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Application Note

Automated Detection of Drug-Induced Lysosomal Cytotoxicity

Automation of the Lyso-ID® Red Assay Using the EL406™ Combination Washer Dispenser

Paul Held, BioTek Instruments, Inc., Winooski, VT
Kheng Newick, University of Vermont, Burlington, VT
Dee Shen and Wayne Patton, Enzo Life Sciences, Farmingdale, NY

Almost all drug compounds will elicit deleterious effects on cells at some concentration. Lysosome perturbation as a result of ion trapping of amine containing compounds has been demonstrated to cause the formation of autophagosomes and autophagic cytopathology. Here we describe the use of the EL406™ Combination Washer Dispenser to automatically aspirate media, wash cells and dispense reagents for the Lyso-ID® Red Detection kit, part of the CELLestial® Live Cell Analysis platform from Enzo Life Sciences.

Introduction

The identification of cytotoxic effects is a critical element in the pre-clinical small molecule drug discovery process. *In-vitro* cell-based assays are typically used early in the process following screening which requires the ability to automate workflows to handle the number of compounds tested. Cytotoxic effects of drug molecules are often first observed as perturbations of normal cellular organelle functionality. For example, lysosome perturbation as a result of ion trapping of amine containing compounds has been demonstrated to cause the formation of autophagosomes and autophagic cytopathology.

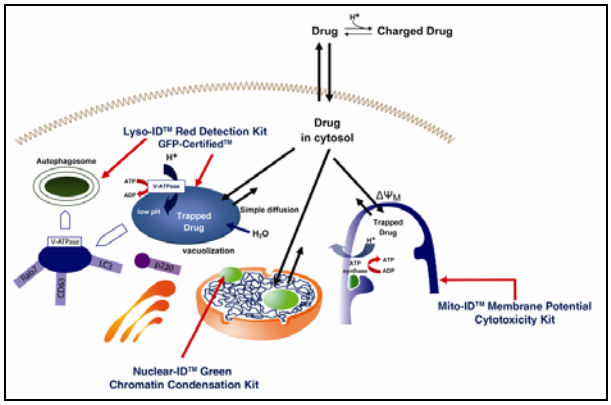

Figure 1. Schematic Organic amine induced ion trapping.

Cationic amphiphilic compounds (i.e. small molecule drug compounds) can be absorbed by cells by simple diffusion and accumulate inside the acidic cellular organelles, a process referred to as lysosomotropism. While many drugs require the presence of a cationic moiety for bioactivity, their accumulation into subcellular organelles can lead to many undesirable effects [1]. Numerous cationic amphiphilic drugs are known to trigger phospholipidosis, which is typified by excessive intracellular accumulation of phospholipids as lamellar bodies [2]. While the origins of these lamellar bodies remain unknown, they appear to be an adaptive response to the presence of the drug [3] (Figure 1). Similarly, autophagy, which can be induced by external or internal stimuli, is a natural pathway that is mediated by lysosomal degradation of sequestered cytoplasmic components. Inappropriate autophagy has been associated with a number of disease states including Huntington's chorea, cancer and cardiac myopathy.

Many drugs cause an accumulation of phospholipids and lysosomes in the cytoplasm. For example amioderone induces an abnormal accumulation of phospholipids that appear as vacuoles with multilamellar inclusions often referred to as autophagosomes [4]. Other organic amines cause vacuolar-ATPase driven ion trapping, which has been associated with vacuolar and autophagic cytopathology.

Lyso-ID® Red dye is a fluorescent reagent that accumulates in lysosomes. An increase in signal is indicative of an increase in the number or size of cellular lysosomes and lysosome-derived vacuoles. In addition to the lysosomal specific dye, the assay also uses Hoechst 33342 nuclear stain. A decrease of 30% or greater of the Hoechst signal is indicative of generalized cytotoxicity. Figure 2 below demonstrates the ability of Lyso-ID® Red to detect cytotoxic lysosomal perturbations from dosing cells with verapamil.

