathepsin S) and cytoplasmic (cathepsin L) compartments, and the class of cathepsin inhibitor determined their ability to move between these compartments: membrane impermeable cathepsin inhibitor such as E64, membrane permeable inhibitor such as E64d, and a lysosomotropic inhibitor, which made up a number of early cathepsin inhibitors that advanced to clinical trials but were unsuccessful^{5,31,34} (Fig. 7a). The steady state cathepsin E64 models were used to parameterize time course models to predict cathepsin dynamics following inhibitor treatment. Inhibitor treatment was modeled as a bolus of 10 μM at time point 24 h, and models were simulated until 48 h.

The dynamic E64 model shared some assumptions with the steady state E64 model, namely that membrane impermeable E64 is endocytosed and accumulates in lysosomal compartments where it can bind cathepsin S, before small amounts of E64 leak into the cytoplasm where E64 can bind cathepsin L. The dynamic model predicted that cells treated with E64 would accumulate cathepsin L-E64 and cathepsin S-E64 complexes, peaking 24 h after treatment, resulting in elevated cathepsin S zymography signals and diminished cathepsin L zymography (Fig. 7b), as was observed experimentally (Fig. 2). Cathepsin L was

FIGURE 7. Dynamic model predicts different classes of cathepsin inhibitors provoke divergent responses by cathepsins L and S. Schematic representation of dynamic models of different inhibitors. Cathepsin S and L are confined to the endolysosomal and cytoplasmic compartments, respectively. Inhibitors can enter the endolysosomal compartment through endocytosis, or enter the cytoplasmic compartment through passive diffusion. Inhibitor can also pass from endolysosomal to cytoplasmic through lysosomal leakage (a). Dynamic model predictions of cathepsin L and S states, and predicted zymography and Western blot results following treatment with E64 (b) E64d (c) or a lysosomotropic inhibitor (d). Steady state model parameters were scaled to create a dynamic model of cathepsin inhibition over time. A treatment of 10 μ M inhibitor was added at 24 h, and cathepsin dynamics were predicted for several days following treatment to allow the system to move toward its steady state. E64 was able to bind to both cathepsins L and S through endocytosis and subsequent leakage out of the endolysosomal compartment and into the cytoplasm. Due to its confinement in the cytoplasm, E64d was much more effective in binding cathepsin L than cathepsin S, which is consistent with previous experimental data showing low dosages of E64d can inhibit cathepsin L while not affecting cathepsin S. E64d also inhibited cathepsin L much more rapidly than E64, since it could cross the cell membrane and enter the cytoplasm directly. Lysosomotropic inhibitor bound both cathepsins L and S more rapidly than E64, resulting in loss of free cathepsins L and S.

predicted to reach peak inhibition in under 1 h, compared to cathepsin S, which did not reach its maximum zymography signal until 12 h after inhibitor treatment.

For E64d, the membrane permeable inhibitor due to addition of an ethyl ester that is removed by cytosolic esterases, effectively trapping E64d in the cytoplasm, the results were different. The model predicted E64d would have little effect on cathepsin S, since it was located in the endolysosomal compartment. However, E64d would be extremely effective at inhibiting cathepsin L due to its ease of crossing the membrane and entering the cytoplasm. According to the simulation, E64d was more effective than E64 at inhibiting cathepsin L (22% inhibition for E64 and nearly 100% inhibition for E64d), but the inhibition subsided more quickly since the greater influx of inhibitor resulted in a more rapid depletion of free inhibitor (Fig. 7c), compared to the limited release of E64 into the cytoplasm

that resulted in a much slower return to baseline for free cathepsin L. As predicted, cathepsin S responded less to E64d than E64 (22% increase in zymography signal for E64 compared to 7% increase for E64d). The predicted greater inhibitory capacity of E64d compared to E64 on cathepsin L and lack of response in cathepsin S are consistent with experiments using E64d in MDA-MB-231 cancer cells, which found 1 μ M E64d inhibited intracellular cathepsin L at a 1 μ M dose, while having no effect on cathepsin S at any dosage.⁴⁰

The third class of lysosomotropic inhibitors were modeled as moving freely across lipid membranes and through compartments, from extracellular to cytosolic to endolysosomal. Lysosomotropic inhibitors showed rapid binding to cathepsins S and L to form complexes. The magnitude of inhibition of cathepsin L by the lysosomotropic inhibitor (85%) was greater than for E64, the membrane impermeable inhibitor (22%).

