Development of Near-Infrared Fluorophore (NIRF)-Labeled Activity-Based Probes for *in Vivo* Imaging of Legumain

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Asparaginyl endopeptidase, also known as legumain, is a lysosomal cysteine protease that was named on the basis of its propensity to cleave protein substrates on the C-terminal side of asparagine residues (1). Legumain is expressed in diverse cell types, and in most cases, its functions are unknown. Recently legumain has emerged as an important enzyme in antigen processing (2, 3) and matrix degradation (4, 5), and it is implicated in various pathological conditions including parasite infection (6, 7), atherosclerosis (8), and tumorigenesis (9, 10). For example, legumain is heavily overexpressed in the majority of human solid tumors such as carcinomas of the breast, colon, and prostate (9). Furthermore, knockdown of legumain in mouse models of cancer resulted in a marked decrease in tumor growth and metastasis (10). More recently, mice lacking legumain developed disorders similar to hemophagocytic syndrome, a form of hyperinflammatory response (11). Despite the mounting evidence of legumain as a therapeutically important target, especially in tumor progression and metastasis, current methods to study legumain function mainly depend on antibodies and genetic modification, making it difficult to study legumain in its native state.

Small molecule chemical tools such as activity-based probes (ABPs) provide a highly versatile means to monitor protease function and regulation in a wide range of biological systems. Typical ABPs utilize irreversible inhibitors that can covalently modify the active site of an enzyme in an activity-dependent fashion. However, only a few legumain-specific inhibitors have appeared in the literature thus far. All of these inhibitors have a Cbz-Ala-Ala-Asn peptide scaffold that is based on the sequence of a known substrate of legumain (12). In addi-

**ABSTRACT** Asparaginyl endopeptidase, or legumain, is a lysosomal cysteine protease that was originally identified in plants and later found to be involved in antigen presentation in higher eukaryotes. Legumain is also up-regulated in a number of human cancers, and recent studies suggest that it may play important functional roles in the process of tumorigenesis. However, detailed functional studies in relevant animal models of human disease have been hindered by the lack of suitably selective small molecule inhibitors and imaging reagents. Here we present the design, optimization, and *in vivo* application of fluorescently labeled activity-based probes (ABPs) for legumain. We demonstrate that optimized aza-peptidyl Asn epoxides are highly selective and potent inhibitors that can be readily converted into near-infrared fluorophore-labeled ABPs for whole body, noninvasive imaging applications. We show that these probes specifically label legumain in various normal tissues as well as in solid tumors when applied *in vivo*. Interestingly, addition of cell-penetrating peptides to the probes enhanced cellular uptake but resulted in increased cross-reactivity toward other lysosomal proteases as the result of their accumulation in lysosomes. Overall, we find that aza-peptidyl Asn ABPs are valuable new tools for the future study of legumain function in more complex models of human disease.

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Received for review November 9, 2009 and accepted December 17, 2009.
Published online December 17, 2009
10.1021/cb900232a
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tion, a number of different reactive electrophilic functional groups including aza-Asn halomethylketones (13), aza-Asn epoxides (7), and aza-Asn Michael acceptors (6) have been used to make irreversible legumain inhibitors. Although these inhibitors are highly potent against legumain in vitro, their potency and, more importantly, their selectivity in vivo have never been tested. We have previously developed a cell-permeable ABP for legumain that is composed of a peptide acyloxy-methyl ketone (AOMK) with a P1 aspartic acid (14). Although this probe is useful to study active legumain in cells, it has overall poor potency and can readily cross-react with caspasps, which also optimally bind to aspartic acid containing AOMKs (15). We therefore decided to develop a new class of legumain inhibitors with faster kinetic properties and increased selectivity for legumain for use in in vivo imaging studies. Herein, we present a new class of aza-Asn epoxide ABPs for legumain that are labeled with Cy5 fluorophore and also tagged with a series of cell-permeabilizing groups. This new generation of legumain probes can be used to image active legumain both in normal tissues and within solid tumors.

