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Active site labeling of cysteine cathepsins by a straightforward diazomethylketone probe derived from the N-terminus of human cystatin C

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ABSTRACT

We designed a straightforward biotinylated probe using the N-terminal substrate-like region of the inhibitory site of human cystatin C as a scaffold, linked to the thiol-specific reagent diazomethylketone group as a covalent warhead (i.e. Biot-$(PEG)_2$-Ahx-LeuValGly-DMK). The irreversible activity-based probe bound readily to cysteine cathepsins B, L, S and K. Moreover affinity labeling is sensitive since active cathepsins were detected in the nM range using an ExtrAvidin®-peroxidase conjugate for disclosure. Biot-$(PEG)_2$-Ahx-LeuValGly-DMK allowed a slightly more pronounced labeling for cathepsin S with a compelling second-order rate constant for association (k_{ass} = 2,320,000 M^{-1} s^{-1}). Labeling of the active site is dose-dependent as observed using 6-cyclohexylamine-4-piperazinyl-1,3,5-triazine-2-carbonitrile, as competitive inhibitor of cathepsins. Finally we showed that Biot-$(PEG)_2$-Ahx-LeuValGly-DMK may be a simple and convenient tool to label secreted and intracellular active cathepsins using a myelomonocytic cell line (THP-1 cells) as model.

1. Introduction

Human cysteine cathepsins (B, H, L, S, C, K, O, F, V, X and W) are proteases related to plant papain (family C1, clan CA) [1]. Apart from their ancillary participation in the lysosomal degradation, these proteases contribute to various biological processes (e.g. apoptosis, angiogenesis, antigen presentation, maturation of peptide hormones) and display potent collagenolytic and elastinolytic activities [2,3]. Their proteolytic activity is specifically regulated and balanced by natural inhibitors of the cystatin family (stefins, cystatins, and kininogens) [4,5]. Cysteine cathepsins have emerged as new players in a wide spectrum of illnesses from atherosclerosis, osteoporosis, adiposity, tumor invasion [6] to lung disorders [7], making them validated and attractive targets for new anti-protease therapies [8]. However their accurate role and their fine regulation remain partly unknown and described. Indeed analysis of global changes in gene transcription and translation by systems-based genomics and proteomics approaches does not provide direct information about protein function. Moreover in some cases, enzymatic activity fails to correlate adequately with transcription or translation levels. Under these circumstances a direct method to broadly assess the activity of proteolytic enzymes is of great utility [9]. Different types of ABPs, mainly having electrophilic properties have been developed (see for review: [10]. Among them, Greenbaum and Bogyo successfully profiled a broad set of cysteine proteases by using L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino) butane (E-64[2]as template [11,12]. Independently cystatin C-derived chemical

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[2] Abbreviations: ABP, activity-based probe; Ahx, 6-aminohexanoic acid; 7-amino-4-methyl coumarin; AMC, 7-amino-4-methyl coumarin; BSA, bovine serum albumin, CA-074, N- (L-3-trans-propylcarbamoyl oxirane-2-carbonyl)-L-isoleucyl-L-proline; DMK, diazomethylketone; DTT, DL-dithiothreitol; ECL, enhanced chemiluminescence; E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino) butane; FBS, Fetal bovine serum; MDM, monocyte-derived macrophage; PBS, Phosphate Buffer Saline; PMA, phorbol myristate acetate; TBS, Tris Buffer Saline; Z, benzyloxycarbonyl.

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probes were engineered to track activity of cysteine cathepsins [13–15]. The N-terminal peptidyl substrate-like region of the inhibitory site of human cystatin C, which is the most potent circulating inhibitor of cysteine cathepsins [4], was used as a scaffold [16,17]. Probes were synthesized by using the core tripeptide recognition portion (i.e. Leu-Val-Gly) ligated to the thiol-specific reagent diazomethylketone group as a covalent warhead. Finally a N-terminal visualization (affinity) tag such as biotin, in the presence or not of a 6-aminohexanoate spacer, was added [14]. Nevertheless the need to improve both the solubility of cystatin C-derived molecules and their detection limit led us to synthesize a new pegylated compound. The covalent and irreversible activity-based probe, Biot-$(PEG)_2$-Ahx-LeuValGly-DMK, inhibited and could label cathepsins B, K, L and S in the nM range. Biot-$(PEG)_2$-Ahx-LeuValGly-DMK was further characterized using a potent and reversible nitrile-based inhibitor of cathepsins (6-cyclohexylamine-4-piperazinyl-1,3,5-triazine-2-carbonitrile) in a well-established model of monocyte-derived macrophages (THP-1 cell line) [18].

