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BODIPY FL-pepstatin A, a fluorescent probe for the subcellular distribution of cathepsin D.

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1.

Abstract

Cathepsin D is an aspartic endopeptidase that is widely distributed in almost all mammalian cells. It plays pivotal roles in intracellular protein degradation, antigen processing and accelerated protein turnover in various pathological conditions. For example, distribution of cathepsin D in phagolysosomes has been suggested to be a prognostic indicator for human breast cancer, and alteration in trafficking of this protease is of functional significance in malignant progression. Here, we have synthesized a fluorescent probe for cathepsin D, where the pepstatin A was covalently conjugated with the BODIPY FL fluorophore. *In vitro*, the fluorescent analog shows a potent inhibitory activity toward cathepsin D, with an IC_{50} of ~10 nM, similar to its non-fluorescent parent compound. The binding specificity of this fluorescent analog was further characterized using a fluorescence polarization measurement. Results showed that the BODIPY FL pepstatin A selectively binds with cathepsin D at the pH 4.5. In live cells, the BODIPY FL-pepstatin A is internalized and transported to lysosomes through an endocytic pathway. The staining in the lysosomes can be competed with non-fluorescent pepstatin A. In fixed cells, BODIPY FL-pepstatin A also stained lysosomes, where it co-localized with cathepsin D. This staining was diminished when cells were co-incubated with non-fluorescent pepstatin A in buffer at pH 4.5. These properties, along with the good photo-stability of BODIPY FL fluorophore, makes this probe a novel tool for the study of the secretion and trafficking of Cathepsin D in live cells during various conditions, such as oxidative stress and apoptosis.

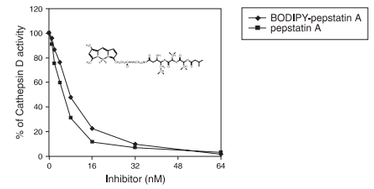
2.

Introduction

Cathepsin D is one of the major endopeptidases in almost all the mammalian cells (Whitaker, J. N. *et al.* 1991. *J. Neurochem*. 57(2): 406–414). This protease degrades various substrates by cleaving on the carboxyl side of aromatic residues (eg. aspartic acid), at a pH of 3.5–5 for its optimal activity (Barrett, A. J. 1977. *Proteinases in Mammalian Cells and Tissues*. pp. 209–248. North Holland Amsterdam). Although free soluble cathepsin D is highly concentrated in lysosomes of normal cells, significant amounts of cathepsin D have been found in endosomes in a membrane-associated form (Diment, S., *et al*., 1988. *J. Biol. Chem.* 263:6901–6907). In the meanwhile, distribution of cathepsin D can be altered by physiological stimulation or in pathological dysfunction. Elevation of intracellular Ca^{+2} in normal rat kidney fibroblasts induces fusion of lysosomes with the plasma membrane. This fusion results in secretion of lysosomal cathepsin D (Rodriguez, A. *et al*. 1997. *J. Cell Biol.* 137(1): 93–104). Cathepsin D is also shown to be secreted at the cell surface in human breast cancer cells at the point of transition between the pre-neoplastic and neoplastic state (Sameni, M. *et al*. 1995. *Pathology Oncology Research* 1:43–53). The distribution of cathepsin D is also affected when cells were subjected to oxidative stress. For example, oxidant treatments in human foreskin fibroblasts and histocytic lymphoma (J.774) resulted in redistribution of the cathepsin D from lysosomes to cytosol (Roberg, K. and K. Ollinger. 1998. *J. Histochem. Cytochem*. 46(3): 411–418). This redistribution was shown to be followed by cell degeneration and, eventually, by cell death. Since enzymatic activities and distribution of the cathepsin D correlates with various important cellular processes, a specific indicator to probe distributions of both extracellular and intracellular cathepsin D is prerequisite for a better understanding of the physiological function of this protease. Fluorescent inhibitors of enzymes are valuable tools in understanding the enzyme-substrate interaction both *in vitro* and *in vivo* systems. Here, we have synthesized a novel fluorescent probe for the cathepsin D, the BODIPY FL pepstatin A. The BODIPY fluorophore is very photostable and pH-insensitive, two important properties for both microscopic studies and enzymatic assay. The inhibitory activity of this fluorescent probe to cathepsin D was first charaterized using an *in vitro* enzymatic assay. Secondly, the selective and active-site directed binding of this inhibitor to cathepsin D were investigated using fluorescence polarization measurements. Finally, the ability of this indicator to probe the subcellular distribution of cathepsin D was demonstrated in both live and fixed macrophages.