RESULTS AND DISCUSSION

Design of Legumain Probes for in Vivo Applications. To develop new tools to study legumain function in vivo, we needed to identify a scaffold that could be used to make probes that were highly selective for legumain with virtually no cross-reactivity for other lysosomal proteases or related CD clan proteases such as caspasps. In the past, our group developed activity-based probes that can be used to label legumain in cell culture models (14). These first generation probes make use of the acyloxyethyl ketone (AOMK) group to covalently modify the active site cysteine and a Pro-Asp peptide for specific recognition by legumain. This peptide sequence was chosen on the basis of the finding that although legumain prefers processing of substrates at asparagine residues, it also binds to probes with a P1 aspartic acid (16). Since P1 Asn AOMKs are highly unstable (17), we originally focused our attention on the P1 Asp AOMK probes. These reagents, while useful for labeling legumain, have overall slow binding properties and generally low potency. In addition, P1 Asp-AOMKs are highly effective labels of caspasps both in vitro and in vivo (18, 19). Recent reports suggest that aza-peptidyl epoxides can be designed to be highly potent inhibitors of legumain with overall low cross-reactivity toward other lysosomal cystein proteases such as the cathepsins (7). The unique aza scaffold also allows incorporation of a P1 Asn residue without causing overall instability of the compound. On the basis of these findings, we envisioned that aza-Asn epoxide should be valuable for use in imaging probes as a result of very low reactivity toward cathepsins and caspasps. We therefore synthesized an activity-based probe LP-1 (Legumain Probe-1) that contains the aza-Asn epoxide and the P2 Pro of the first generation AOMK probe, as well as a Cy5 fluorophore for in vivo imaging applications (Figure 1, panel a). We also synthesized a Cy5-labeled version of the previously reported probe Biotin-PD-AOMK (LP-0, Figure 1, panel a) for direct comparison with LP-1. LP-1 was synthesized via a previously reported solid-phase synthesis technique (20), and the Cy5 fluorophore was conjugated to the purified peptide at the final step (Scheme 1). To directly compare enzyme specificity and kinetics between LP-1 and the previously described AOMKs, we also synthesized acetyl-capped inhibitor versions of LP-1 and LP-0 (LI-1 and LI-0, respectively; Figure 1, panel a).

Selectivity and Potency of Legumain Inhibitors and Probes. To determine the overall potency and selectivity of the aza-epoxide and AOMK inhibitors, we performed inhibition studies for both compounds against recombinant legumain, cathepsin B, cathepsin L, and caspase-3 (Table 1). Simple IC_{50} determination showed that LI-1 (IC_{50} = 11.5 nM) is 70-fold more potent than LI-0 (IC_{50} = 704 nM) against legumain, whereas both compounds showed very weak activity against cathepsin B (IC_{50} = 390 μM for LI-1 and >1 mM for LI-0) and cathepsin L (IC_{50} = 220 μM for LI-1 and >1 mM for LI-0). Importantly, LI-0 showed a significant inhibitory effect (IC_{50} = 2.8 μM) on caspase-3, whereas LI-1 showed nearly no inhibition (IC_{50} = 890 μM). To further evaluate the kinetics of inhibition of legumain by the two classes of inhibitors, we also measured second-order rate constants (k_{obs}/[I]) for both compounds (Table 1). As expected, LI-1 (k_{obs}/[I] = 72,352 M^{-1} s^{-1}) inhibited legumain approximately 50-fold faster than LI-0 (k_{obs}/[I] = 1586 M^{-1} s^{-1}). These results confirmed that incorporation of P1 Asn via an aza-peptidyl scaffold greatly enhanced efficiency and specificity of inhibition by LI-1 compared to that of the P1 Asp AOMK scaffold. The rapid inhibition kinetics of the aza-Asn epoxide scaffold is advantageous for in vivo imaging as it allows
rapid binding to legumain, thus providing a better signal-to-noise ratio even for probes with relatively short half-lives in vivo. This allows the use of lower overall doses of probe and prevents extended circulation that can possibly cause cross-reactivity with other proteases.

