EXPERIMENTAL BIOLOGY 2008
TODAY'S RESEARCH: TOMORROW'S HEALTH

1059.1

Metabolic Lysosomal Enzyme Probes

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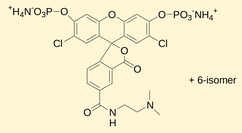
Introduction

Lysosomes are acidic cytoplasmic organelles that are present in all nucleated mammalian cells. Lysosomes have been found to be involved in a variety of cellular processes including repair of the plasma membrane, defense against pathogens, cholesterol homeostasis, bone remodeling, metabolism, apoptosis, and cell signaling. Defects in lysosomal enzyme activity have been associated with a variety of diseases including Parkinson's, Tay-Sachs, Sandhoff, Krabbe, Wolman, and Gaucher syndromes. New lysosomal staining compounds that are useful for labeling lysosomes in a live-cell format and are capable of monitoring lysosomal metabolic activity have been developed. These new targeted substrates are based upon fluorescent probes that have a low pKa value for optimum fluorescence at the lower physiological pH values found in the lysosomes as well as targeting groups to direct their accumulation to the lysosomes using a live-cell staining format. Application to the staining of human and murine fibroblast cells are presented. In addition the ability to monitor the effect of secondary therapeutic agents on staining is presented. This work was supported by NIH Grant 5R43MH079542-02.

Figure 1: Lysosomal Enzyme Substrates

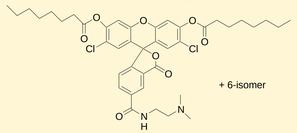
a.) Substrate for Acid Phosphatase



+ 6-isomer

M1347

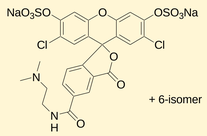
b.) Substrate for Acid Lipase



+ 6-isomer

M1344

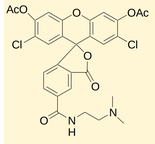
c.) Substrate for Aryl Sulfatase



+ 6-isomer

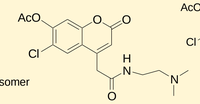
M1359

d.) Substrates for Acid Esterases

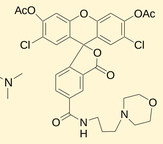


M1268

+ 6-isomer



M1271



+ 6-isomer

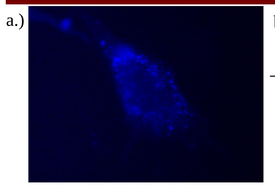
M1299

Materials and Methods

- Primary Human Fibroblasts from a Gaucher I patient (F0381999) were obtained from Istituto Giannina Gaslini (Genova, Italy). Cells were cultured in RPMI 1640 Medium.
- Primary Human Fibroblasts from a healthy donor (GM03440) were obtained from the Coriell Institute for Medical Research (Camden, NJ). Cells were cultured in Minimum Essential Medium Eagle (Lonza).
- CRE BAG 2 murine fibroblasts were obtained from ATCC (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagles Medium.
- Under normal growth conditions, all media was supplemented with 9% FCS, 100U/mL penicillin, 100µg/mL streptomycin, and 0.25µg/mL amphotericin B.
- Cell labeling solutions were prepared by diluting substrate stock solutions in the appropriate medium for the cell line (see above). Media used in cell labeling experiments was unsupplemented. DAPI was added to a final concentration of 2µg/mL for use as a nuclear stain. Cells were washed with Phosphate Buffered Saline prior to adding labeling solutions to remove residual serum and antimycotic/antibiotic compounds.
- Cell lines were incubated at 37ºC, 5% CO_2 humidification.
- Labeled cells were washed 3x with Phosphate Buffered Saline prior to imaging. Cells were imaged using a Nikon Labophot 2 fluorescence microscope and Sony Cyber-shot DSC-S85 digital camera.
- CellProfiler cell image analysis software was obtained from the Broad Institute at the Massachusetts Institute of Technology.
- LysoTracker® Red DND-99 was obtained from Invitrogen (Carlsbad, CA).

