Cell penetrable, clickable and tagless activity-based probe of human cathepsin L

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ABSTRACT

Human cathepsin L is a ubiquitously expressed endopeptidase and is known to play critical roles in a wide variety of cellular signaling events. Its overexpression has been implicated in numerous human diseases, including highly invasive forms of cancer. Inhibition of cathepsin L is therefore considered a viable therapeutic strategy. Unfortunately, several redundant and even opposing roles of cathepsin L have recently emerged. Selective cathepsin L probes are therefore needed to dissect its function in context-specific manner before significant resources are directed into drug discovery efforts. Herein, the development of a clickable and tagless activity-based probe of cathepsin L is reported. The probe is highly efficient, active-site directed and activity-dependent, selective, cell penetrable, and non-toxic to human cells. Using zebrafish model, we demonstrate that the probe can inhibit cathepsin L function *in vivo* during the hatching process. It is anticipated that the probe will be a highly effective tool in dissecting cathepsin L biology at the proteome levels in both normal physiology and human diseases, thereby facilitating drug-discovery efforts targeting cathepsin L.

1. Introduction

Cellular proteolysis is a highly dynamic, complex, and yet well-organized event orchestrated by more than 550 human proteases [1,2]. The basic process involves catalytic hydrolysis of a specific peptide bond of protein targets under a wide range of cellular conditions (e.g. pH, specific organelle, cytoplasmic vs extracellular). These enzymes play critical roles in maintaining homeostasis, growth, differentiation, and survival. Among these are cysteine cathepsins, a small group of 11 proteolytic enzymes that usually function in acidic cellular environment (e.g. in endosomes/lysosomes), and employ a Cys-dependent covalent nucleophilic catalysis. Originally, cysteine cathepsins were considered a group of house-keeping enzymes whose primary function was to merely degrade unwanted cellular proteins in lysosomes [3]. As a result, their functional roles were often considered redundant. Recent studies, however, strongly point to individual members of cysteine

cathepsins having a specific and non-redundant function in various cell types [4,5]. They can regulate specific physiological processes such as antigen presentation in the immune response pathway, neuropeptide and hormone processing, apoptosis, autophagy, and bone development and remodeling [6–9]. Consistently, their localization is not limited to only in the endo-lysosomal compartment; they are also found in other intracellular organelles such as cytosol, nucleus, secretary vesicles, and in the extracellular matrix. The substrate preferences of various cathepsins using a large synthetic library as well as cellular proteome have revealed that both a relatively broad tolerance and a stricter preference exist for amino acids at distinct positions around the scissile peptide bond [10,11]. One of the main challenges in cathepsin biology remains to assign function to individual members of cysteine cathepsins. This task is especially difficult since cathepsins are initially synthesized as zymogens (inactive enzyme), and stored in specialized organelles. They are often activated by post-translational modifications

(e.g. proteolysis, glycosylation and phosphorylation), and transported to various cellular compartments for function [12]. For such reasons, robust functional assignment of enzymes such as cathepsins have proven challenging, and have led to datasets where estimate of cellular enzyme activities have been off by orders of magnitude [13–15]. Other complexities also exist in defining specific cathepsin function because of the existence of (a) endogenous cysteine cathepsin inhibitors (e.g. cystatins and stefins), and (b) existence of alternative splice variants of enzymes, and overlapping substrate specificities.

A prominent member of the cysteine cathepsin protease family is cathepsin L, which is involved in a variety of critically important cellular processes [5,9,16–28]. Aberrant expression and activation of cathepsin L has been implicated in many types of human diseases [5,18,29–46]. These include promoting highly invasive cancer, cardiovascular, lung, immune, and metabolic disorders [4,28,41,42,47–51]. In addition to its involvement in human biology, cathepsin L has been shown to promote deadly infections arising from viral and parasitic pathogens; some of these include, Ebola, and Severe Acute Respiratory Syndrome (SARS) viruses [52,34]. Despite cathepsin L's involvement in several human diseases, many of its function in appropriate cellular contexts (i.e. different cell types) remain poorly understood [5,16,18,53]. This problem has been further exacerbated since both contradictory and redundant roles of various cathepsin enzymes have recently emerged. For example, while cancer-promoting roles of cathepsin L are well documented, recently in mouse model cathepsin L served as an epidermal tumor suppressor by terminating the growth factor signaling [54]. In another example, cathepsin L seems to be involved in both bone growth and bone loss processes [55,56]. Another challenge related to functional dissection of cathepsin L is due to presence of an analogous cathepsin B which also shares redundant as well as contradictory function. For example, in the inflammatory response signaling pathways, both cathepsin B and L can serve in a mutually compensatory role during the synthesis and activation of the key cytokine molecule, IL-1β [57]. In contrast, processes involved in regulating the release of digestive proteases from pancreas for food digestion, cathepsin B and L seem to play opposing (and non-redundant) roles; while cathepsin B activates the key protease, trypsin, for initiating the digestive proteolysis cascade [58,59], cathepsin L seem to function so as to keep trypsin activation in check [60]. Similarly, in primary hippocampal neuronal signaling during the amyloid precursor protein processing by γ-secretase, cathepsin B elevated while cathepsin L decreased the γ-secretase activity [61]. Cathepsin L is postulated to be a homeostatic protease in cardiac signal transduction but what, if any, role does cathepsin B play in these events remains an open question [62,63]. Small molecule cathepsin L probes that can selectively (over cathepsin B) report on cathepsin L activity in cells with high sensitivity can thus help expedite functional assignments of cathepsin L in both normal biology and diseased states.

Several small molecule probes of cysteine cathepsins have been developed in the recent years [64–82]. These probes can broadly be classified into three major categories. The first type is substrate-based probes (e.g. compound **1** and **2**, see Fig. 1). They are turned over like a substrate at a specific amide bond (in blue) producing enhanced fluorescence readout. The fluorescence enhancement is either due to a relief of quenching mechanism (probe **1**), or by production of a highly fluorescent chemical moiety (probe **2**) upon turnover [64,65]. Although substrate-based probes produce an activity-dependent fluorescence readout, they often suffer from few major limitations: They do not covalently label their target cathepsins; thus it becomes almost impossible to conclude if the optical signal is directly coming from the active intracellular target cathepsin. This becomes especially an issue when overlapping substrate specificities exists, making the quantification of intracellular activity problematic for precise functional assignments. Finally, they can be bulky in nature (hence reduced affinity), and often possess high charge density (hence poor cell penetrability). The second category of probes are photoaffinity probes which have