Figure 1. Legumain inhibitors and probes. a) Structures of Aza-Asn epoxide legumain inhibitor, LI-1, and legumain probe, LP-1, compared to Asp-AOMK inhibitor, LI-0, and probe, LP-0. b) Direct labeling of legumain in intact cells by LP-1 and LP-0. Intact monolayers of NIH-3T3 fibroblasts (top) or RAW 264.7 macrophages (bottom) were pretreated with the cathepsin inhibitor JPM-OEt (10 μM; first column) and the legumain inhibitors LI-0/LI-1 (10 μM; second and third columns) and labeled by addition of LP-1 and LP-0 at the indicated concentrations.
We next wanted to verify labeling of active legumain in intact cells. Therefore, we treated intact NIH-3T3 and RAW 264.7 cells with LP-1 and LP-0, respectively, and monitored protein labeling using SDS–PAGE followed by scanning of the gel to detect the Cy5 fluorescence (Figure 1, panel b). Both probes selectively labeled active legumain in NIH-3T3 fibroblasts. As previously observed in the enzyme kinetic assays, LP-1 labeled active legumain more efficiently than LP-0 at low probe concentrations and showed overall stronger labeling signals. Interestingly, when the two probes were used to label RAW 264.7 macrophages, both showed some degree of cross-reactivity toward lysosomal cathepsins. The identity of these off targets as cathepsins was con-

### Table 1. Inhibition of various cysteine proteases by LI-1 and LI-0

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC$_{50}$</th>
<th>$k_{cat}$/[I]</th>
<th>Cathepsin B IC$_{50}$</th>
<th>Cathepsin L IC$_{50}$</th>
<th>Caspase-3 IC$_{50}$</th>
</tr>
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<tbody>
<tr>
<td>LI-1</td>
<td>11.5 nM</td>
<td>72352 M$^{-1}$ s$^{-1}$</td>
<td>390 µM</td>
<td>220 µM</td>
<td>890 µM</td>
</tr>
<tr>
<td>LI-0</td>
<td>704 nM</td>
<td>1586 M$^{-1}$ s$^{-1}$</td>
<td>&gt;1 mM</td>
<td>&gt;1 mM</td>
<td>2.8 µM</td>
</tr>
<tr>
<td>JPM-OEt$^a$</td>
<td>N/D</td>
<td>0.78 µM</td>
<td>2.98 µM</td>
<td>N/D</td>
<td>0.13 µM</td>
</tr>
<tr>
<td>Z-DEVD-FMK$^b$</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
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</table>

$^a$JPM-OEt is a broad-spectrum cathepsin inhibitor. $^b$Z-DEVD-FMK is a caspase-3 specific inhibitor.
Confirmed by pretreatment of cells with the broad-spectrum cathepsin inhibitor JPM-OEt (21) and further verified by immunoprecipitation (Supplementary Figure S1). Although some degree of cross-reactivity of AOMK probes toward cathepsins has been reported (15), attempts to inhibit cathepsins with various aza-epoxide inhibitors have been unsuccessful (22). Therefore, the labeling of cathepsins by LP-1 was particularly surprising. These data suggest that even though compounds may have very low potency toward a particular protease target in vitro, when added to cells that actively accumulate the probes in their lysosomes, such as macrophages, they are able to react with other abundant proteases. Furthermore, when cells were pretreated with legumain inhibitors, we observed more intense cathepsin labeling. This could be explained by the fact that cathepsins are known to be substrates for legumain (3, 23); legumain inhibition thus could result in increased levels of cathepsins and therefore increased nonspecific labeling by the legumain probes. Although the cathepsin cross-reactivity is not ideal, by using lower probe concentrations and more potent inhibitor scaffolds, it should be possible to obtain selective labeling of legumain in vivo. On the basis of these results, we decided that LP-1 would be the optimal reagent for in vivo imaging studies, as it has faster kinetics and overall higher potency, resulting in less background labeling of cathepsins and caspas.

