A Novel Fluorescence-Based Lysosomal Degradation Pathway Assay Suitable for Analysis of Live Cells Using a Microplate Reader

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ABSTRACT

Lysosomes digest materials internalized by the cell from the external environment (heterophagy) as well as those originating within the cell's own cytoplasm (autophagy). Materials from both sources are ultimately incorporated into the same membrane-bounded compartments as the lysosomal enzymes. In heterophagy, cells internalize materials by the process of endocytosis, engulfing them in membrane-bound vesicles or vacuoles that are formed at the cell surface. The endocytosed material enters lysosomes via endosomes. In autophagy, organelles, proteins and portions of the cytoplasm to be degraded are first enveloped inside a specialized organelle, the autophagosome, which subsequently fuses with lysosomal vesicles and delivers the engulfed materials for degradation. Drug-induced phospholipidosis is characterized by intracellular accumulation of phospholipids within lamellar bodies, likely arising from impaired phospholipid metabolism within the lysosome. Few studies have addressed whether phospholipidosis can be classified as an autophagic stress response, akin to amino acid deprivation or starvation, or should more properly be categorized as a heterophagic sequesteration event. We describe a novel microplate-based live cell assay based upon a red fluorescent probe that rapidly sequesters into acidic organelles by a mechanism that likely involves protonation and retention within their membranes. By careful selection of titratable groups on the probe, labeling has been extended into the vacuoles of cells pre-treated with weakly basic, cell-permeant drugs, such as the anti-malarial drug chloroquine. Using the assay in parallel with a fluorescent phospholipid-based probe facilitates differentiation between autophagosome accumulation and phospholipidosis.

INTRODUCTION

The accumulation of cationic amphiphilic drugs, toxic agents and other basic compounds inside acidic subcellular organelles is referred to as lysosomotropism. While many drugs require the presence of a cationic moiety for intrinsic bioactivity, their accumulation into subcellular organelles can also lead to undesirable tissue distribution, alkalinization of lysosomes, phospholipidosis and aberrant pharmacokinetic disposition (Ikeda et al BBRC 377 (2008) 268–274). Over fifty cationic amphiphilic drugs, including antibiotics, antidepressants, antipsychotics, antimalarial and antiarrhythmic agents are known to trigger phospholipidosis, which is typified by the excessive intracellular accumulation of phospholipids within lysosomes as lamellar bodies (Anderson and Borlak *FEBS Lett.* 580 (2006) 5533–5540). The origins of these lamellar bodies remain unresolved, though they appear to be generated by autophagic or heterophagic processes. Some commonly prescribed drugs known to perturb lysosomes by inducing phospholipidosis include amiodarone, azithromycin, chlorpromazine, chloroquine, gentamicin, imipramine, phenobarbital, propranolol and tamoxifen.

We highlight a 96-well cell-based assay that provides a rapid and quantitative high-throughput approach for determining drug- or toxic agent-induced lysosomal perturbation in live cells, offering throughput advantages relative to previously described methods based upon electron microscopy, fluorescence microscopy or flow cytometry. Early secondary screening of candidate drugs for potential lysosome-perturbing activity in the drug discovery phase could predict later risks in drug development arising from drug safety issues. Such a screening approach could aid in selecting a successful candidate compound with low or weak lysosome-perturbing activity for further drug development efforts, as well as provide preliminary benchmarking of dosing limits in preclinical toxicity studies.

Absorbance and Fluorescence Emission Spectra

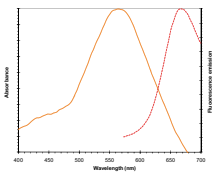


FIGURE 1: Absorbance and Fluorescence emission spectra of Lyso-ID® Red dye (Abs. = 568 nm, Em = 667 nm (A). Spectra were determined in 1 X phosphate-buffered saline. This red-emitting dye is compatible with blue-emitting fluorophores , such as Hoechst 33342, and green-emitting fluorophores, such as GFP and FITC.