chemical functionalities suitable for a reversible binding to their target enzymes, and contain an additional photoactivatable group. These probes can be activated for a covalent bond formation with their target enzymes upon exposure to photons. An example of this kind of probe is probe **3** (Fig. 1), developed for monitoring cathepsin L activity. Unfortunately this probe also contains a bulky photoactivatable benzoylphenylalanyl group that renders it only modestly potent (K_i = 3.7 μM), and poorly selective (against cathepsins B) [66]. Finally, such probes may also suffer from non-specific labeling of surrounding proteins, often due to less-than-desirable affinity [83,84]. The third category of probes are ABPs which exploit a conserved catalytic residue of the target cathepsin (the key nucleophilic catalytic residue Cys25), thereby labeling the enzyme covalently and irreversibly in an activity-dependent manner. Probe **4A** and **4B** (Fig. 1) are two examples of such cysteine cathepsin-targeted activity-based probe (ABP). The probe **4A**'s response to the cellular activity of cathepsin L enzyme relies on relief of the quenching mechanism (as in probe **1**) (thus the name qABPs) of the installed fluorophore [68,69,79]. While several highly interesting findings have recently emerged from studies involving qABPs, these probes suffer from less-than-desirable selectivity toward cathepsin L (with respect to cathepsin B) and sensitivity. Using an elegant positional scanning substrate approach, HyCoSuL, Poreba *et al.* developed an efficient activity-based cathepsin L-selective probe **4B**; however this probe also contained a bulky (molecular weight = 1410) and charged Cy5 fluorophore, and exhibited only 412-fold selectivity over cathepsin B [85]. Herein, we report the development of a new class of clickable and tagless activity-based probe (ctABP) of human cathepsin L. We show that the probe is ~10,000-fold selective toward cathepsin L (over cathepsin B), and demonstrate its utilization in probing cathepsin L activity in human MDA-MB-231 breast cancer cell lines. Furthermore, we demonstrate that that this probe can be utilized for perturbation of previously reported cathepsin L function *in vivo*.

2. Results and discussion

2.1. Rationale for the design

The motivation for the current work stemmed from our hypothesis that development of a cathepsin L ABP with a strategically-positioned small clickable moiety without bulky and charged functional groups (e.g. fluorophore tag and quencher moiety) could provide a highly effective probe with enhanced cell penetrability, higher affinity and selectivity and sensitivity of detection. Such clickable and tagless activity-based probe (ctABP) could prove to be highly useful for monitoring activity profile of human cathepsin L in living cells at the proteome levels. Recently using a hybrid-design approach, we reported the development of a highly potent, cell permeable, and non-basic inhibitor, KD-1, of human cathepsin L (Fig. 2) [86]. KD-1's non-basic nature was especially important so that homogenous distribution of inhibitor in the cell can be accomplished; else an undesired accumulation would occur in acidic environment of the lysosome [87–89]. This inhibitor was active-site directed, rapidly inhibited cathepsin L activity in a covalent and irreversible manner, and exhibited very high selectivity preference over cathepsin B [86]. This could therefore serve as an excellent starting point for acquiring cathepsin L ABP. Since the carbobenzyloxy (cbz) group of KD-1 likely sat in the S3 pocket and knowing that S3 pocket of cathepsin L could accommodate bulkier aromatic groups, we thought it possible to incorporate a minimally perturbing small clickable ethynyl group on the cbz moiety [10]. The main advantages of this design approach were envisioned to be 2-fold: (i) The neutral ethynyl group inclusion, while anticipated to minimally affect the kinetic parameter of cathepsin L inactivation, was designed to retain or perhaps augment the cell penetrability, and (ii) The ethynyl group would prove to be a highly valuable tool in detection of labelled cathepsin L from living cells using aqueous-friendly well-established click chemistry protocols [90]. This led to the conception of a ctABP of cathepsin L, KDP-1 (Fig. 2).
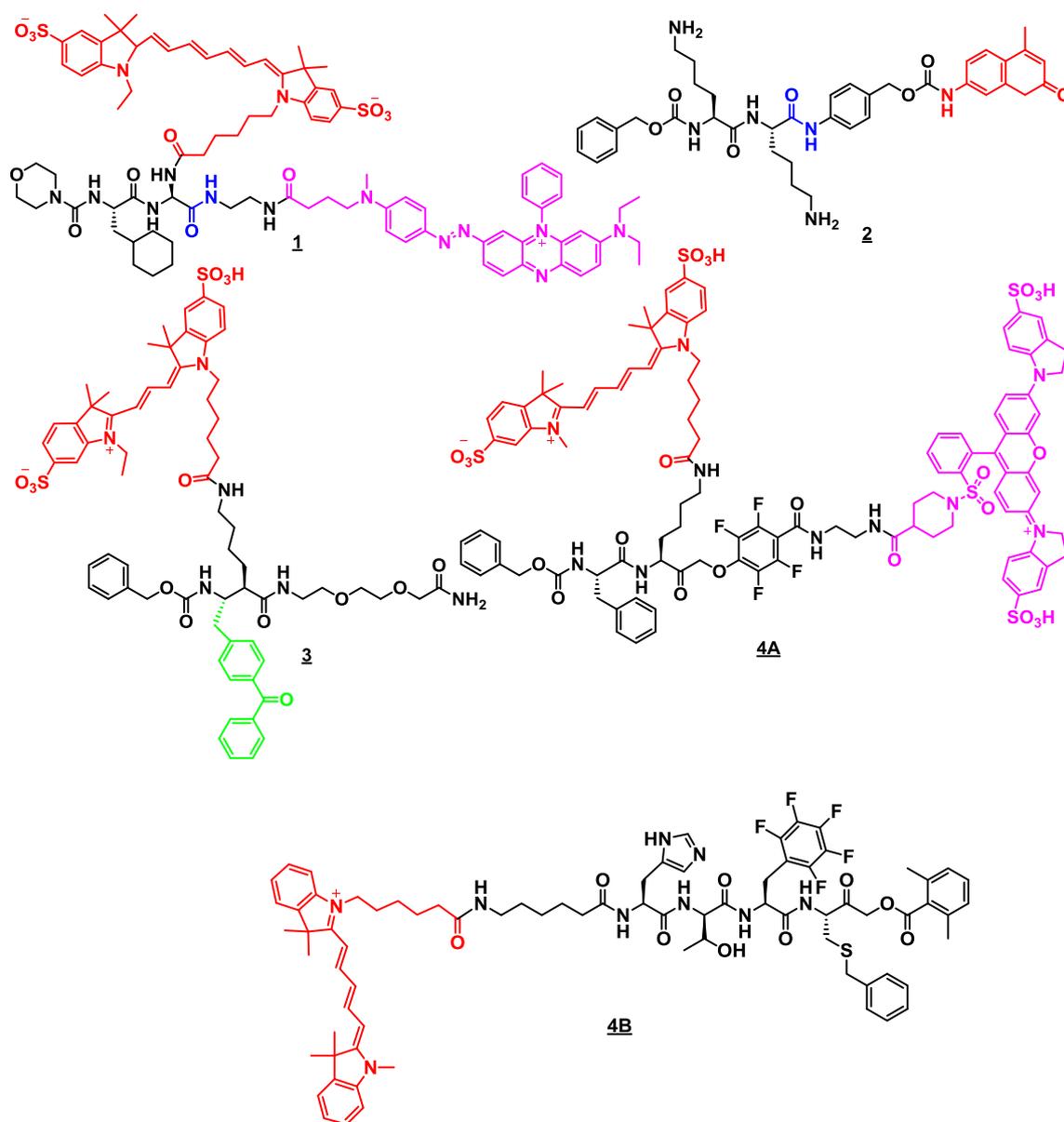
Fig. 1. Chemical structures of the representative small molecule probes developed for cysteine cathepsins. Red: fluorescence reporter group; Pink: quencher; Blue: hydrolyzing amide bond; Green: photoactivatable group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
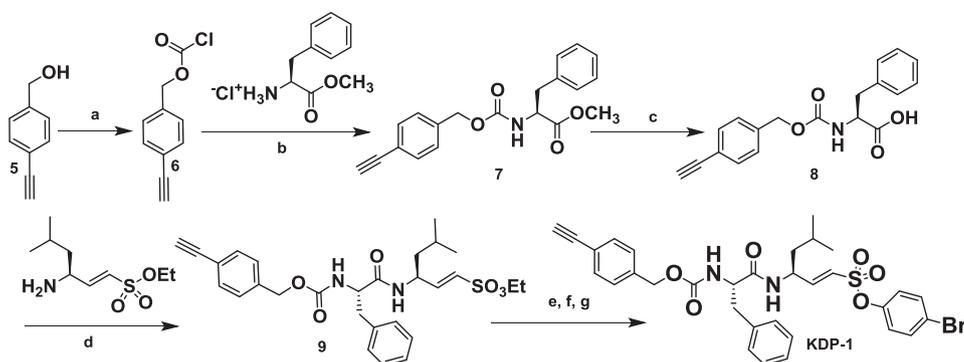