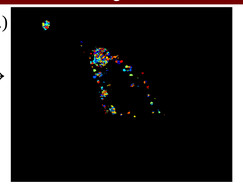
Figure 2: Acid Esterase Activity in Cytoplasmic Acidic Organelles

a.)



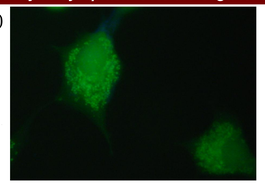
Human Fibroblast (Gaucher I, F0381999)
250µM M1271, 20 min. incubation
XF06 filter (Omega Optical), 1s shutter speed

b.)



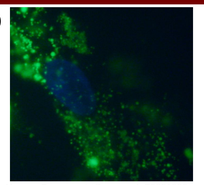
CellProfiler Identification of labeled organelles

c.)



CRE BAG 2 (murine fibroblast)
2µM M1299, 20 min. incubation
B-2A filter (Nikon), 1s shutter speed

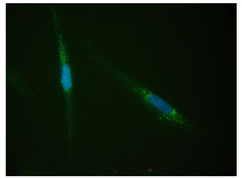
d.)



Human Fibroblast (Gaucher I, F0381999)
50µM M1268, 20 min. incubation (DAPI counterstain)
XF68 multi-band filter (Omega Optical), 1s shutter speed

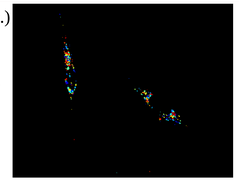
Figure 3: Aryl Sulfatase Activity in Cytoplasmic Acidic Organelles

a.)



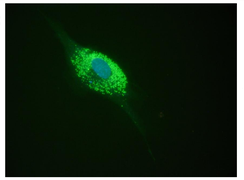
Human Fibroblast (healthy donor, GM03440)
200µM M1359, Overnight incubation (DAPI counterstain)
XF68 multi-band filter (Omega Optical), 2s shutter speed

b.)



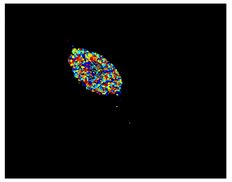
CellProfiler Identification of labeled organelles

c.)



GM03440 60 h. incubation in 100mM sucrose
200µM M1359, Overnight incubation (DAPI counterstain)
XF68 multi-band filter (Omega Optical), 2s shutter speed

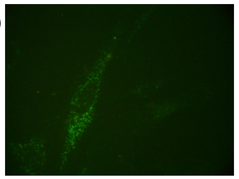
d.)



CellProfiler Identification of labeled organelles

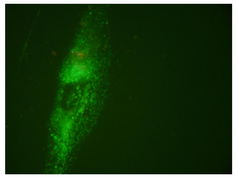
Figure 4: Acid Phosphatase Activity in Cytoplasmic Acidic Organelles

a.)



Human Fibroblast (healthy donor, GM03440)
200µM M1347, Overnight Incubation
XF68 multi-band filter (Omega Optical), 5s shutter speed

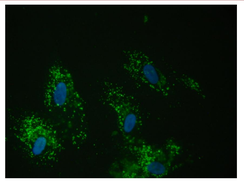
b.)



GM03440 5 day incubation in 100mM sucrose
200µM M1347, Overnight incubation
XF68 multi-band filter (Omega Optical), 5s shutter speed

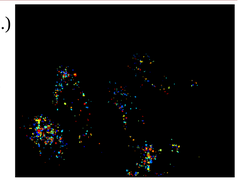
Figure 5: Acid Lipase Activity in Cytoplasmic Acidic Organelles

a.)



Human Fibroblasts (healthy donor, GM03440)
10µM M1344, 90 min. Incubation (DAPI counterstain)
XF68 multi-band filter (Omega Optical), 2s shutter speed

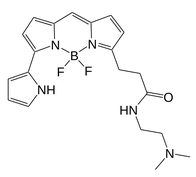
b.)