In Vivo Imaging of Legumain Using the Aza-Epoxide Probe LP-1. To examine in vivo properties of LP-1 and monitor active legumain levels noninvasively, we performed imaging experiments using a simple tumor xenograft model. Mice bearing C2C12/ras xenograft tumors were IV injected with LP-1 (top) or LP-1-ctrl (bottom) probes and imaged at the indicated time points. Images are presented using a colorimetric scale based on photons per second per centimeter square per steradian (p s⁻¹ cm⁻² sr⁻¹) overlaid on bright light images. b) Tumor to normal tissue signal ratio calculated from the mice labeled with LP-1 (circle symbols with a solid line) and LP-1 Ctrl (square symbols with a dotted line). Ratios were calculated from multiple mice (n = 3 for LP-1 and n = 2 for LP-1 Ctrl) and represent mean ± standard error. c) Ex vivo imaging of organs and protein labeling analyzed by SDS–PAGE. Fluorescently labeled proteins were visualized by scanning of the gel using a flatbed laser scanner. Each column represents an organ from an individual mouse collected after in vivo imaging experiments.
nograft model (C2C12/Hras1) (24). To verify that the in vivo fluorescent signal from LP-1 is legumain-specific, we also used a control probe (LP-1 ctrl, see Supporting Information) that lacks the reactive epoxide group and therefore does not covalently bind to legumain. Mice were injected with the probes via tail vein, and fluorescent images were collected over the course of 5 h. As expected, LP-1 rapidly accumulated in tumor tissues, whereas LP-1 ctrl did not show such accumulation (Figure 2, panel a). Quantification of the tumor to normal tissue ratio from the fluorescent images showed that LP-1 accumulated in tumors with a maximum signal to background ratio obtained at around 90 min (Figure 2, panel b). Furthermore, the specific legumain signal declined over time but remained significantly higher than the signal observed for the control probe even at the later time points. This labeling pattern is in contrast to the previously reported cathepsin probes, which only provide contrast after 8–12 h, and signals are retained beyond 48 h. These data further support our hypothesis that using a more potent and kinetically fast binding scaffold allows selective labeling of legumain in vivo.

Use of a tat Peptide To Increase Cellular Uptake. Previously we reported that conjugating the tat peptide to an activity-based probe that targets caspases enhanced cell permeability; however, it also increased lysosomal uptake due to its positive charge. Thus the use of the tat carrier inadvertently increased cross-reactivity toward legumain (18). Inspired by this result, we conjugated the tat peptide to LP-1 to further improve its in vitro and in vivo reactivity toward legumain. To our surprise, when tested against intact RAW 264.7 macrophages, tat conjugated LP-1 (tLP-1; Figure 3, panel a) labeled not only legumain but also multiple cathepsins, even at low concentrations (Figure 3, panel b). We believe that this cross-reactivity results from increased lysosomal uptake and is not the result of a loss of specificity caused by addition of the tat peptide. To test this hypothesis, we labeled RAW cell lysates with LP-1 and tLP-1 (Figure 3, panel c). SDS–PAGE analysis confirmed that there is no difference in reactivity toward legumain for these two probes. We also performed a competition assay by pre-treating RAW lysates with each probe and labeling with the general cathepsin probe, I$^{125}$-DCG04 (25). These results further confirmed that both LP-1 and tLP-1 are equally poor inhibitors of the cathepsins (Figure 3, panel d). Thus, the increased cross-reactivity toward
cathepsins is likely due to significant accumulation of
tLP-1 in the lysosome.