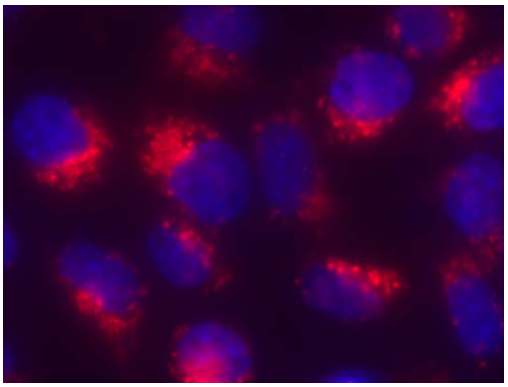

Figure 2. Image of lysosomal staining by Lyso-ID® Red dye. Increase in lysosome volume and number resulting from 200 µM verapamil**.**

Thiostrepton is an antibiotic related to Siomycin A, first described by Donivick et. al. in 1955 [5] . This thiazole compound, while typically used in topical antibiotic mixtures due to its poor water solubility, has been found to target breast cancer cells through inhibition of forkhead box M1 expression [6]. Because tumor cells are selectively targeted as compared to normal cells and the chemical structure of thiostrepton contains several amino groups (see figure 3) we sought to examine whether or not this compound is differentially partitioned into lysosomes depending on cellular transformation.

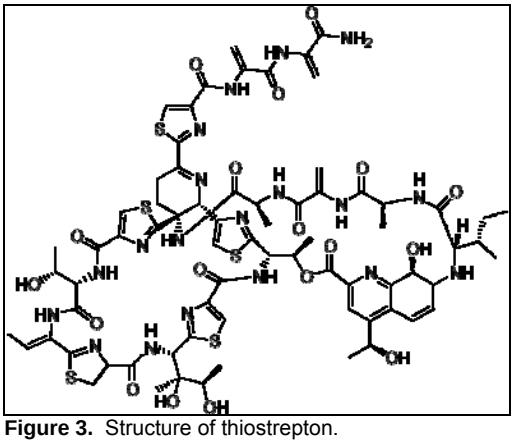

Figure 3. Structure of thiostrepton.

Materials and Methods

H-mesothelioma (H-Meso) cells were seeded at 20,000 cells per well and allowed to attach overnight. The following morning the cells were treated with increasing doses of chloroquine, verapamil or thiostrepton. After an 18-hour exposure to the drugs the cells were washed and Lyso-ID® Red and Hoechst 33342 dyes were added using the EL406 Washer Dispenser. After a 30 minute incubation, excess dye was removed by washing 3 times with wash solution, followed by a final addition of 80 µL of wash solution. The fluorescence was then determined using a Synergy Mx Reader. Lyso-ID® Red dye (red fluorescence) was measured using an excitation of 540 nm and an emission of 680 nm, while Hoechst dye (blue fluorescence) was determined with an excitation of 340 nm and an emission of 480 (Figure 4).

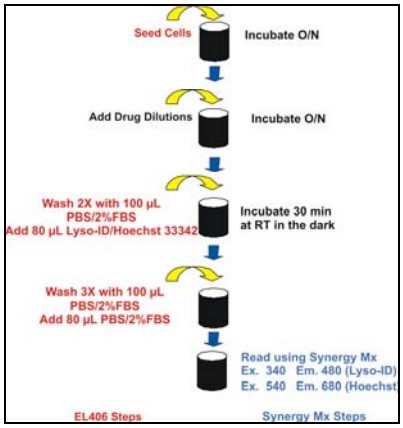

Figure 4. Schematic of the automated Lyso-ID® Red process carried out by the EL406™ Washer Dispenser and Synergy™ Mx Reader.

Two BioTek instruments were used in these studies. The EL406 was used to automate many of the fluid handling tasks, while the Synergy Mx was used to make fluorescent determinations. The EL406 Combination Washer Dispenser provides full plate washing with three reagent dispensers in one instrument. The EL406 offers fast, accurate media removal and cell washing capabilities through its Dual-Action™ manifold. It also offers reagent dispensing capabilities through the use of its peristaltic or syringe pumps, with volumes ranging from 1-3000 µL/well. The instrument was used to plate cells, remove media, and dispense reagents to the 96-well cell plates. The Synergy Mx Monochromator-based Multi-mode Microplate Reader was used to perform fluorescence measurements. The Ultra Fine-Tuned™ technology of the Synergy™ Mx incorporates a quadruple monochromator system which selects wavelengths with a repeatability of plus or minus 0.2 nm. The optical head can focus up and down on samples with a 100 µM resolution.

It also uses a dedicated optical system, separate from the fluorescence optics, for high-performance luminescence detection. Ultra low noise digital photon integration system and high-quality optics ensure the best sensitivity available today.

Results

Initial experiments focused on the response of human mesothelioma cells to agents known to cause drug-induced phospholipidosis. When H-meso cells were treated with various concentrations of either chloroquine or verapamil a dose dependent increase in Lyso-ID® Red signal is observed (Figure 5). The presence of 100 µM Chloroquine increases Lyso-ID® Red fluorescence approximately 3-fold, while 100 µM verapamil resulted in a 2-fold increase in fluorescence. This indicates that these drugs cause an increase in the number of cellular lysosomes and/or an increase in lysosomal volume in H-Meso cells. As both compounds have been shown to elicit this response in other cell types this finding was not unexpected. Staining with Hoechst 33342, a monitor for cytotoxic cell loss, did not change over the tested concentrations.