Fig. 2. Chemical structures of (a) the highly potent and selective inhibitory compound KD-1 that inhibits human cathepsin L activity in a covalent and irreversible manner, and (b) the developed clickable and tagless activity-based human cathepsin L probe, KDP-1.

2.2. Chemical synthesis and steady state enzymology of inhibition kinetics

The probe KDP-1 was synthesized as outlined in Scheme 1 [91]. Briefly, this synthesis started with construction of (((4-ethynylbenzyl) oxy)carbonyl)-ʟ-phenylalanine intermediate 8 in three steps, starting from commercially available (4-ethynylphenyl)methanol. Intermediate 8 was then condensed with ethyl(S,E)-3-amino-5-methylhex-1-ene-1-sulfonate to generate the vinylsulfonate ethyl ester intermediate 9.

Deprotection of ethyl group followed by reaction with 4-bromophenol yielded KDP-1. The next step was to assess if the time-dependent inhibition kinetics of KDP-1 probe with human cathepsin L was still robust, and if the developed probe maintained its selectivity profile. Steady state enzymology experiments revealed that KDP-1 exhibited a strong time dependent inactivation of cathepsin L activity, although with ∼10-fold lower rate (Fig. 3) than KD-1; this perhaps could be due to some unfavorable interaction within the S3 pocket of cathepsin L enzyme and may require structural studies in future. Furthermore, KDP-1 still maintained a strong selectivity preference for cathepsin L compared to cathepsin B (∼10,000 fold) and homologous cathepsin K (∼29-fold), and displayed no reactivity towards cathepsin H, also a cysteine cathepsin. A ∼10,000-fold Cathepsin L selectivity over cathepsin B is especially noteworthy since the probe can potentially be utilized for sorting functional redundancies between the two in diverse cellular signaling events; a recently reported human cathepsin L ABP only exhibited ∼412-fold selectivity over human cathepsin B [85]. Aspartyl cathepsin D and serine cathepsin G also exhibited no

noticeable reactivity toward KDP-1, indicating the absolute requirement of a suitably positioned active site Cys25 residue. Further, no cross reactivity was observed against a low pK_a Cys containing protein tyrosine phosphatase 1B enzyme, and the serine protease trypsin.

2.3. Mass spectrometry-based characterization of inhibition

To establish that active site Cys25 was the target for covalent labeling, mass spectrometric analysis was performed on human cathepsin L labelled with KDP-1 probe (Fig. S2, Supporting Information). The MS^2 spectrum at $m/z = 2230.7$ from a chymotryptic digest of KDP-1-reacted Cathepsin L, corresponding to residues 16–30 ([16]VTPVKNQGQCGS-CWA[30]), contains major peaks consistent with neutral loss of KDP-1-related fragments, as well as an *N*-terminal backbone fragment with the label still attached (b^*_{12}, cleavage between Ser27 and Cys28). This latter fragment indicates that the modification is at Cys25 rather than Cys28. These experiments together indicated that the developed cathepsin L ctABP is a selective active-site directed probe.

2.4. Activity-dependent labeling of human cathepsin L

The next task in hand was to demonstrate if (a) KDP-1 was a suitable probe for labeling active human cathepsin L, (b) the ethynyl moiety was amenable for post-labeling quantification of active enzyme using click chemistry protocol, and (c) the enzyme labeling reaction was active site-directed, covalent and irreversible in nature. To investigate this, active recombinant human cathepsin L was incubated with KDP-1

probe in an acidic buffered solution. After the labeling, a click chemistry protocol was performed using TAMRA-azide. A varying amount of TAMRA-KDP-1-cathepsin L complex was resolved using SDS-PAGE and the gel was analyzed using Typhoon 9400 scanner. The scanning experiment revealed that as low as 40 femtomoles (1 ng) of labeled active cathepsin L could be detected with this probe (Fig. 4A), making it a highly sensitive probe, suitable for cellular studies; a covalent and irreversible nature of labeling was also established from this experiment. No labeling was observed when heat-denatured cathepsin L was allowed to react with KDP-1 and undergo identical click chemistry procedure (Fig. 4B left); this experiment indicated an activity-dependent labeling. Finally, when active cathepsin L reaction with KDP-1 was performed in presence of a competitive inhibitor, a significantly reduced amount of labeling was observed, indicating active-site directed chemistry of labeling.

2.5. Assessment of cytotoxicity, cell-penetrability, and cell-migratory potential using triple-negative human MDA-MB-231 cell lines