The lysosomotropic inhibitor provoked the largest increase in cathepsin S zymography signal (43% for lysosomotropic vs. 22% for E64). These simulations are consistent with studies of lysosomotropic inhibitors that have shown high efficacy in binding target and off-target cathepsins and side effects caused by these potent inhibitors.^{3,5,7}

DISCUSSION

This work shows treatment with a cathepsin inhibitor can increase different active cathepsins in multiple cell types and primary tissue, suggesting there is a common mechanism among these cell types that should be considered when designing inhibitor treatment regimens. *Ex vivo* tumor biopsy specimens from different patients displayed significant person-to-person heterogeneity in endogenous active cathepsins and in response to inhibitor treatment. The presence of stromal cells, such as tumor associated macrophages, could explain some of the variability observed in the primary tumor samples as macrophages are known to have donor-specific variation in active cathepsin expression.²³ Successful clinical treatment of cancer metastasis with cathepsin inhibitors will require better understanding of the proteolytic interactions, in order to effectively suppress target proteases contributing to metastasis, while avoiding impacting proteases that would provoke unexpected side effects. Additionally, these results underscore the importance of assaying active cathepsins, in addition to total cathepsins, during inhibitor clinical trials. The models we developed were able to differentiate between inhibitor-bound, active, and inactive cathepsin species, which are difficult to measure experimentally and can confound *in vivo* and *in vitro* experiments. Finally, the dynamic models of different classes of cathepsin inhibitors that have been deployed in clinical trials, demonstrate how inhibitor trafficking and access to subcellular compartments can have drastically divergent effects on active cathepsins located in different intracellular compartments.

Cathepsins are attractive targets for multiple diseases including cancer, osteoporosis and atherosclerosis, but off-target effects and unexpected responses to cathepsin inhibitor treatments have prevented their clinical adoption. This work sought to explain a previously documented non-intuitive response to cathepsin inhibitor treatment in breast cancer cells, a simultaneous increase in active cathepsin S and decrease in cathepsin L following E64 treatment. Several different cells related to breast cancer tumors were experimentally tested to determine the distribution of

the non-intuitive E64 treatment response, including different transformed and non-transformed breast cancer cell lines, primary tumor and patient matched normal tissue samples, and a human macrophage cell line.

This work focused on the small molecule inhibitor E64 as a model cysteine cathepsin inhibitor with cross-reactivity. To test the effects of E64 on primary tissue, patient matched normal and tumor samples were incubated with E64. The patient samples displayed diverse endogenous cathepsin signatures, including cathepsins L, S and V. This high degree of proteolytic variability agrees with our previous findings of a high degree of person-to-person variability in endogenous active cathepsin and cystatin expression among monocyte derived macrophages isolated from healthy donors.³⁰ On average, E64 treatment decreased active cathepsins in tumor samples. However, several normal tissue samples had increased active cathepsins following E64 treatment. These results highlight the difficulty in designing effective cathepsin inhibitor regimens due to high patient variability in endogenous proteases. Additional experimentation will be required to determine the roles of different cells in complex *in vivo* systems, including tumor tissue, which is composed of many different cells including cancer-associated fibroblasts, tumor associated macrophages, other leukocytes, endothelial cells and adipose cells, many of which are known producers of cysteine cathepsins.²⁰ Additionally, breast cancer cells are known to be highly heterogeneous even within the same primary tumor,²⁹ further underscoring the need for *in silico* tools to study variation in proteolytic inhibitor responses.

To explore the effects of E64 on other cancer associated cells, macrophages were treated with E64 and active cathepsins were quantified. Tumor associated macrophages are abundant in breast tumors, comprising up to 50% of the tumor mass.¹⁵ Cysteine cathepsins produced by tumor associated macrophages have been shown to promote tumor growth and metastasis, angiogenesis, and even to suppress chemotherapy.^{27,41} E64 treatment significantly increased active cathepsin S and active cathepsin V in the Thp-1 human macrophage cell line. Increased active cathepsins in Thp-1 macrophages suggests this effect is conserved across cells of multiple lineages, including epithelial cells derived from the endoderm and macrophages derived from the mesoderm. Additionally, the increase in active cathepsin V indicates this response is not limited to cathepsin S, but could also affect other proteases, particularly other cysteine cathepsins due to the high degree of protein identity shared between family members.