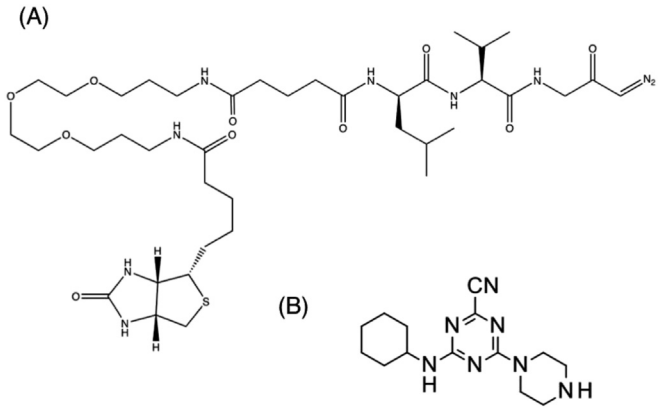

Fig. 1. Structures of the pegylated activity-based probe and of the nitrile-based inhibitor. (A) Biot-$(PEG)_2$-Ahx-LeuValGly-DMK (probe 2). (B) 6-cyclohexylamine-4-piperazinyl-1,3,5-triazine-2-carbonitrile (I-25).

2. Material & methods

2.1. Enzymes and reagents

DTT was supplied by Bachem (Bubendorf, Switzerland). E-64, CA-074 and EDTA were supplied by Sigma–Aldrich (Saint-Quentin Fallavier, France). Z-Phe-Arg-AMC and Z-Leu-Arg-AMC were purchased from Bachem. Human cathepsin K was kindly provided by Dr Dieter Brömme (University of British Columbia, Vancouver, Canada). Human cathepsins B, L and S were purchased from Calbiochem (VWR International, Pessac, France). Papain was purchased from Boehringer (Roche Molecular Biochemicals, Mannheim, Germany). Aspartic cathepsin D was supplied by R & D research (Minneapolis, USA). Human MMP-12 was a kind gift from Anne-Sophie Lamort (INSERM UMR1100, CEPR, University F. Rabelais, Tours, France). Trypsin and chymotrypsin were purchased from Euromedex (Strasbourg, France). Human neutrophil elastase (HNE) was supplied by BioCentrum (Krakow, Poland). All other reagents were of analytical grade.

2.2. Synthesis of peptidyl activity-based probes and of 6-cyclohexylamine-4-piperazinyl-1,3,5-triazine-2-carbonitrile

Biot-Ahx-LVG-DMK (probe 1) was prepared according to a former report [14]. Biot-$(PEG)_2$-Ahx-LeuValGly-DMK (probe 2) was synthesized using the same procedure except that a hydrophilic biotinylated linker (N-Biotinyl-NH-$(PEG)_2$-COOH, Novabiochem, Merck KGaA, Darmstadt, Germany) was introduced (mass spectrometry: theoretical Mw, m/z = 854.07; experimental Mass, m/z = 853.47) (see Fig. 1A). Synthesis of the 4,6-diamino-substituted cyanotriazine (6-cyclohexylamine-4-piperazinyl-1,3,5-triazine-2-carbonitrile, so called I-25), previously developed by Organon (Lanarkshire, UK) [20] (Fig. 1B), was detailed in the supplementary file 1.