Efficient activity-based probes can be a very valuable tool for functional studies as well as for assessing *in vivo* target engagement of a drug/probe [92,93] It is therefore imperative that they be non-toxic and biocompatible. Since future studies will involve the use of KDP-1 in cellular studies, we investigated its toxicity in cell culture using human MDA-MB-231 breast cancer cells and the CytoScan WST–1 cell viability assay (G-Biosciences Inc.) [94]. This assay is based on determining metabolically active cells by monitoring reduction of tetrazolium salt
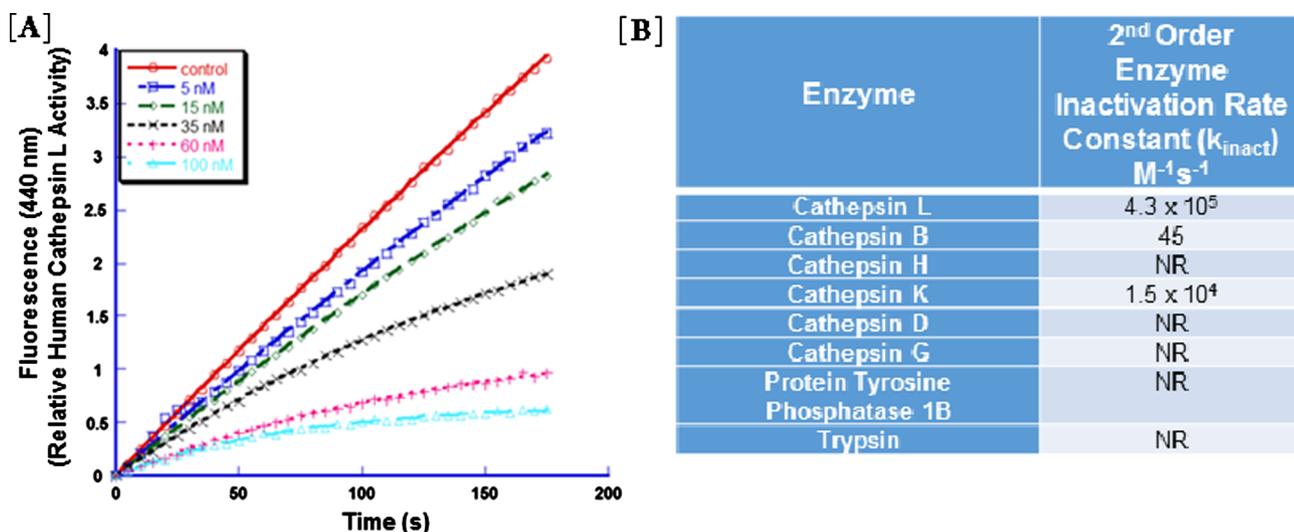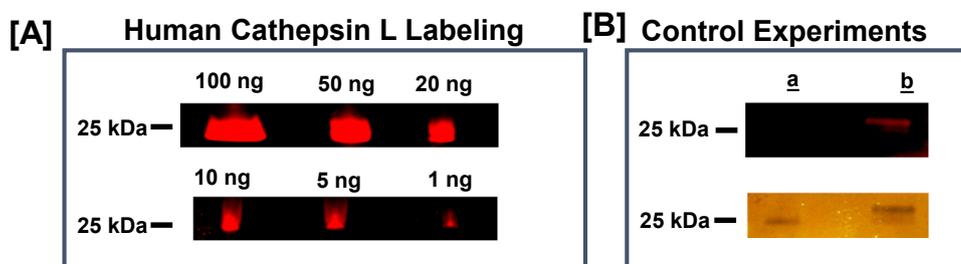

Enzyme	2nd Order Enzyme Inactivation Rate Constant (k_{inact}) $M^{-1}s^{-1}$
Cathepsin L	4.3×10^5
Cathepsin B	45
Cathepsin H	NR
Cathepsin K	1.5×10^4
Cathepsin D	NR
Cathepsin G	NR
Protein Tyrosine Phosphatase 1B	NR
Trypsin	NR

Fig. 3. Kinetic evaluation of cathepsin L probe, KDP-1: [A] Demonstration of time-dependent inhibition of human cathepsin L activity by KDP-1 probe in a dose dependent manner. The progress curves is acquired by monitoring the hydrolysis of Z-FR-AMC substrate in presence of varying concentration of KDP-1 probe and control (DMSO). [B] Selectivity profile of KDP-1 probe against a panel of cathepsins, a low pKa containing Cys enzyme, protein tyrosine phosphatase 1B, and a serine protease, trypsin. NR = No reactivity at 50 μM KDP-1.

Fig. 4. Imaging of active human cathepsin L, labeled with ctABP, KDP-1. [A]: Purified and activated recombinant human cathepsin L was incubated with KDP-1 probe. After reaction was quenched, the labeled enzyme was clicked with 5-TAMRA-azide, resolved using SDS-PAGE, and detected using Typhoon 9400 scanner (ex: 532 nm, em: 580 nm). The experiment indicates that as little as 1 ng (40 fmol) of active human cathepsin L can be detected. [B] Control experiments: (a) heat-denatured inactivated human cathepsin L was subjected to identical labeling and click chemistry procedures; no labeling was observed (50 ng loaded), indicating KDP-1 is an activity-based probe. (b) Identical labeling and detection procedures were performed as in [A], but in presence of a competitive cathepsin L inhibitor KD-1. Significantly reduced amount of human cathepsin L labeling was observed, as expected. The bottom panel is the silver-stained image of the same fluorescence gel. A representative of three independent experiments is shown.
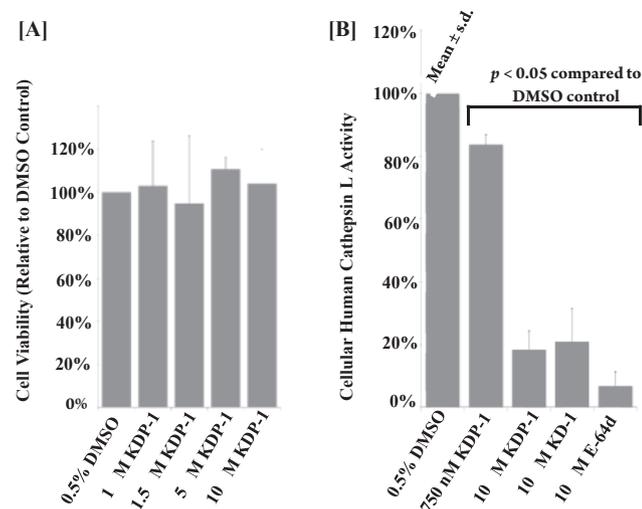

Fig. 5. Assessment of KDP-1 probe's biocompatibility and its ability to inhibit human cathepsin L activity in human MDA-MB-231 breast cancer cell lines. [A] Live MDA MB–231 cells were treated (24 h) with KDP–1 (1–10 µM) and control (DMSO). Cell viability was assessed using colorimetric CytoScan WST–1 cell proliferation assay in a microplate reader. No apparent cellular toxicity is observed, even at a concentration as high as 10 µM. [B] Live MDA MB–231 cells were treated (24 h) with appropriate concentration of DMSO (−ve control), cathepsin L probe KDP-1, cell permeable cathepsin L inhibitor KD-1 (+ve control), and a pan cell permeable cathepsin L inhibitor E-64d (+ve control). After wash, cells were permeabilized with digitonin, and cathepsin activities was measured using fluorescence-based cathepsin substrate, Z-FR-AMC. Dose-dependent loss of cathepsin activities indicates that KDP-1 is cell penetrable, and is thus suitable for activity-based labeling of human cathepsin L at the proteome levels.