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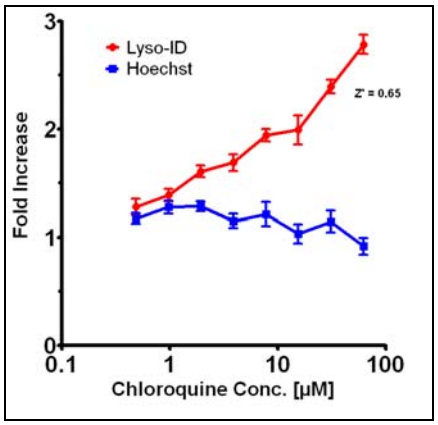

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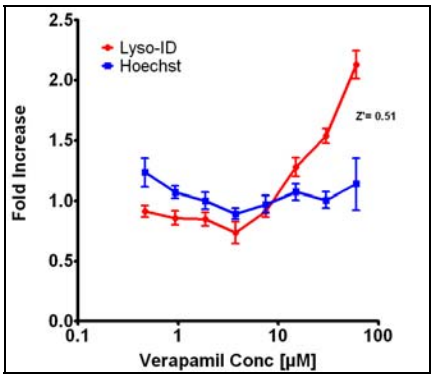

Figure 5. Dose dependent drug induced increase in Lysosomal content as measured by increase in Lyso-ID® Red fluorescence.

The effect of thiostrepton on lysosomal content was tested with several different mesothelioma cell lines, as well as control LP9 mesothelial cells. With all cell lines tested, the verapamil dose response curves were run in parallel as a positive control. As seen in figure 6, verapamil elicited an approximately 2-fold increase in Lyso-ID® staining in all cell lines at higher drug concentrations with no increase in Hoechst signal.

This indicates that these cell lines are capable of responding to agents known to cause phospholipidosis, resulting in increased lysosome content. When the effect of thiostrepton was examined, only the non-transformed cell line LP9 was found to respond with increased lysosome content as a result of exposure to the compound. Lyso-ID® Red dye fluorescence increased almost 3-fold with the addition of 5 µM thiostrepton in LP9 cells. Some malignant transformed cell lines showed a small response, while others displayed no increase as a result to exposure to thiostrepton (Figure 6).

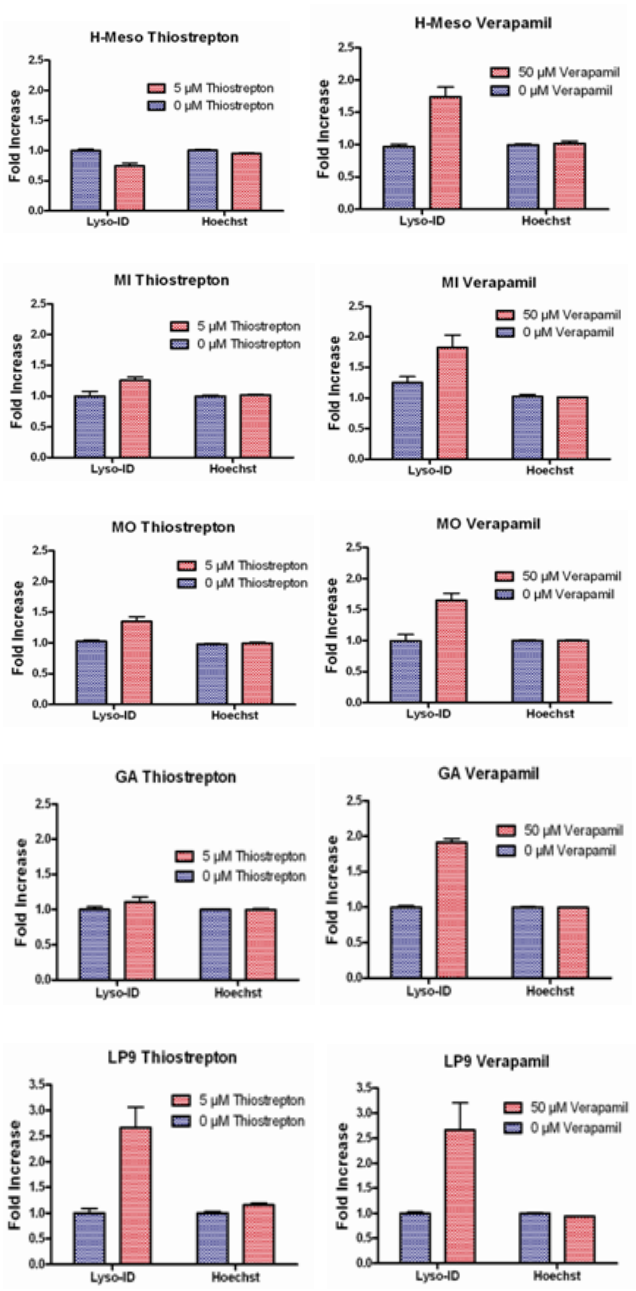

Figure 6. Response change in Lyso-ID® Red dye fluorescence caused by thiostrepton and verapamil in several different mesothelial cell lines.