2.3. Enzymatic assays

Assays for cathepsins B, K, and L were carried out at 37 °C in their activity buffer (0.1 M sodium acetate buffer, pH 5.5, containing 2 mM DTT and 0.01% Brij35), using Z-Phe-Arg-AMC as substrate (spectromicrofluorimeter SpectraMax Gemini, Molecular Devices, Saint Grégoire, France; λ_{ex} = 350 nm, λ_{em} = 460 nm). The same protocol was handled for cathepsin S, except that Z-LR-AMC was used as substrate. Active site concentrations of cysteine cathepsins were determined using E-64 [19]. Alternatively activity buffers were 0.1 M Tris/HCl buffer, pH 8.0, 50 mM $CaCl_2$, 100 mM NaCl for trypsin and chymotrypsin, 0.05 M HEPES buffer, pH 7.4, NP40 0.05% 150 mM NaCl for HNE, 0.05 M HEPES buffer, pH 7.5, 150 mM NaCl, 8 mM $CaCl_2$ and 0.05% Brij35 for MMP-12, 0.1 M sodium citrate buffer, pH 4.0 for aspartic cathepsin D, respectively.

2.4. Kinetics

Cathepsins B, K, L and S (2 nM) were incubated in the activity buffer (0.1 M sodium acetate buffer, pH 5.5, containing 2 mM DTT and 0.01% Brij35) in the presence of increasing concentrations of I-25 (0–250 nM) for 30 min, before measurement of the residual enzymatic activity, using fluorogenic AMC-derived substrates (10 μM) (spectromicrofluorimeter SpectraMax Gemini, Molecular Devices, Saint Grégoire, France; λ_{ex} = 350 nm, λ_{em} = 460 nm). Assays were performed in triplicate and repeated three times. Slopes were calculated and average values of IC_{50} determined (software Softmaxpro, Molecular Devices). Otherwise inactivation studies by probes 1 and 2 were performed using the method designed by Tian and Tsou [21]. Briefly, the inactivation of papain (0.2 nM) was monitored continuously in the presence of 25 μM Z-Leu-Arg-AMC (Kontron SFM25 spectrofluorimeter; λ_{ex} = 350 nm, λ_{em} = 460 nm), allowing determination of k_{ass}, the second-order rate constant for association of the DMK inhibitor as described elsewhere in details [13]. Assays were performed under experimental conditions where less than 10% of the substrate was hydrolyzed before the protease was fully inhibited (n = 3). Experiments were repeated with cathepsin S (0.2 nM).

2.5. Affinity labeling of cathepsins by biotinylated cystatin-derived probes

Cysteine proteases (0–50 nM) were incubated in the activity buffer (0.1 M sodium acetate buffer, pH 5.5, containing 2 mM DTT and 0.01% Brij35) with a large excess of peptidyl activity-based probes (1 μM) for 1 h at 30 °C. Samples were further subjected to 12% SDS-PAGE under reducing conditions before blotting [14]. The nitrocellulose sheet was incubated with ExtrAvidin®-peroxidase (1:500) (Sigma–Aldrich) for 2 h at room temperature. Finally the peroxidase activity was revealed with by chemiluminescence (ECL kit, Amersham Pharmacia Biotech, Buckinghamshire, England).

2.6. Cell culture

Human THP-1 cells (LGC Promochem, Molsheim, France) were cultured at 37 °C, in 5% CO2, with Gibco RPMI-1640 (Fisher Scientific, Illkrich, France), containing 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, 0.05 mM 2-β-mercaptoethanol. Culture medium was supplemented by 10% FBS, 50 units/ml of penicillin, and 100 μg/ml of streptomycin (InVitrogen, Cergy-Pontoise, France). Monocytes were differentiated into macrophages by addition of 162 nM PMA (Sigma–Aldrich) according to [18]. At day 2, culture medium was removed, and substituted by FBS-free medium. At day 6, cell-free supernatants and MDM hydrolyzates (following three cycles of freezing/thawing lysis) were prepared according to [22]. Protein concentration was determined using the Bradford Protein Assay (BioRad, Marnes-La-Coquette, France). Supernatants were incubated for 1 h, at 30 °C with increasing concentrations of I-25 (0–100 μM), before addition of probe 2 (2 μM). CA-074 (10 μM) was used as control. Aliquots were removed and residual cathepsin activity was measured using Z-Phe-Arg-AMC as substrate as described above. Alongside supernatant samples were subjected to electrophoresis on 12% SDS-PAGE under reducing conditions, electro transferred to a nitrocellulose membrane, incubated with ExtrAvidin®-peroxidase (1:500) for 2 h at room temperature. Finally the peroxidase activity was revealed with ECL. The same procedure was used for THP-1 lysates. Alternatively I-25 (0–100 μM) was added to the culture medium and incubated for 6 and 24 h, respectively, in order to evaluate its internalization. Briefly culture medium was removed and MDM lysates were prepared by freezing/thawing cycles, then residual peptidase activity corresponding to lysosomal cathepsins was recorded. In a second step I-25-treated lysates were incubated with Biot-$(PEG)_2$-Ahx-LeuValGly-DMK (2 μM), in order to disclose by chemiluminescence (see above) the presence of remaining active cathepsins.