Since tLP-1 showed high cross-reactivity toward
cathepsins in cells, we wanted to monitor its in vivo
distribution and labeling kinetics compared to those of
LP-1. We performed in vivo imaging and found that
although the tat peptide enhanced overall uptake of the
probe, tLP-1 failed to show the selective uptake in tu-
mors observed for LP-1. In addition, the tat peptide dra-
matically increased overall probe distribution and re-
duced the rate of clearance (Figure 4, panel a). As a
result, tLP-1 showed increased nonselective labeling in vivo relative to that of LP-1 (Figure 4, panel b).

**Use of Additional Carrier-Conjugates on Legumain Probes.** Cell-penetrating peptides (CPPs) and
membrane-targeting moieties have proven to be useful
delivery methods for various biological reagents (26,
27). In addition to the tat peptide, we also decided to
test several additional carrier molecules with our legu-
main probes. We chose octa-arginine (r8) and penetra-
tin because of their widespread use as carriers. We also
 chose cholesterol since it has high affinity for mem-
brane raft domains and has been used to enhance
membrane permeability (28) (Figure 5, panel a).

To compare differences in cell permeability and label-
ing efficiency between these probes, we treated intact
cells with each conjugate and analyzed labeling by SDS–PAGE. As previously observed for tLP-1, all the carrier-conjugates showed increased cellular uptake resulting in stronger labeling but also more cross-reactivity (Figure 5, panel b). All CPP conjugated probes, tLP-1, r8 LP-1, and penetratin LP-1 showed almost identical protein labeling profiles, indicating that all of these probes are delivered by similar mechanisms and are enriched in lysosomes. Cholesterol LP-1 showed enhanced legumain labeling with less cross-reactivity, suggesting that membrane anchoring cholesterol helped selective delivery of LP-1. Next, we carried out in vivo imaging experiments with penetratin LP-1 and cholesterol LP-1 to compare their in vivo distribution to LP-1 (Figure 6, panel a). Although the carrier labeled probes accumulated in tumors to some extent, their slow clearance resulted in low tumor to background levels (Figure 6, panel b). Ex vivo imaging of the collected organs followed by analysis of lysates by SDS–PAGE confirmed that carrier labeled probes suffered from increased cross-reactivity with cathepsin proteases (Figure 6, panel c). Overall, LP-1 showed the most legumain labeling in tumors as well as the highest levels of legumain-specific fluorescent signal, whereas penetratin LP-1 and cholesterol LP-1 showed nonspecific distribution in most organs and much higher cross-reactivity toward cathepsins. We believe that enhanced cellular delivery of these probes adversely affects overall circulation of the probe, resulting in less useful imaging reagents. Furthermore, all of the carrier molecules caused increased association with tissues other than the target tumors, suggesting that legumain ABPs are more effective as free probes that do not contain a carrier peptide.

In conclusion, we have developed a NIRF-labeled legumain probe, LP-1, based on a highly potent and selective inhibitor. The probe contains a Pro-Asn-aza epoxide scaffold that is distinct from the previously reported legumain inhibitors. When LP-1 was used for noninvasive imaging applications, we were able to monitor legumain activity both in normal tissues and in solid tumors. Its favorable reactivity and clearance resulted in high contrast in tumors soon after probe injection. We were also able to track whole body distribution of the probe as well as the level of active legumain in organs by ex vivo imaging and SDS–PAGE. In addition, we tested a series of cell-permeabilizing moieties as a delivery strategy for ABPs. Although some of these moieties improved cell permeability and legumain labeling in cells, they also increased off-target labeling via enhanced lysosomal uptake and extended circulation times in vivo. We conclude that LP-1 is a valuable new imaging probe with desirable in vitro and in vivo characteristics. While this probe can be used for noninvasive imaging studies, it also has great potential value for invasive applications in which direct assessment of levels of active legumain in whole tissues or cells in vivo are required. This new imaging agent and its corresponding inhibitor are likely to prove valuable for future in vivo studies of legumain function.