WST-1 to formazan by cellular dehydrogenases. No apparent cellular toxicity was observed, even as high as 10 µM of the probe (Fig. 5A). Ubiquitously expressed human cathepsin L has been shown to have several cytoplasmic and nuclear function, in addition to its roles in extracellular matrix (ECM) space [5,95,96]. To demonstrate that developed cathepsin L ctABP, KDP-1 was capable of labeling intracellular cathepsin L in living cells, we sought to establish that it is cell permeable. Human MDA-MB-231 breast cell line was chosen for this study since herein cathepsin L is known to be overexpressed [97,98]. Live cells were treated with KDP-1 probe (with DMSO as negative control) and cells were washed and permeabilized under non-denaturing conditions. Cellular cathepsin activity was measured using the Z-Phe-Arg-AMC fluorogenic cysteine cathepsin substrate. A dose dependent loss of activity was observed, thereby indicating that KDP-1 was cell penetrable and was likely able to block cellular cathepsin L activity (Fig. 5B). In positive control experiments, cells treated with cell permeable cathepsin L inhibitor KD-1 and pan cysteine cathepsin inhibitor E-64d also exhibited significantly diminished cathepsin L

activity [86,99,100]. These studied together suggest that KDP-1 is a nontoxic probe, and can be utilized to label active cellular cathepsin L at the proteome level in the living cells from diverse origin.

We further investigated if the developed probe, KDP-1 was biologically relevant; i.e. if it could successfully perturb cathepsin L function in cellular settings and *in vivo*. In several biological processes involving ECM remodeling, cathepsin L plays important roles [23,101–103]. For example in highly metastatic cancers, overexpressed cathepsin L promotes cell motility by degrading the adherens junction protein, E-cadherin [4]. To investigate if indeed the inhibitory KDP-1 probe reduced the migratory phenotype promoted by cathepsin L, we carried out experiments to assess cell motility in live cells. A wound-healing assay was thus performed on highly motile human MDA-MB-231 breast cancer cells (Fig. 6). Compared to cell incubated in solvent control (0.5% DMSO), cells incubated with 1.25 µM KDP-1 had a 5% decrease in cell migration rate, while 3.75 µM KDP-1 reduced the migration rate 20% (independent samples two-tailed *t*-test p_{obs} = 0.15). This dose-dependent reduction in cell motility is consistent with the inhibition of cathepsin L in ECM space, and supports the notion that KDP-1 can indeed be utilized to probe biological function of cathepsin L in distinct cellular environment.

2.6. Demonstration of functional perturbation of cathepsin L activity in vivo by cathepsin L probe, KDP-1

in Finally, the inhibitory effect of KDP-1 *in vivo* was investigated in a zebrafish system. The hatching process in zebrafish involves a major tissue remodeling. To carry out this function, overexpressed cathepsin L in hatching gland is secreted to degrade proteinaceous chorion [104,105]. Activity of cathepsin L is thus required for the hatching process to begin, and serves as an excellent marker for studying mesoderm and axis induction during the hatching process. A group of zebrafish embryos post-fertilization were treated with KDP-1 at appropriate concentrations and embryos hatched at different hours post-fertilization (hpf) were counted. A dose-dependent decrease in hatching was observed between 49 and 51 hpf compared to control (DMSO) (Fig. 7 II). In positive control, a high concentration of pan cysteine cathepsin inhibitor E64 (10 µM) delayed the hatching process considerably with only 50% hatching, even at 54–72 hpf. This experiment further demonstrates that the developed probe KDP-1 is amenable for studying cathepsin L-mediated *in vivo* function.

3. Conclusion

The development of a highly efficient, cell-penetrable and clickable probe (KDP-1) of human cathepsin L is reported. Steady-state enzymology, labeling, and mass-spectrometric experiments indicate that the probe is highly sensitive, selective, and active-site directed. Cell-biology experiments involving human MDA-MB-231 breast cancer cell lines reveal that the probe is non-toxic, can block cathepsin L activity, and reduce cell migratory phenotype. Finally using live zebrafish
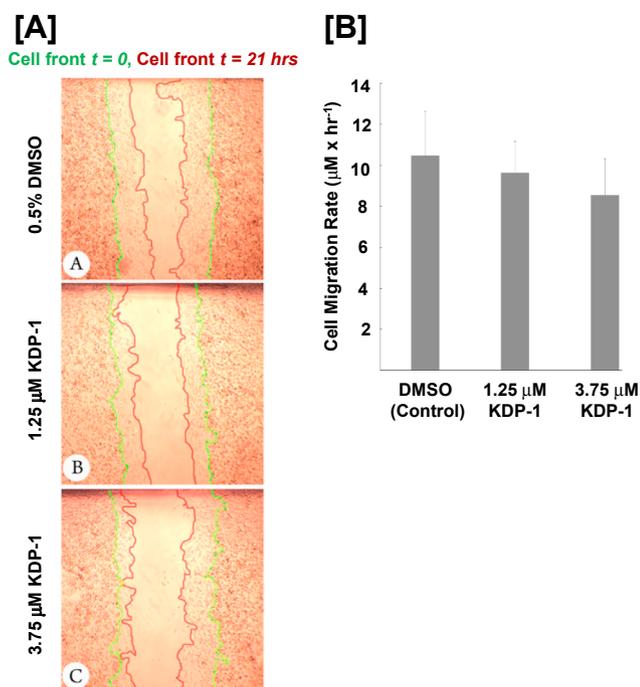
Fig. 6. Phenotypic evaluation of cathepsin L probe, KDP-1 in human MDA-MB-231 breast cancer cells. [A] Wound-healing assay for assessing cell migratory potential where living cells grown to 95% confluency were treated with control (DMSO) and KDP-1. A wound (green borders, t = 0 h) was created and the filling of the wound (cell front) was assessed after 21 h (red borders). Migratory potential of the cells are reduced when treated with KDP-1, consistent with cathepsin L physiological function. [B] Quantitative assessment of the cell migration rate when cells are treated with KDP-1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

embryos, we demonstrate that presence of KDP-1 can delay hatching – a biological process where cathepsin L activity is required. We anticipate that this ctABP cathepsin L probe will find extensive applications in both functional proteomics and in cathepsin L-targeted drug discovery processes.

4. Experimental

4.1. Organic synthesis

4.1.1. General

^{1}H NMR spectra were recorded at either 400 or 500 MHz using CDCl$_3$ or DMSO as the solvent. ^{13}C NMR spectra were recorded at either 125 or 100 MHz using CDCl$_3$ or DMSO as the solvent. Chemical shifts (δ) are reported in parts per million (ppm) and referenced to CDCl$_3$ (7.26 ppm for ^{1}H and 77.0 ppm for ^{13}C), DMSO (2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C). Coupling constants (*J*) are reported in Hertz (Hz) and multiplicities are abbreviated as singlet (s), doublet (d), doublet of doublets (dd), triplet (t), triplet of doublets (td), and multiplet (m). The mass spectra were acquired using a 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS (Agilent Technologies, Santa Clara, CA, USA). 4-ethynylbenzyl alcohol was purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) was purchased from peptide international (Louisville, Kentucky, USA). All anhydrous solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other materials were purchased from Fisher Scientific Inc. (Pittsburgh, PA, USA) unless otherwise mentioned in the text.