The data contained in this poster is now published in "Probing the Cathepsin D Using a BODIPY FL–Pepstatin A: Applications in Fluorescence Polarization and Microscopy." Chen CS, Chen WN, Zhou M, Arttamangkul S, Haugland RP. *J Biochem Biophys Methods* 42: 137-151 (2000).

3.



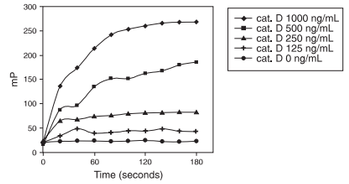
Inhibitory effect of pepstatin A and BODIPY FL pepstatin A on cathepsin D activity.
Cathepsin D (EC 3.423.5, 50 nM) was pre-incubated with the indicated concentration of inhibitors in 50 mM citrate buffer, pH 3.5 at 37°C for 30 min. The substrate (BODIPY TR-X casein) was added into the above mixtures at the final concentration of 15 µg/ml. Following 90 min incubation, fluorescence was measured using a filter set for excitation and emission at 590±10 and 645±20 nm, respectively. The relative cathepsin D activities are expressed as the percentage of the activity relative to the control reaction without inhibitors.

4.

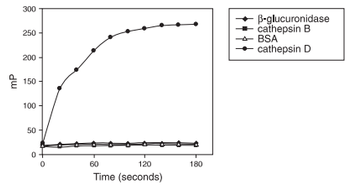
Fluorescence polarization assays for the detection of interaction between BODIPY FL pepstatin A and cathepsin D

FPM-1™ Fluorescence Polarization Analyzer (Jolley Consulting and Research Inc., Grayslake, IL) was used to measure the fluorescence polarization change of the BODIPY FL pepstatin A under various treatments. In general, all assays were performed at 37°C in the kinetic mode. A stock solution of BODIPY FL pepstatin A was prepared in DMSO (10 mM). Two microliter of the BODIPY FL pepstatin A was then added to the appropriate reaction buffer (2 mL) to yield a final concentration of 2 nM. At the beginning of each measurement, a blank buffer reading was made, which was substracted from following sample measurements. A zero time reading was taken, serial dilutions of the cathepsin D or of other proteases was added, and polarization readings were taken every 20 seconds.

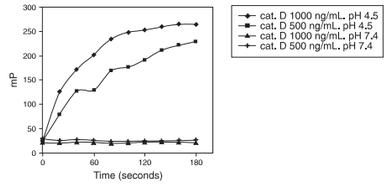
A. Detection of cathepsin D binding to BODIPY FL pepstatin A at pH 4.5.



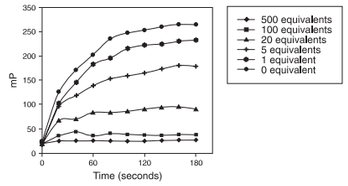
B. The selective binding of BODIPY FL pepstatin A to cathepsin D at pH 4.5.



C. Detection of pH-dependent binding of BODIPY FL pepstatin A to cathepsin D.

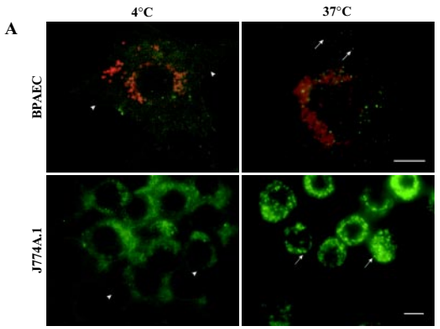


D. Binding between BODIPY FL pepstatin A and cathepsin D can be competed by unlabeled pepstatin A.



5.

Trafficking of BODIPY FL pepstatin A in live cells.

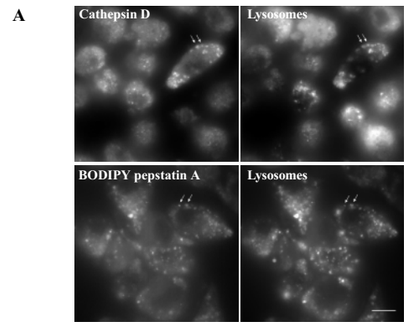


BODIPY FL pepstatin A is internalized into live cells through endocytic pathway.
Monolayers of bovine pulmonary artery endothelial cells (BPAEC) and murine macrophage-like cells (J774A.1) were first pulse-labeled with Alexa Fluor 594-conjugated hydrazide to stain lysosomes. The cell monolayers were then incubated with 1 µM BODIPY FL pepstatin A in a complete culture medium at either 4°C or 37°C for 30 min. As shown, plasma membrane staining was predominant when cells were incubated at 4°C (as indicated by arrowheads). When the temperature was increased to 37°C, BODIPY FL pepstatin A entered cells through endocytosis, as indicated by the appearance of endosome-like structures (see arrows). The red fluorescence in the BPAEC represents lysosomal compartments stained by the Alexa Fluor 594-conjugated hydrazide.
Bar, 10 µm.