METHODS

General Methods. Unless otherwise noted, all resins and reagents were obtained from commercial suppliers and used without further purification. All solvents used were HPLC-grade and also purchased from commercial suppliers. Reactions were analyzed by LC-MS performed on an Agilent 1100 liquid chromatography system with an API 150EX single quadrupole mass spectrometer (Applied Biosystems). HPLC purifications were carried out using an AKTA explorer 100 (Amersham Pharmacia Biotech) with C18 reversed-phase columns (Waters). Mobile phase consisted of 95:5:0.1 = water/acetonitrile/trifluoroacetic acid (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). High-resolution mass spectrometry (HRMS) was performed using an LTQ-FITMS (Thermo Fisher Scientific). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed using a Bruker Autoflex TOF/TOF mass spectrometer (Bruker). IC50 measurements and enzyme kinetics assays were performed on a Spectramax M5 fluorescent plate reader (Molecular Devices). Fluorescent gels were scanned with
a Typhoon 9400 flatbed laser scanner (GE Healthcare). Male BALB/c nude mice (4–8 weeks old) were obtained from Charles River and housed in the research animal facility at the Stanford University Department of Comparative Medicine. All animal protocols were approved by the Stanford Administrative Panel on Laboratory Animal Care, and the procedures were performed in accordance to their guidelines. In vivo imaging experiments were performed using the IVIS 200 imaging system (Xenogen), and ex vivo imaging experiments were performed using the FMT 2500 system (VisEn Medical).

Figure 6. In vivo imaging and biochemical analysis using penetratin LP-1 and cholesterol LP-1. a) Comparison of in vivo fluorescent images of mice labeled with LP-1 and carrier-conjugated probes. Mice bearing C2C12/ras xenograft tumors were IV injected with LP-1 (top) or Penetratin LP-1 (middle) and Cholesterol LP-1 (bottom) probes and imaged at the indicated time points. Images are presented using a colorimetric scale based on photons per second per centimeter square per steradian (p s⁻¹ cm⁻² sr⁻¹) overlaid on bright light images. b) Ex vivo imaging of whole organs and SDS–PAGE analysis from the corresponding organ extracts. Fluorescent probe labeled proteins were visualized by scanning of the gel using a flatbed laser scanner. The location of legumain (Leg) is shown. c) Quantification of fluorescent signal in tumors (left) compared to tumor to normal tissue signal ratios (right).
Synthesis and Characterization. Peptidyl Aza-Asn epoxide was synthesized by following the previously reported procedure (20) on a Rink SS resin (Advanced ChemTech). Peptidyl Asp AOMK was synthesized by the previously reported procedure (25). Each carrier-probe conjugate was synthesized by following the previously reported procedure (18). The tetrapeptide and the penetratin peptide were custom synthesized by the Stanford PAN peptide synthesis facility. All synthesized peptides were cleaved from resin by applying cleavage cocktail containing 95% TFA and purified by HPLC. The purified peptides were then coupled with Cy5-NHS (1 equiv) in DMSO with DIEA (5 equiv) for 1 h and purified by HPLC. The purity and identity of all compounds were assessed by LC-MS and HR-MS. Detailed procedures (5 equiv) for 1 h and purified by HPLC. The purity and identity of all compounds were assessed by LC-MS and HR-MS. Detailed synthetic procedures and characterization data of final compounds can be found in Supporting Information.