4.1.2. Synthesis protocol

The Synthesis of the <u>c</u>lickable and <u>t</u>agless <u>a</u>ctivity-<u>b</u>ased <u>p</u>robe (ctABP), KDP-1 was achieved following the scheme outlined in Scheme 1:

(i) 4-ethynylbenzyl carbonochloridate (**6**): The reaction was carried out by following a literature supported protocol [106]. First, 2.25 g
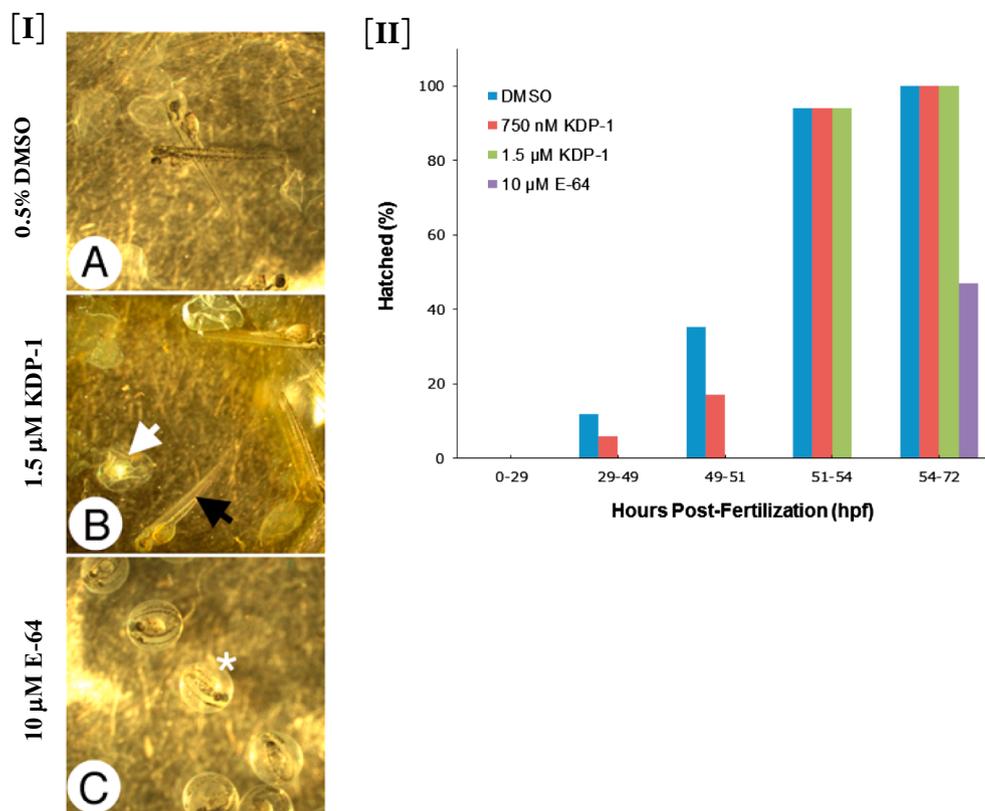

Fig. 7. Inhibition of zebrafish embryo hatching from 29 to 72 h post fertilization by cathepsin L probe, KDP–1 and a pan cysteine cathepsin inhibitor, E–64. [I] Phase contrast micrographs of zebrafish embryos 53 h post hatching and 29 h post treatment with (A) Control (0.6% DMSO), (B) 1.5 µM KDP–1, and 10 µM E–64, a pan cysteine cathepsin inhibitor. The white arrow indicates an empty chorion and the black arrow indicates a hatched embryo. The asterisk indicates an embryo still in the chorion. [II] A group of embryos were exposed to probe KDP-1 (750 nM, and 1.5 µM), and E-64 (10 µM) post-fertilization, and the number of embryos hatched were counted and plotted. It is evident that hatching is delayed when embryos are treated with inhibitory probe KDP-1 (29–51 hpf), compared to control (0.6% DMSO). A high concentration of pan cysteine cathepsin inhibitor E64 (10 µM) delays the hatching process significantly (only about 50% hatched), even at 54–72 hpf, and serves as a positive control.

(7.57 mmol, 2 equiv) of triphosgene was dissolved in a minimal amount of anhydrous toluene in the presence of 1.88 g (17.7 mmol, 9 equiv) of oven dried sodium carbonate (Na_2CO_3) under inert atmosphere at 0 °C. After allowing the mixture to stir for 35 min, 0.500 g (3.78 mmol, 1 equiv) of 4-ethynylbenzyl alcohol dissolved in dry toluene was added dropwise. Then, the mixture was stirred overnight at room temperature. The reaction mixture was filtered, evaporated, and the resulting 4-ethynylbenzyl carbonochloridate was used without purification.

(ii) Methyl (((4-ethynylbenzyl)oxy)carbonyl)phenylalaninate (**7**): The reaction was carried out by following a literature reported protocol with slight modification [107]. To a stirred suspension of L-phenylalanine methyl ester hydrochloride (815 mg, 3.8 mmol, 1 eqv.) in 5 ml of toluene was added 4-ethynylbenzyl carbonochloridate (735.6 mg, 3.8 mmol, 1 eqv.) in an icebath. To this was added 5.3 ml of 1 M sodium carbonate solution. The reaction mixture was kept stirring for 3 h while the temperature was maintained at less than 10 °C. The reaction mixture was diluted with ethyl acetate and washed with 0.1 M HCl, saturated sodium bicarbonate solution and brine successively. The organic layer was dried over sodium sulfate and purified by silica gel column chromatography using hexanes and EtOAc in a 95:5 ratio, yielding a white solid (R_f = 0.44, Hexanes/EtOAc (80:20), 46.3% yield). The characterization was found to be consistent with the previously reported NMR spectra [108].

(iii) (((4-ethynylbenzyl)oxy)carbonyl)-L-phenylalanine (**8**): Subsequent deprotection to the carboxylic was carried out by following the literature reported protocol and its characterization was found to be consistent with the previously reported NMR spectra [108].

(iv) Ethyl (E)-3-amino-5-methylhex-1-ene-1-sulfonate: This compound was synthesized by following a literature reported protocol [109].

(v) Ethyl (S,E)-3-((S)-2-(((4-ethynylbenzyl)oxy)carbonyl)amino)-3-phenylpropanamido)-5-methylhex-1-ene-1-sulfonate (**9**): This compound was synthesized by following an analogous literature reported protocol [109]. ^{1}H NMR (400 MHz CDCl$_3$) δ ppm: 0.87 (m, 6H), 1.31 (m, 2H), 1.38 (t, J = 7.0 Hz, 3H), 1.50 (m, 1H), 3.02 (dd, J = 13.5 and 8.1 Hz, 1H), 3.12 (dd, J = 13.5 and 8.1 Hz, 1H), 3.09 (s, 1H), 4.14 (m, 2H), 4.36 (m, 1H), 4.62 (m, 1H), 5.08 (m, 2H), 5.33 (b, 1H), 5.75 (b, 1H), 5.94 (d, J = 15.2 Hz, 1H), 6.60 (dd, J = 15.2 and 5.1 Hz, 1H), 7.18 (d, J = 7.0 Hz, 2H), 7.30 (m, 5H), 7.48 (d, J = 8.2 Hz, 2H). ^{13}C NMR (100 MHz CDCl$_3$) δ ppm: 14.9, 21.8, 22.6, 24.6, 38.5, 42.7, 48.1, 56.6, 66.7, 66.9, 77.8, 83.2, 122.2, 124.6, 127.6, 127.9, 129.1, 129.2, 132.4, 135.9, 136.6, 147.6, 155.9, 170.4.