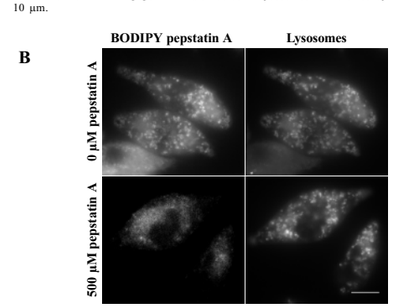
Competition of pepstatin A with the BODIPY FL pepstatin A in live J774A.1 cells.
The competition effect of pepstatin A on the staining of BODIPY FL pepstain A was studied when cells were labeled with 1 µM BODIPY FL pepstatin A in the presence or absence of excess non-fluorescent pepstatin A (100 µM). As shown in the upper panels, pepstatin A selectively competed with the BODIPY FL pepstatin A in lysosomes, which resulted in the disappearance of lysosomal staining. This decrease in the total fluorescence intensity was further quantified, as shown in the lower panel. Bar, 10 µm.

6.

BODIPY FL pepstatin A selectively binds to lysosomal cathepsin D in fixed J774A.1 cells.



Co-localization of BODIPY FL pepstain A with cathepsin D in lysosomes.
Cellular lysosomal compartments were prelabeled with fixable Cascade Blue-conjugated dextran (10K). The immunofluorescent staining of cathepsin D was performed using a rabbit anti-human cathepsin D as the primary Ab (1:400 dilution) and the Alexa Fluor 594-conjugated anti-rabbit IgG as the secondary Ab. The staining of BODIPY FL pepstatin A in fixed cells was performed as followed. Cell monolayers were fixed and permeabilized in 3% formaldehyde and 0.1% glutaldehyde in sodium acetate buffer containing 0.1% Brij 35 (pH 4.5-5, see Matthews *et al*., 1981. *Biochem. J.* 199:611–617). After several wash in the sodium acetate buffer, cells were incubated in the same buffer containing 1 µM BODIPY FL pepstain A at room temperature for one hour. As shown, both cathepsin D and the BODIPY FL pepstatin A distributed in lysosomes as indicated by arrows. Bar, 10 µm.



Competition effect of pepstatin A with the BODIPY FL pepstatin A staining.
Fixed cells were stained with BODIPY FL pepstatin A as previously described, except a 500 fold higher concentration (500 µM) of the non-fluorescent pepstatin A or equal amount of DMSO was added. As shown, pepstatin A completely depleted the punctate staining of the BODIPY FL pepstatin A in the lysosomes. Bar, 10 µm.

7.

Summary

A novel fluorescent inhibitor for cathepsin D, BODIPY FL-pepstatin A, was synthesized. Its inhibitory and selective binding activities toward cathepsin D were investigated. Meanwhile, this fluorescent probe was used to study the distribution of cathepsin D in live and fixed macrophage-like cell line. The results are summarized herein:

1. The BODIPY FL-pepstatin A is a potent inhibitor for cathepsin D. It inhibits the protease activity of cathepsin D with an IC_{50} ~50 nM;
2. The binding of BODIPY FL-pepstatin A with cathepsin D can be studied using fluorescence polarization measurements. The binding is selective to cathepsin D, but not to other proteases, including cathepsin B and β-glucuronidase. Furthermore, BODIPY FL-pepstatin A only binds with cathepsin D at acidic pH, indicating that this binding is active-site directed;
3. BODIPY FL-pepstatin A can be used to probe the distibution of cathepsin D in fixed cells. The fluorescent staining concentrates in the lysosomal compartment, where it co-localizes with cathepsin D. This staining can be competed with non-fluorescent pepstatin A;
4. In live cells, BODIPY FL-pepstatin A enters cells through an endocytic pathway. It is eventually transported to lysosomes.

The development and application of this novel fluorescent probe may open new insight for the distribution and secretion of cathepsin D in live cells under various physiological conditions.

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