The failure of our original model assumption, that cathepsins bound by E64 are undetectable by cathepsin zymography, to explain the observed increase in active cathepsin S following E64 treatment led us to question this assumption and test it experimentally. Treated lysate with additional E64 did not affect the resulting zymography signal, suggesting E64 loses its inhibitory capacity during non-reducing electrophoresis. We suspect E64 is playing a stabilizing role on cathepsin S during E64 incubation, where free cathepsin S becomes bound to E64 instead of naturally inactivating due to changes in intracellular compartment pH or oxidative conditions. Once E64 binding is interrupted during electrophoresis, the cathepsin S bound by E64 may regain its ability to degrade substrate to generate the elevated zymography signal observed. E64 does not provide the same stabilizing effect intracellularly as it provided to cathepsin S, resulting in loss of active cathepsin L signal, following cellular treatment with E64. This could be caused by differences in the intracellular environments of the two enzymes in MDA-MB-231 cells, since cathepsin L is dominantly in the cytoplasm in these cells while cathepsin S was confined to the endolysosomal compartment.⁴⁰ Cathepsin S is also known to tolerate neutral pH more effectively than cathepsin L, which could be quickly denatured in the more neutral pH of cytoplasm or basic pH of lysis buffer, regardless of E64 binding.³³

Dynamic simulations of different classes of cathepsin inhibitors showed the importance of intracellular trafficking mechanisms on the specificity and strength of inhibition. Lysosomotropic inhibitors elicited large responses from both cathepsins L and S due to high cellular uptake and accumulation in the lysosomes, consistent with previous results showing high intracellular potency, but poor selectivity among lysosomotropic cathepsin inhibitors.^{7,31,34}

Previous studies of cathepsin inhibition in rodent models have shown the ability of cathepsin inhibitors to affect the expression and activity of cysteine cathepsins. Cathepsin B, H and L had increased half lives in the livers of rats treated with the potent cysteine cathepsin inhibitor EP-475 (E-64c).¹ While cathepsin L or cathepsin S protein were not increased following E64 treatment, it is possible that the protein could accumulate over a period of time longer than 24 h. Treatment with the weaker binding, reversible inhibitor leupeptin elicited increased cathepsin B and other lysosomal hydrolase activity in rat fibroblasts and mouse bone tissue, measured with reporter substrates.²¹ However, enzyme activity was abolished when the rat fibroblasts were treated with E64.

This work suggests inhibitors can protect specific enzymes resulting in long-term increases in activity following the unbinding of an inhibitor. Small molecule inhibitor interactions or interference with cystatins, the endogenous protein inhibitors of cathepsins, must also be included. Previous work in our lab has shown cystatin C can elicit the same preservation of active cathepsin S, while interestingly failing to inhibit cathepsin L.⁴⁰ Cathepsin stability is greatly affected by binding substrates and effector molecules such as glycosaminoglycans, the presence of which can increase cathepsin K half-life at neutral pH from 7 to 190 min.²⁵ Similarly, binding to E64 could preserve cathepsin S activity, creating a reservoir of active cathepsins capable of degrading substrate following inhibitor unbinding. Our model results suggest even broad spectrum inhibitors can have diametrically opposed enzyme-specific impacts, preserving or inhibiting activity depending on properties of the enzyme and its intracellular location. The exact effector molecules and mechanisms responsible for this differential response to cathepsin inhibitor binding will be the subjects of future studies.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (<https://doi.org/10.1007/s12195-019-00580-5>) contains supplementary material, which is available to authorized users.

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CONFLICT OF INTEREST

W Andrew Shocke, Christopher A. Kieslich, Catera L. Wilder, Valencia Watson, and Manu O. Platt declare that they have no conflicts of interest.

ETHICAL STANDARDS

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

RESEARCH INVOLVED IN HUMAN AND ANIMAL RIGHTS

No animal studies were carried out by the authors for this article.

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