(vi) 4-bromophenyl (S,E)-3-((S)-2-((((4-ethynylbenzyl)oxy)carbonyl) amino)-3-phenylpropanamido)-5-methylhex-1-ene-1-sulfonate (KDP-1): This compound was synthesized by following an analogous literature reported protocol [109]. ^{1}H NMR (400 MHz CDCl$_3$) δ ppm: 0.82 (m, 6H), 1.22 (m, 2H), 1.41 (m, 1H), 2.99 (dd, J = 13.5 and 8.4 Hz, 1H), 3.10 (dd, J = 13.5 and 8.4 Hz, 1H), 3.10 (s, 1H), 4.37 (m, 1H), 4.53 (m, 1H), 5.06 (m, 2H), 5.44 (d, J = 7.1 Hz, 1H), 5.89 (d, J = 5.2 Hz, 1H), 6.04 (d, J = 15.1 Hz, 1H), 6.47 (dd, J = 15.1 and 5.3 Hz, 1H), 7.08 (m, 2H), 7.13 (m, 2H), 7.28 (m, 5H), 7.49 (m, 4H). ^{13}C NMR (100 MHz CDCl$_3$) δ ppm: 21.8, 22.5, 24.6, 38.4, 42.3, 48.2, 56.6, 66.7, 77.8, 83.2, 120.8, 122.2, 123.8, 124.4, 127.6, 127.9, 129.1, 129.1, 132.3, 132.9, 135.8, 136.5, 148.3, 149.9, 155.9, 170.5. HRMS (*m/z*): [M +H]$^+$ for molecular formula C$_{32}$H$_{33}$BrN$_2$O$_6$S: calculated 653.1315; found 653.1318.

4.2. Steady state inactivation kinetics

4.2.1. General

The following enzymes and their substrates were purchased from Enzo Life Sciences (Farmingdale, NY, USA): cathepsin L (BML-SE201), cathepsin K (BML-SE553), cathepsin B (BML-SE198), cathepsin D (BML-SE199), cathepsin S (BML-SE453), cathepsin H (BML-SE200), cathepsin G (BML-SE283), human PTP1B (BML-SE332), Z-Arg-Arg-pNA (BML-P138), Z-Gly-Pro-Arg-AMC (BML-P142), Z-Phe-Arg-AMC (BML-P139), H-Arg-AMC.2HCl (BML-P135), Suc-Ala-Ala-Pro-Phe-pNA (BML-P141), Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH$_2$ (BML-P145), Z-Val-Val-Arg-AMC (BML-P199). *N*-alpha-Benzoyl-DL-arginine-p-nitroanilide (BAPNA) (B4875), trypsin (T1426), *para*-nitrophenylphosphate were purchased from Sigma–Aldrich (St. Louis, MO, USA). All the biological grade buffers were used and were purchased from Sigma–Aldrich (St. Louis, MO, USA). All enzyme kinetics experiments (unless otherwise stated) were carried out at 30 °C in appropriate buffer condition with 5% DMSO concentration. For measuring the initial rates of enzyme catalyzed reaction, a temperature-controlled steady-state arc lamp fluorometer equipped with Felix 32 software (Photon Technology Instrument, Birmingham, NJ, USA) and a UV–Vis spectrophotometer (Model Lambda 25; Perkin Elmer Inc., Waltham, MA, USA) was used.

4.2.2. Determination of second order inactivation rate constant of KDP-1 reactivity with human cathepsin L and its selectivity profile

A previously reported cathepsin L assay procedure was adopted with suitable modifications [86]. Briefly, recombinant human liver cathepsin L (Enzo Life Sciences, USA) was first activated by incubation in sodium acetate buffer (50 mM, pH 5.5) for 30 min at 30 °C. The inactivation reaction at pH 5.5 was then continuously monitored for 3 min under pseudo first order condition ([I] ≫ [E]; Net [E] 160 pM; [I] 5–100 nM) in a fluorescence (excitation:365 nm; emission: 440 nm) quartz cuvette containing Z-FR-AMC substrate (net 5 µM) maintained at 30 °C. The progress curves thus obtained at various KDP-1 concentration were fitted using a non-linear regression method to the following equation [110]:

$$P = v_z[1 - \exp(-k_{obs} * t)]/k_{obs}$$

where [P] is the product, v_z is the initial velocity (at time zero), and k_{obs} is the pseudo-first order rate constant.

The pseudo-first order rate constants k_{obs} thus obtained from the fit above were plotted against corresponding inhibitor concentrations [I]. The 2nd order enzyme inactivation rate constant, k_{inact}, was calculated (Fig. S1, Supporting Information) from the slope of the plot according to following equation [110]:

$$k_{obs} = k_{inact}[I]/[1 + ([S]/K_M)]$$

where k_{obs} is the pseudo-first order rate constant, [I] is the corresponding inhibitor concentration, [S] is the substrate concentration with a given K_M. KaleidaGraph (version 3.52) was used for plotting and analyzing the data. All assays involving selectivity profile of various enzymes were performed following the earlier published protocols [86,111,112].

4.3. Tandem Mass-spectrometric analysis of human cathepsin L labelled with KDP-1

A 10 µg KDP1-reacted and 5 µg unreacted human Cathepsin L samples were run on a 4–12% % SDS-PAGE gel and stained with colloidal coommassie blue (Thermo Scientific). Bands were excised manually, and digested by sequencing grade chymotrypsin (Promega) at 27 °C for 6 h. Chymotrypsin was added at the beginning of the digestion to a final substrate-to-enzyme ratio of ∼30:1 in a buffer containing 100 mM Tris, 10 mM CaCl$_2$, pH 8.0 and supplemented at a substrate-to-enzyme ration of ∼60:1 halfway through the reaction. After digestion, samples were prepared as previously described [113]. Briefly, POROS 20 R2 resin (Applied Biosystems) was added into the digested Cathepsin L samples for extraction at 4 °C for 4 h. Prior to MALDI mass spectrometric analysis, the digests were desalted using ZipTip (Millipore) and eluted from ZipTip resins directly onto a MALDI plate with a saturated α-cyano-4-hydroxycinnamic acid (Sigma) MALDI matrix solution trifluoroacetic acid/acetonitrile/water (0.1:70:29.9, v/

v/v), and the solvent evaporated at room temperature. The resulting MALDI crystals were analyzed by hypothesis-driven MS^2 at m/z values corresponding to potentially-modified chymotryptic peptides (Fig. S2, Supporting Information) [114,115]. Mass spectrometry was performed using a Thermo LTQ XL linear ion trap mass spectrometer (Thermo Scientific) equipped with a vacuum MALDI source. MS^2 spectra were acquired using the supplied N$_2$ laser (Thermo Scientific) at a power of 5 μJ with a repetition rate of 10 Hz, using an isolation width = 4.0 m/z, activation Q = 0.25 and activation time = 100 ms. Each spectrum was acquired for approximately 2.25 min. The resulting spectra were analyzed manually with the assistance of PAWS sequence analysis software (Genomic Solutions) and ProteinInfo (Rockefeller University, NY; http://prowl.rockefeller.edu) to calculate theoretical molecular weights.