Simple Protocol

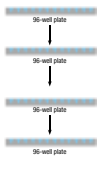


Plate cells, allow to attach and grow with appropriate culture medium over night.

Add test compound for desired time period at 37°C. (Generally 4 to 24 hours)

Remove the medium and add Lyso-ID® Dual detection reagent for 30 min at room temperature

Wash the cells and analyze with a fluorescence microplate reader. If the Blue nuclear signal decreases by more than 30%, the compound is generally cytotoxic. The remaining samples can be evaluated for their activity on the lysosome.

FIGURE 2: Generalized workflow for analyzing compounds that induce changes in lysosomal number and volume using a live cell fluorescence microplate assay for high-throughput screening. If the blue nuclear counterstain signal decreases by more than 30% in a well, the compound is considered generally cytotoxic. Increased red emission in a well indicates an expansion of lysosome number and/or volume in the cell population.

Chloroquine Increases Lysosomal Size and Number in HeLa Cells

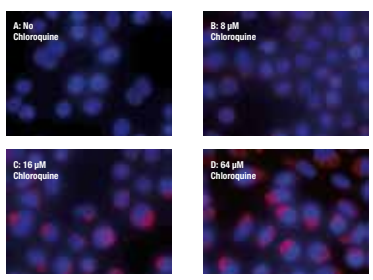


FIGURE 3: Lyso-ID® Red dye is a cell-permeant fluorescent probe that selectively associates with lysosomes and other acidic organelles (shown in A). When cells are pre-treated for 20 hours with weakly basic cell-permeant compounds, such as chloroquine, a dramatic increase in lysosome-like vesicle number and volume is observed (shown in B-D). This confirms Lyso-ID® Red dye partitions with the lysosomal compartment. Nuclei are counter-stained with Hoechst 33342 in the images.

EC_{50} estimate of Chloroquine, as Determined by the Fluorescence Microplate Assay

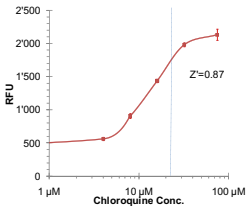


FIGURE 4: Using a conventional fluorescence microplate reader, the half maximal effective concentration (EC_{50}) of chloroquine in HeLa cells was estimated. The high Z-factor (0.87) obtained using the assay demonstrates excellent signal-to-noise and signal-to-background ratios. The error bars denote the standard deviation of at least six determinations.

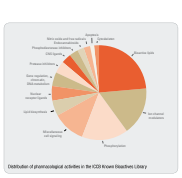
Monitoring Changes in Lysosomal Number and Volume in a Compound Library Screen

ICCB Known Bioactives Library

- 480 compounds with defined biological activity
- Ready-to-screen

Enzo Life Sciences has created a novel library of biologically active small organic molecules in cooperation with the Harvard Institute of Chemistry and Cell Biology (ICCB; now the Broad Institute Chemical Biology Program (ICBP) and ICCB-Longwood). Known active compounds are indispensable tools for target validation, biological mechanism profiling and assay validation. The ICCB Known Bioactives Library serves as a widely used standard set of actives for high content screening.

Includes compounds that affect most cellular processes and drug targets*	
GPCR ligands	Second messenger modulators
Nuclear receptor ligands	Actin & tubulin modulators
Kinase inhibitors	Protease inhibitors
Ion channel blockers	Gene regulation agents
Lipid biosynthesis inhibitors	Phosphodiesterase inhibitors
... many other classes	



*Zhang et al Proc Natl Acad Sci U S A. (2007) 104(48):19023-8.

Enzo's ICCB Known Bioactives compound library panel was screened using the Lyso-ID® Red microplate cell-based assay. 480 compounds were evaluated.