CellProfiler Identification of labeled organelles

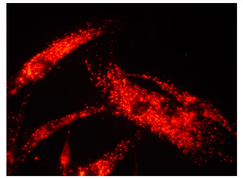
Figure 6: Co-localization of Metabolic Probe with Pre-Existing Lysosomal Probe

a.)



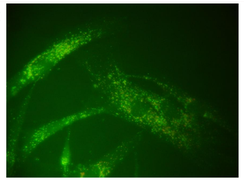
LysoTracker® Red DND-99 (Invitrogen)

b.)



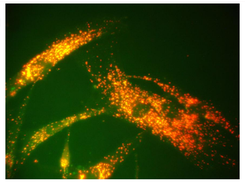
Human Fibroblasts (healthy donor, GM03440)
LysoTracker® Red DND-99 (Invitrogen)
XF40 filter (Omega Optical), 1s shutter speed

c.)



Human Fibroblasts (healthy donor, GM03440)
M1359
B-2A filter (Nikon), 1s shutter speed

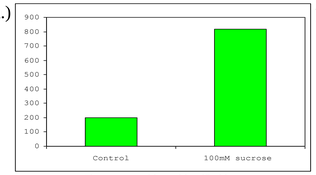
d.)



LysoTracker® Red DND-99/M1359 Merge

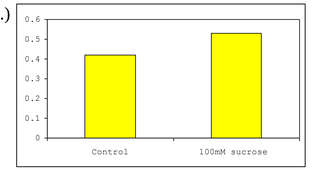
Figure 7: Analysis of Lysosomal Enzyme Activity using CellProfiler

a.)



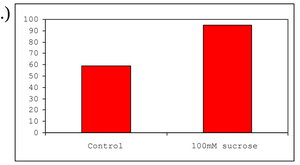
Fluorescent objects identified by CellProfiler software in images of cells grown for 60 h. in medium containing 100mM sucrose vs. control medium (Figure 3). Cells were incubated with 200µM M1359 overnight prior to imaging.

b.)



Mean intensity of fluorescent objects identified by CellProfiler software in images of cells grown for 60 h. in medium containing 100mM sucrose vs. control medium (Figure 3). Cells were incubated with 200µM M1359 overnight prior to imaging.

c.)



Integrated Intensity of images determined by CellProfiler software. Images were of cells grown for 60 h. in medium containing 100mM sucrose vs. control medium (Figure 3). Cells were incubated with 200µM M1359 overnight prior to imaging.

Discussion and Conclusions

- We synthesized novel enzyme substrates for several lysosomal enzymes that are targeted to acidic organelles *in vivo* with high specificity.
- The released products of enzyme cleavage show strong fluorescence emission in the acidic environment of the lysosome.
- Enzyme activities and increased lysosomal storage can be induced with addition of sucrose to growth medium.
- Co-localization can be visualized with LysoTracker® Red DND-99, an existing fluorescent lysosomal probe.
- Differences in lysosomal storage and lysosomal enzyme activity are detectable using CellProfiler cell image analysis software.
- Work is underway to synthesize glycosidase substrates specific for several enzymes deficient in patients with Lysosomal Storage Diseases (LSDs).
- Work is underway to synthesize long-wavelength substrates capable of fluorescence at acidic pH to enable analysis of multiple lysosomal enzymes simultaneously.
- Assay Kits utilizing these lysosomal substrates will be available commercially from Marker Gene.

References

- Carpenter AE,Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J, Golland P, Sabatini DM (2006) "CellProfiler: image analysis software for identifying and quantifying cell phenotypes." Genome Biology 7:R100.
- Karageorgos LE, Isaac EL, Brooks DA, Ravenscroft EM, Davey R, Hopwood JJ, Meikle PJ (1997) "Lysosomal Biogenesis in Lysosomal Storage Disorders" Experimental Cell Research 234, 85-97.
- Koenig H (1963) "Vital staining of lysosomes by acridine orange." J. Cell Biol 19, 87A.