4.4. Labeling and detection of recombinant active human cathepsin L using click chemistry

First, the recombinant human Cathepsin L (2.2 μg, 5 μL of 17 μM supplied stock, R&D Systems Inc.) was fully activated by incubating for 30 min at 30 °C in 13.5 μL NaOAc buffer (50 mM, pH = 5.5) containing 4 mM DTT. Subsequently, cathepsin L labeling was initiated with addition of 1.5 μL KDP-1 (net 25 μM in the total 20 μL reaction volume) and the labeling reaction was allowed to occur for 2 h at 30 °C. To detect the amount of KDP-1-labeled human cathepsin L, a click chemistry protocol was planned using 5-TAMARA azide (Click Chemistry Tools, Inc., cat # 1245). The labeling reaction mixture was brought to near neutral pH by addition of 2 μL HEPES buffer (1000 mM, pH 8.0) before performing the click chemistry reaction. To begin click chemistry the following was added to the reaction mixture: 2 μL of TAMRA-Azide solution (5 mM stock in DMSO), 2 μL Sodium Ascorbate (20 mM stock in dH$_2$O), 1 μL CuSO$_4$ solution (10 mM stock in dH$_2$O), 1 μL THPTA (10 mM stock solution in dH$_2$O), and 2 μL of deionized water. The click reaction mixture was gently shaken for 2 h at 30 °C. The reaction was quenched with the addition of 15 μL of 4x Laemmli sample buffer and the resulting sample was vortexed and centrifuged. Appropriate concentrations of labeled and clicked human cathepsin L were loaded in 4–20% gradient SDS-PAGE gel (Bio-Rad Inc.) and the protein was resolved from other components. For control experiments, identical labeling and click chemistry protocols were utilized except that (i) heat-denatured and inactivated human cathepsin L was utilized, and (ii) the labeling with KDP-1 was performed in presence of the highly potent competitive inhibitor KD-1 (net 15 μM).

4.5. Cell biology studies

4.5.1. Biocompatibility assay

MDA MB–231 (ATCC ® HTB–26™) human breast cancer cells were plated in 96-well tissue culture plates at 0.5×10^4 cells per well in 100 μL complete medium (DMEM supplemented with 10% FCS and 10% penicillin/streptomycin, nonessential amino acids, and glutamine). The next day, the medium was aspirated from the cells and 100 μL complete medium was added also containing various concentrations of KDP–1 or 0.5% DMSO (solvent control). After 24 h of treatment, the relative number of metabolically–active cells in each condition was measured using the colorimetric CytoScan WST–1 cell proliferation assay [94] (G-Biosciences Inc., USA) in a microplate reader. Average of three independent assays were taken into consideration on separate days (biological replicates) and each individual day assay was the average of 3 wells (technical replicates). The degree of biocompatibility was calculated as the percentage of cells alive in presence of KDP–1 relative to the number alive in the presence of the solvent:

$$\%survival = (ABS_{cells+KDP-1} - ABS_{KDP-1\,no\,cells})$$
$$/(ABS_{cells+DMSO} - ABS_{DMSO\,no\,cells})$$

4.5.2. Assessment of human cathepsin L activity in MDA-MB-231 live cells treated with KDP-1

The method of Repnick was used [116]. MDA MB–231 cells were plated at $10–15 \times 10^4$/well in complete medium cells in black transparent bottom 96–well plates. The next day, fresh media was added containing one of the following: 0.5% DMSO, 0.75 μM KDP-1, 10 μM KDP-1, 10 μM KD-1 or 10 μM E-64d (a known broad range, cell-permeable cysteine protease inhibitor). After 24 h, soluble extracellular inhibitors were removed by aspirating the media and washing the cells with phosphate–buffered saline. Next a cellular lysate was made by solubilizing the plasma membrane and intracellular membranes by adding 50 μL of 200 μg/mL digitonin in acetate buffer (50 mM sodium acetate pH 5.6, 150 mM NaCl, 0.5 mM EDTA) to the wells containing the cells and incubating on ice for 12 min. We next added 50 μL of the Omnicathepsin fluorogenic substrate Z-FR-AMC in sodium acetate buffer directly to the lysates in the wells for a final concentration of 30 μM Z–FR–AMC and 10 mM DTT. We quantified the relative amount of substrate cleavage in each well using SpectraMax M4 multi-mode microplate reader at 380 nm excitation and 460 nm emission. The experiments were repeated in 3 independent trials and each trial represented a technical replicate of two. Because of slight variability in amount of substrate or the number of cells in different trials, we normalized the fluorescence generated in each trial relative to the DMSO control from that trial. A one-way ANOVA was conducted to compare the effect of the various inhibitors on intracellular cathepsin. At 0.75 and 10 μM KDP–1 and 10 μM of the inhibitors KD-1, and E-64d, there was a significant inhibition of Z-FR-AMC cleavage at the $p < 0.05$ level. Because these data were proportional (percentages), significance was assessed by first transforming the percentage data using an arcsine transformation followed by a t Test with Bonferroni correction.

4.5.3. Wound healing assay for assessment of the effect of probe KDP-1 on cell migration

Approximately 3×10^5 MDA MB–231 cells were added to 24– well culture plates containing 1 ml of complete medium and allowed to attach and spread to confluency for 24 h. A 200 μL Pipetteman tip was then used to wound the monolayer and form a cell–free area. Any detached cells and cell debris were removed by washing the well with complete medium and then 1 ml of complete medium was added to the well. Next, 1.25 μM or 3.75 μM KDP–1 or 0.5% DMSO as added to the wells. Phase contrast photographs of the scratched area were taken as a time zero reference for cell position. The cells were returned to 37 °C for 21 h and then photographs were taken to determine how far the cells had moved into the scratch. Images from the two time points were overlaid and the distance between the initial and final front were measured at 6 points along the fronts to determine distance of migration. The data represent the average of four independent trials.

4.6. In vivo zebra fish hatching experiment

Zebrafish embryos were a gift from the laboratory of Dr. Nathalia Glickman Holtzman, Queens College. Approximately 18–20 embryos were place in 6–well plates containing 1 ml of embryo water containing 0.5% DMSO. Subsequently, 0.75 or 1.54 μM KDP–1 or 10 μM of the pan cysteine cathepsin inhibitor E64 were added. The embryos were incubated at 28.5 °C and the number hatching from the chorion (egg envelope) at various timepoints were then counted under a dissecting scope.

Author contributions

The manuscript was written through valuable contributions of all

authors. All authors have given approval to the final version of the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.02.032.

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