3. Results and discussion

3.1. Efficient affinity labeling of cysteine cathepsins

Both biotinylated inhibitors bound to human cathepsins B, K, L and S and were revealed by an ExtrAvidin®-peroxidase conjugate after blotting (Fig. 2). The staining, however, was more pronounced with the longer and more hydrophilic Biot-$(PEG)_2$-Ahx-LeuValGly-DMK (probe 2) than with Biot-Ahx-LVG-DMK (probe 1). Compared to cathepsins B, K and S, diazomethylketone probes more weakly labeled human cathepsin L. A similar observation was observed with rat cathepsin L [14]. Interestingly cathepsin S was detected to a concentration as low as 5 nM following incubation with Biot-$(PEG)_2$-Ahx-LeuValGly-DMK. A similar detection level was observed for papain, the archetypal member of the C1 family (data not shown). It should be noted that the introduction of the polyethylene glycol linker, in addition to allowing a better solubilization, improves the detection limit compared to those of commercial hydrophobic diazomethylketone probes (usually approx. 0.1 μM), potentially via improved presentation of the reporter group to the ExtrAvidin®-peroxidase conjugate.

3.2. Selective inhibition of cathepsins B, K, L and S by active site targeting

Nitrile-based compounds have been identified as inhibitors of cathepsins and have received a lot of attention. Among them 6-cyclohexylamine-4-piperazinyl-1,3,5-triazine-2-carbonitrile (I-25) was reported as a potent cathepsin K inhibitor [20]. However in addition to cathepsin K, I-25 inhibited also cathepsins B, L, and S,

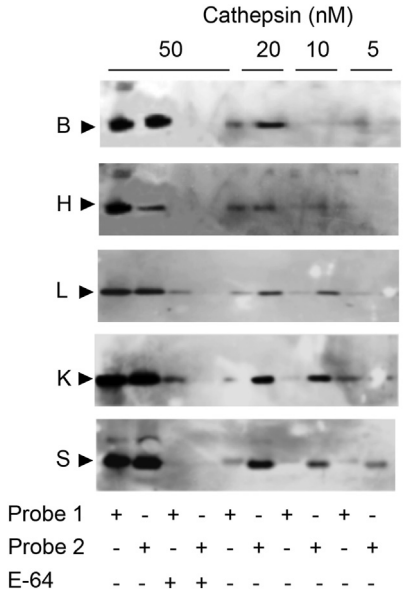

Fig. 2. Labeling of cysteine cathepsins by biotinylated probes. Cathepsins (0–50 nM) were incubated in the activity buffer with probes 1 and 2 (1 μM) for 1 h at 30 °C prior to 12% SDS-PAGE under reducing conditions and blotting. After incubation with an ExtrAvidin®-peroxidase conjugate, revelation was performed by chemiluminescence. E-64 (1 μM) was used as control.

suggesting that I-25 is a broad-spectrum inhibitor of endopeptidase cathepsins. Conversely I-25 did not impair the peptidase activity of cathepsin D, an aspartyl-protease, of macrophage metalloelastase (MMP-12), a matrix metalloproteinase, and of serine proteases (i.e. trypsin, chymotrypsin and neutrophil elastase), corroborating that nitrile-based inhibitors are selective inactivators of cysteine proteases (Fig. 3A). Experimental IC_{50} values were: 136 nM for cathepsin B, 57 nM for cathepsin K, 45 nM for cathepsin L, and 23 nM for cathepsin S, respectively. Following preincubation of cathepsins with I-25, labeling by Biot-$(PEG)_2$-Ahx-LeuValGly-DMK is impaired (Fig. 3B). According to inhibition of cathepsins by electrophilic nitriles is achieved by formation of a covalent, reversible thioimidate between Cys25 (papain numbering) and the carbon–nitrogen triple bond [19], data endorse the specific targeting of the nucleophilic thiol of the active site by peptidyl diazomethylketones.