Compounds Causing an Increase in Lysosomal Dye Signal

Name	Classification	Action	Concentration in assay	Relative lysosomal staining	Hoechst staining relative to untreated
Ro 20-1724	Inhibitors	Phosphodiesterase (PDE4) inhibitor	18 µM	190%	53%
Bafilomycin A1	Inhibitors	Vacuolar ATPase inhibitor	1 µM	176%	58%
E6 Berbamine	**Inhibitors**	**Calmodulin inhibitor**	**7 µM**	**230%**	**100%**
Ro 31-8220	**Kinase inhibitors**	**PKC inhibitor**	**9 µM**	**146%**	**89%**
Propranolol (S-)	**CNS receptor ligands**	**Adrenoceptor antagonist (beta)**	**17 µM**	**142%**	**88%**
SB-431542	**Kinase inhibitors**	**ALK4, ALK5, ALK7 inhibitor**	**13 µM**	**154%**	**101%**
Rottlerin	Kinase inhibitors	PKC delta inhibitor	10 µM	187%	34%
GF-109203X	**Kinase inhibitors**	**PKC inhibitor**	**12 µM**	**197%**	**87%**
Tyrphostin 9	Kinase inhibitors	PDGF-R tyrosine kinase inhibitor	18 µM	200%	31%

TABLE 1: The compounds listed above induced changes in lysosomal staining that were greater than 4 standard deviations different from untreated cells. The percentage cells remaining after treatment was determined by the amount of blue nuclear staining. Loss of cells indicates general cytotoxicity at the compound concentration evaluated.

Treatment of U-2-OS Cells with Specific Members of the ICCB Known Bioactives Compound Library Increases Lysosomal Volume and Number

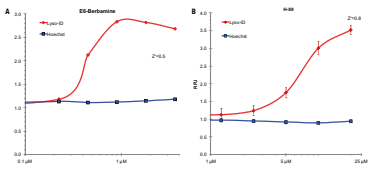


FIGURE 5: Using a conventional fluorescence microplate reader, the observed lysosomal fluorescence signal increased in a dose-dependent manner : (A) E6 Berbamine (B) H-89. Nuclei were counter-stained with Hoechst 33342. Hoechst 33342 is used to monitor false positives in the microplate assay, due to compounds that are generally cytotoxic at the screening concentration, as indicated by a significant decrease in overall cell number. The high Z-factor (>0.5) obtained using the assay demonstrates excellent signal-to-noise and signal-to-background ratios.

Treatment of U-2-OS Cells with Certain Members of the ICCB Library Resulted in Loss of Cells, as Flagged by the Declining Hoechst 33342 Signal.

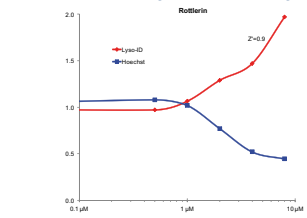


FIGURE 6: False positives can be generated with the fluorescence microplate assay when a compound is used at concentrations that lead to a >-30% decrease in cell number (as monitored by a decline in Hoechst 33342 signal). Rottlerin is an example of this condition. Monitoring the Hoechst signal along with the Lyso-ID red signal solves the problem of potential false positives. Microscopic examination indicated that generally cytoxic agents lead to diffuse cytoplasmic staining with Lyso-ID® Red dye.

Fluorescence Microscopy Confirms the Intracellular Increase in Lysosome Size and Number by Certain Members of the ICCB Known Bioactives Compound Library

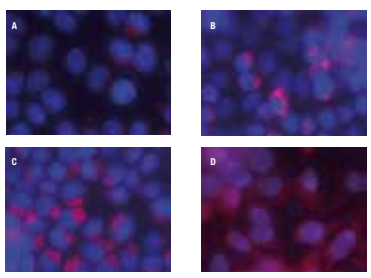


FIGURE 7: Treatment of U-2-OS cells with compounds causes a specific intracellular pattern of lysosome signal increase compared to untreated cells by fluorescence microscopy (A) Untreated cells (B) E6 Berbamine, 1.8 µM (C) H-89, 10 µM and (D) Rottlerin, 4 µM. In all cases, nuclei are stained with Hoechst 33342. Fluorescence microscopy demonstrated a punctuate lysosomal staining pattern for Panels A-C, which is different from the diffuse cytoplasmic staining arising from cytotoxicity (Panel D).