Discussion

These data indicate that Lyso-ID® Red stain can detect an increase in lysosomal content in live mesothelioma cells when exposed to known phospholipidosis inducers such as chloroquine and verapamil. In addition, it appears that there is a differential susceptibility of cells to respond to thiostrepton exposure with respect to increases in their lysosomal content. Interestingly, LP9 cells are less sensitive to the cytotoxic effects of this chemotherapeutic agent when tested in viability assays. One possibility is that the drug is sequestered in lysosomes reducing the effective drug concentration. The loss of adaptive responses by tumor cells to outside stimuli has been shown to occur under a number of different conditions. Thiostrepton is an amine-containing compound that is a candidate for treatment of mesothelioma. The inability of many of these tumors to sequester thiostrepton might be exploited with other drug treatments.

The identification of drugs that induce phospholipidosis is driven primarily by regulatory pressure. This is considered an adverse event for a number of reasons. Phospholipidosis has been association with some genetic conditions such as Niemann-Pick disease [7]. There is a relationship between specific tissue phospholipidosis and toxicity in the same tissue. Most notable is highlighted by kidney phospholipidosis caused by gentamycin and its associated renal tubular toxicity. In addition there is a certain amount of uncertainty as to the harm caused by phospholipidosis, despite the great deal of data that seems to indicate that it does no harm. Despite the innocuous nature of phospholipidosis it is important to determine the potential liability of a candidate drug compound. Situations where effective drug concentrations may be reduced due to lysosomal sequestration would be particularly important with toxic agents such as cancer chemotherapies.

The Lyso-ID® Red kit in conjunction with the EL406 Combination Washer Dispenser provides an optimized workflow to investigate and screen compounds for phospholipidosis. The Lyso-ID® Red fluorescent assay kits provide a much easier way to screen for this potential problem than the gold standard method of electron microscopy. The EL406 Washer Dispenser provides the liquid handling capabilities to automate many of the tedious fluid handling steps required of this assay. The EL406 instrument has the ability to quickly and reliably add and remove fluid from 96- or 384-well microplates containing live cells, replacing numerous manual pipetting and plate washing tasks. In addition the device can dispense accurate amounts of Lyso-ID® Red reagent as needed.

References

1. Ikeda , K., M. Hirayama, Y. Hirota, E. Asa, J. Seki and Y. Tanaka (2008) Drug-induced phospholipidosis is caused by blockade of mannose 6-phosphate receptor-mediated targeting of lysosomal enzymes, *BBRC*, **377:**268-274.

2. Anderson, N. and J. Borlak (2006) Drug-induced phospholipidosis, *FEBS Lett*. **580:**5533-5540.

3. Reasor, M.J. and S. Kacew (2001) Drug-induced phosopholipidosis. FEBS Lett. 580:5533-5540.

4. Lewis,J.H., F. Mullick, , K.G. Ishak, R.C. Ranard, B. Ragsdale, R.M. Perse, E.J. Rusnock, A. Wolke, S.B. Benjamin, L.B. Seeff, H.J. Zimmerman. (1990) Histopatholic Analysis of Suspected Amiodarone hepatoxicity. Hum. Path. **21:**59-67.

5. Donovick R, Pagano JF, Stout HA, Weinstein MJ (1955). Thiostrepton, a New Antibiotic I. In. vitro Studies Antibiotics Annual **3**: 554–9.

6. Kwok, J. M-M., S.S. Myatt, C.M. Marson, R.C. Coombies, D. Constantinidou, and E. W-F. Lam (2008) Thiostrepton Selectively Targets Breast Cancer Cells through Inhibition of Forkhead box M1 Expression. Mol. Cancer Ther. 7(7):2022-2032.

7. McGovern, M.M., A. Aron, S.E. Brodie, R.J. Desnick, and M.P. Wasserstein (2006). Natural History of Type A Niemann-Pick Disease: Possible Enpoints for Therapeutic Trials. Neurology. 66:228-232.

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