Determination of $k_{cat}$ against Cysteine Proteases and Second-Order Rate Constants ($k_{diss}$/[I]) for Legumain. Activity of legumain was measured with the fluorogenic substrate, Cbz-Ala-Ala-Asn-AMC (Anaspec), cathepsin B and cathepsin L were measured with Cbz-Phe-Arg-AMC (Bachem), and caspase-3 was measured with Caspase-3 Substrate VII (Calbiochem). Assay buffers consist of 20 mM citric acid, 60 mM disodium hydrogen orthophosphate, 1 mM EDTA, 0.1% CHAPS, 4 mM DTT, pH 5.8 for legumain; 50 mM dihydrogen sodium orthophosphate, 1 mM EDTA, 5 mM DTT, pH 6.25 for cathepsin B and cathepsin L; and 100 mM Tris, 0.1% CHAPS, 10% sucrose, 10 mM DTT, pH 7.4 for caspase-3. Concentrations of substrates during the measurement were 10 μM (legumain, cathepsin L, and caspase-3) and 50 μM (caspase-3). Each enzyme was incubated with inhibitor concentrations ranging from 1 nM to 1 mM in the presence of the substrates. The increase in fluorescence was continuously monitored every 3 s for 2.5 h with a Spectramax M5 fluorescent plate reader (Molecular Devices), and inhibition curves were recorded. $k_{cat}$ values were calculated by plotting the normalized enzyme activity against the inhibitor concentration at 60 min for legumain and at 30 min for caspase-3, cathepsin B, and cathepsin L using nonlinear regression analysis (GraphPad Prism). $k_{cat}$ values with known broad-spectrum cathepsin inhibitor (Z-DEVD-FMK) and caspase-3 inhibitor (Z-DEVD-FMK) were also measured for comparison. All measurements were performed in triplicate, and the average values were reported.

Second-order inhibition rate constants were determined by following the previous described method in the literature (6). The pseudo-first-order rate constants ($k_{cat}$) were obtained from plots of ln [v]/v versus time where $k_{cat}$ is the rate of hydrolysis of fluorogenic substrate and $k_{cat}$ is the rate of hydrolysis of substrate in the presence of the inhibitor. The second-order inhibition rate constants were calculated using the following equation: second-order rate constant = ($k_{cat}$/[I])/(1 + [S]/$k_{cat}$).

Direct Labeling of Endogenous Legumain in Intact Cells and Cell Lysates. RAW 264.7 cells (250,000 cells well$^{-1}$) and NIH-3T3 cells (200,000 cells well$^{-1}$) were seeded in a 24-well plate 24–30 h prior to labeling. Cells were pretreated with appropriate inhibitors for 1.5 h and labeled by addition of each probe for 1 h; the final DMSO concentration was maintained at ~0.2%. Cells were washed with PBS buffer and lysed by addition of sample buffer. Crude lysates were collected and separated by 12.5% SDS—PAGE. Labeled proteins were analyzed by scanning the gel with a Typhoon flatbed laser scanner. In Vivo/ex Vivo Imaging and SDS—PAGE Analysis of Organ Lysates. Tumor-bearing mice were prepared by following the previously described method (24). C2C12/Hras1 or MDA-MB-231 MFP cells (2 × 10⁶ cells mouse$^{-1}$) were injected subcutaneously in 4–8 week old male BALB/c nude mice. Fourteen days after transplantation, each probe (25 nmol in 100 μL of sterile PBS) was injected via the tail vein into tumor-bearing mice. Mice were imaged at various time points after injection using the IVIS 200 imaging system equipped with a Cy5.5 filter. Relative fluorescence of equal-sized areas of tumor and background were measured using Living Image software (Caliper life science). Upon finishing the last time point of imaging, mice were anesthetized and sacrificed by cervical dislocation. Tumors, livers, kidneys, and spleens were collected and imaged ex vivo by using the FMT 2500 with a Cy5 filter. After ex vivo imaging, organs were lysed by a dounce homogenizer in muscle lysis buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.2% sodium azide in PBS, pH 7.2). Total protein extracts (100 μg each) were separated by SDS—PAGE and visualized by scanning the gel with a Typhoon flatbed laser scanner.

Acknowledgment: We thank G. Blum and L. Edgington for valuable advice and technical assistance for initial animal experiments. We thank S. Verhelst and V. Albro for helpful discussions about chemical synthesis. We also thank E. Deu Sandovall for help with enzyme assays, A. Guzzetta for HR-MS analysis, and F. Yin and M. Paulick for helpful discussions about cathepsins and assistance with the immunoprecipitations. This work was supported by a National Institutes of Health National Technology Center for Networks and Pathways grant U54 RR020843 (to M.B.), NIH grant R01-EB00511 (to M.B.).

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

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