A Screen of Compounds Known to Induce Phospholipidosis Also Demonstrates an Increase in Lysosomal Volume and Number

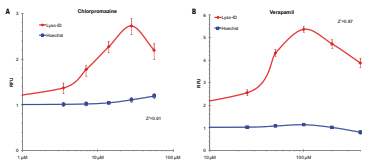


FIGURE 8: Using a conventional fluorescence microplate reader, known drug-induced phospholipidosis correlated with increased lysosome-associated fluorescence signal in a dose-dependent manner. (A) Chlorpromazine (B) Verapamil. Both compounds are cationic and amphiphilic, and are known to induce abnormal accumulation of phospholipids within lysosomes, resulting in formation of lamellar bodies. Hoechst 33342 is used to monitor for false positives in the microplate assay, arising from compounds present at cytoxic doses. The high Z-factor (>0.5) obtained using the assay demonstrates excellent signal-to-noise and signal-to-background ratios.

Fluorescence microscopy Confirms an Increase in Lysosome Volume and Number by Cationic & Amphipathic Drug Molecules

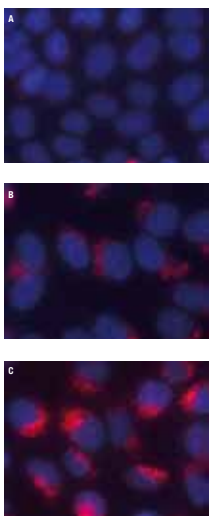


FIGURE 9: Treatment of U-2-OS cells with phospholipidosis-inducing drugs causes a an increase in lysome number and volume, as demonstrated by fluorescence microscopy. (A) Untreated cells (B) Chlorpromazine 28 µM (C) Verapamil, 200 µM. These two compounds are cationic and amphiphilic, and known to induce abnormal accumulation of phospholipids within lysosomes, resulting in lamellar bodies. Nuclei are counter-stained with Hoechst 33342 dye.

Lyso-ID® Red Dye Detects more than Phospholipidosis

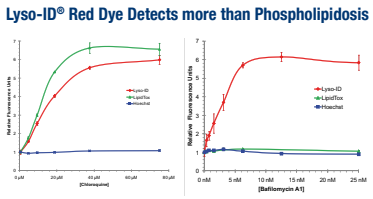


FIGURE 10: U-2-OS cells were treated with various concentrations of chloroquine (induces phospholipidosis) or bafilomycin A1 (prevents acidification of lysosomes and prevents fusion of lysosomes with autophagosomes) for 24 hours. Cells stained with LipidTox dye (a fluorescent phospholipid that detects phospholipidosis, green line) were incubated in the presence of the dye for 24 hours during treatment with the drugs. Cells stained with Lyso-ID® Red dye (red line) and Hoechst 33342 (blue line) were stained for 15 minutes post drug incubation.

LipidTox only detects phospholipidosis induced by chloroquine. Lyso-ID® Red can detect phospholipidosis induced by chloroquine and it also detects the effects induced by bafilomycin A1.

CONCLUSION

- The described lysosome-targeting dye is shown to be a suitable indicator of lysosome perturbation arising from drug or toxic agent treatment, suggesting potential application in *in vitro* toxicology investigations
- The microplate-based assay provides a rapid and robust method, with potential for serving as an *in vitro* high throughput screening assay for preclinical drug safety assessment
- The described assay is rapid (4-24 hour drug incubation), sensitive and specific. It is also compatible with standard high-throughput microplate-based screening workflows.
- Current phospholipidosis assays require electron microscopy or a long term incubation (48-72 hours) with a fluorescent phospholipid, followed by fixation of the cells. The described high-throughput method for screening of phospholipidosis-inducing drugs is much more rapid, and requires fewer processing steps.
- The Lyso-ID® Red dye detects perturbations by bafilomycin A1 that are not detected using fluorescent phospholipids.