3.3. Kinetics

We have previously shown that the progress curves of inactivation in the presence of different concentrations of model cystatin-derived diazomethylketones (i.e. Z-RLVG-DMK and GlcA-QVVA-DMK) have the same initial slopes, indicating that no significant non-covalent enzyme-probe binding occurs during the course of competitive, irreversible inhibition [23], despite that fact that it is usually reported that probe labeling is a two-step process [10]. This was further verified by plotting k_{obs} against [I], which gave a straight line [13,14]. Biot-$(PEG)_2$-Ahx-LeuValGly-DMK was assayed kinetically on papain and its second-order rate constant for association (k_{ass}) was compared with those obtained with Biot-Ahx-LeuValGly-DMK. Addition of the hydrophilic $(PEG)_2$ spacer significantly increased the rate of inhibition (k_{ass} = 2,750, 000 M^{-1} s^{-1} for probe 2 vs k_{ass} = 800, 000 M^{-1} s^{-1} for probe 1) (average value of three independent experiments). Beside papain, we repeated kinetic measurements with cathepsin S, the enzyme with the lowest detection limit in the presence of both biotinylated probes. Rate constants of inactivation are almost identical with

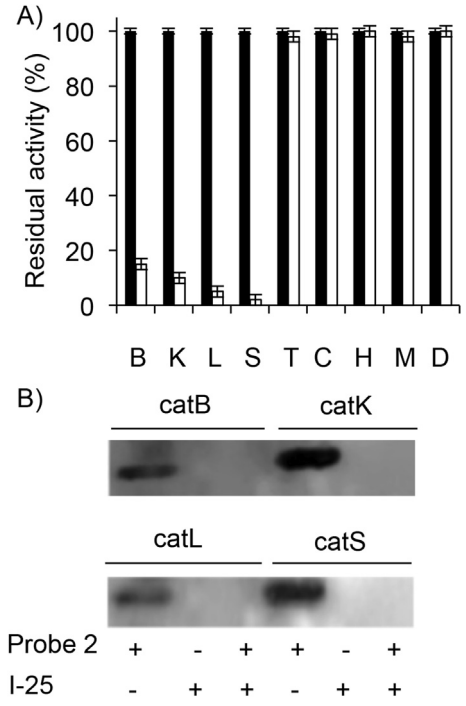

Fig. 3. Active site targeting of cathepsins by Biot-$(PEG)_2$-Ahx-LeuValGly-DMK. (A) Inhibition of cysteine cathepsins by I-25. Proteases (0.5 nM) were incubated in their respective activity buffer alone (black bar) or in the presence of I-25 (10 μM, white bar) before measurement of the residual enzyme activity as described in the experimental section (n = 3). B, catB; K, catK; L, catL; S, CatS; T, trypsin; C, chymotrypsin; H, CatH; M, MMP-12; D, CatD (b) Cathepsins (20 nM) were incubated for 20 min with I-25 (100 μM) before adding Biot-$(PEG)_2$-Ahx-LeuValGly-DMK (probe 2, 1 μM). Samples were further submitted to 12% SDS-PAGE under reducing conditions and blotting. Revelation was completed as reported above.

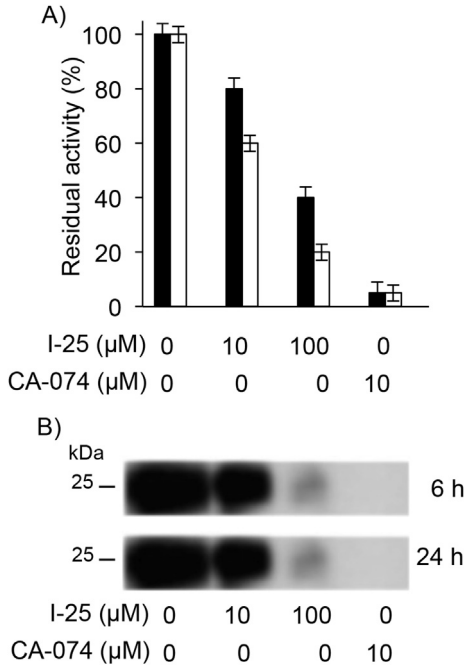

Fig. 4. Internalization of I-25 and subsequent inhibition of intracellular cathepsins. (A) Differentiated THP-1 cells were incubated for 6 h and 24 h in the presence of I-25 (0–100 μM). After cell lysis, the residual intracellular peptidase activity was assayed using Z-Phe-Arg-AMC (20 μM) and is expressed in percentage (black bar, 6 h; white bar, 24 h) (n = 3). (B) Alternatively, probe 2 (2 μM) was added immediately after cell lysis and incubated for 1 h at 30 °C. Lysate samples were further subjected to 12% SDS-PAGE and transfer to a nitrocellulose sheet, incubated with ExtrAvidin®-peroxidase, then revealed as explained before. CA-074 was used as control.

k_{ass} = 680, 000 M^{-1} s^{-1} for probe 1, and k_{ass} = 2,320, 000 M^{-1} s^{-1} for probe 2. Present data support that addition of the hydrophilic spacer both improved the sensitivity threshold of affinity labeling and the rate of inhibition (circa 3-fold). However it should be noted that a possible disadvantage of a robust electrophilicity (as reflected by the second-order rate constant for association) is that an excessive reactivity may induce a decrease in the selectivity and an increase in off-targeting and background labeling [10].

3.4. Labeling of cathepsins expressed by differentiated THP-1 cells

We used as model a myelomonocytic cell line (THP-1 cells) to assess the use of Biot-$(PEG)_2$-Ahx-LeuValGly-DMK for affinity labeling of cathepsins. We have previously validated this cell line for studying the resistance of secreted cysteine cathepsins to peroxide oxidation [18]. Briefly following addition of phorbol myristate acetate (PMA, day 0), THP-1 monocytes differentiated into macrophages (day 3). No endopeptidase activity of cathepsins was monitored in monocyte supernatants. Conversely proteolytic activity was found in supernatants of differentiated THP-1 cells, with a maximum reached at day 6. Western blot analysis correlated with measurements of enzyme activity since secreted cysteine cathepsins were detected only 72 h after the addition of PMA with strongest immunostaining at day 6. I-25 inhibited in a dose-dependent manner cathepsin activity in supernatants, as attested by a dose-dependent decrease in the labeling by Biot-$(PEG)_2$-Ahx-LeuValGly-DMK (i.e. probe 2) (data not shown). Furthermore cells were incubated for 6 h and 24 h in the presence of I-25 before removal of the culture medium, lysis and subsequent addition of probe 2. The results showed that I-25 was rapidly internalized by macrophages and could inhibit lysosomal cathepsins (Fig. 4A). This was again verified by the dose-dependent decrease in labeling by Biot-$(PEG)_2$-Ahx-LeuValGly-DMK (Fig. 4B). Interestingly patterns are identical after 6 h and 24 h of incubation, supporting that I-25 was readily cell-permeable. Moreover labeling was impaired by CA-074, which confirms that cathepsin B is the prominent cysteine cathepsin expressed by THP-1 cells, in agreement with an earlier paper [18].

In conclusion, we have developed a sensitive affinity probe (employing the diazomethylketone group as warhead) with a simple scaffold based on the N-terminal substrate-like region of human cystatin C. Compared to anterior cystatin C-derived inhibitors, a $(PEG)_2$ spacer was added. Besides an enhanced solubility in buffer, presence of this short pegylated arm improves rates of inactivation (k_{ass}). Biot-$(PEG)_2$-Ahx-LeuValGly-DMK that binds covalently to the nucleophilic cysteine of the active site allows detection of cysteine cathepsins B, K, L and S at low concentrations (nM range). We confirmed that the affinity labeling is dose-dependent as observed using a potent competitive inhibitor, 6-cyclohexylamine-4-piperazinyl-1,3,5-triazine-2-carbonitrile.
Finally its utility in revealing active extracellular and lysosomal cathepsins has been validated on THP-1 cells, a monocytic lineage model. Taken together with an other report using human lung CCD-19Lu fibroblasts [22], the present report supports that Biot-$(PEG)_2$-Ahx-LeuValGly-DMK may represent a useful substitute and facile tool for labeling cysteine cathepsins in cell extracts.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.020.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.020.

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