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A high content screening assay for identifying lysosomotropic compounds

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ABSTRACT

Lysosomes are acidic organelles that are essential for the degradation of old organelles and engulfed microbes. Furthermore, lysosomes play a key role in cell death. Lipophilic or amphiphilic compounds with a basic moiety can become protonated and trapped within lysosomes, causing lysosomal dysfunction. Therefore, high-throughput screens to detect lysosomotropism, the accumulation of compounds in lysosomes, are desirable.

Hence, we developed a 96-well format, high content screening assay that measures lysosomotropism and cytotoxicity by quantitative image analysis. Forty drugs, including antidepressants, antipsychotics, antiarrhythmics and anticancer agents, were tested for their effects on lysosomotropism and cytotoxicity in H9c2 cells. The assay correctly identified drugs known to cause lysosomotropism and revealed novel information showing that the anticancer drugs, gefitinib, lapatinib, and dasatinib, caused lysosomotropism. Although structurally and pharmacologically diverse, drugs that were lysosomotropic shared certain physicochemical properties, possessing a *C*log*P* > 2 and a basic p*K*a between 6.5 and 11. In contrast, drugs which did not lie in this physicochemical property space were not lysosomotropic. The assay is a robust, rapid screen that can be used to identify lysosomotropic, as well as, cytotoxic compounds, and can be positioned within a screening paradigm to understand the role of lysosomotropism as a contributor to drug-induced toxicity.

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1. Introduction

Lysosomes are conventionally known as the cell's recycling center as they are essential for the digestion of old organelles and engulfed microbes. Three processes, endocytosis, phagocytosis and autophagy, are involved in delivering macromolecules to lysosomes, in which lie enzymes that function at pH 4.5 (de Duve, 1983; Luzio et al., 2007; Turk and Turk, 2009). More than 50 lysosomal enzymes are responsible for the breakdown of lipids, phospholipids, glycolipids, proteins, nucleic acids and sugars. Genetic disorders involving defects in lysosomal enzymes can affect many organs and tissues and can sometimes be fatal (Walkley, 2009). Remarkably, lysosomes are also important players in triggering programmed cell death since lysosomal enzymes known as cathepsins are involved in both mitochondria-dependent apoptosis and mitochondria-independent programmed cell death (Kroemer and Jaattela, 2005; Kirkegaard and Jaattela, 2009). Furthermore, rupture of lysosomes leading to release of cathepsins can cause necrosis (Kirkegaard and Jaattela, 2009; Groth-Pedersen and Jaattela, 2010).

Compounds that accumulate in lysosomes are called lysosomotropic agents, and their accumulation within lysosomes is known as lysosomotropism (de Duve et al., 1974). Compounds with both a lipophilic moiety and a basic moiety have a propensity to cause lysosomotropism (de Duve et al., 1974). Within the neutral pH of the cytoplasm, these compounds are free to penetrate membranes including those of lysosomes, but once inside the acidic environment of lysosomes, they become protonated and are unable to cross the lysosomal membrane to re-enter the cytoplasm. Hence, these compounds become trapped, leading to intra-lysosomal compound concentrations which are much higher than those found in the cytoplasm (Kaufmann and Krise, 2007). Many drugs given in the treatment of central nervous system disorders are cationic amphiphilic compounds and it is, thus, not surprising that they have been reported to be lysosomotropic (Daniel, 2003).

How lysosomotropism might cause drug-induced toxicity is not well understood. Cationic amphiphilic compounds have been known to cause phospholipidosis, a phenomenon in which excessive accumulation of phospholipids occurs within lysosomes (Reasor et al., 2006). Phospholipidosis occurs predominantly in the brain and in organs which are lysosome-rich such as the lungs,

Abbreviation: HCS, high content screening.

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liver, and kidney (Kodavanti and Mehendale, 1990). However, it is not clear if phospholipidosis is a manifestation of toxicity or merely an adaptive response to drugs (Reasor et al., 2006).

A potential mechanism by which lysosomotropic compounds might cause toxicity is via inhibition of autophagy. Autophagy is a homeostatic process by which old or damaged intracellular components are enveloped in double-membraned vesicles called autophagosomes which, then, fuse with lysosomes to form autophagolysosomes (Rubinsztein et al., 2007). The contents of autophagolysosomes are subsequently degraded by lysosomal acid hydrolases (Rubinsztein et al., 2007). Lysosomotropic compounds could conceivably inhibit the degradation of autophagolysosomes by increasing the pH of lysosomes and thereby impairing lysosomal acid hydrolases. Indeed, the lysosomotropic drug, chloroquine, has been reported to inhibit the degradation of autophagolysosomes (Walls et al., 2010).

Early safety assessments of new chemical entities are becoming prevalent in the pharmaceutical industry as a means to reduce compound attrition caused by toxicity. Although there is a limited understanding of how lysosomotropism contributes to toxicity, an association between the two has been observed (Walls et al., 2010), and therefore assays that identify lysosomal impairment are desired. Hence, the aim of this study was to develop a high content screening (HCS) assay that identifies compounds which cause lysosomotropism. The assay was multiplexed so that cytotoxicity could also be measured. Two commercially available fluorescent dyes, LysoTracker® Red DND-99 and Hoechst 33342, were used to measure lysosomotropism and cytotoxicity, respectively, in a rat cardiomyocyte-derived cell line, H9c2. Under physiological conditions, LysoTracker® Red, a fluorophore linked to a weak base, readily enters acidic organelles such as lysosomes. However, in the presence of either a lipophilic basic compound or cationic amphiphilic compound, competition between the compound and the LysoTracker dye occurs, resulting in a pH-independent decrease in the fluorescence signal of the dye (Lemieux et al., 2004). We tested the effect of forty drugs including antidepressants, antipsychotics, anti-arrhythmics, and anticancer agents on H9c2 cells in the multiplexed HCS assay. The assay correctly identified several drugs that are known to be lysosomotropic agents and also revealed novel information showing that some, but not all, of the tyrosine kinase inhibitors that we tested caused lysosomotropism. The HCS assay was robust, rapid, and sensitive, and can be implemented in large-scale compound screening to identify lysosomotropic, as well as, cytotoxic compounds.

2. Materials and methods

Chemicals were obtained from Axxora LLC (San Diego, CA), Toronto Research Chemicals (Toronto, Canada), Sigma (St. Louis, MO) and Pfizer's chemical sample bank (Groton, CT). Cell culture media and supplements were from Invitrogen (Carlsbad, CA).

The fluorescent dyes, LysoTracker® Red DND-99 and Hoechst 33342, were from Invitrogen.

2.1. H9c2 cell culture conditions

The rat cardiomyocyte-derived cell line, H9c2 (catalog# CRL 1446), was obtained from the American Type Culture Collection (Manassas, VA) and sub-cultured less than 18 times. Cells were grown in a culture medium containing high-glucose Dulbecco's Modified Eagle's medium (Invitrogen, 11995-065) supplemented with 10% fetal bovine serum, 2 mM glutamine, 5 mM HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid), 100 units/ml penicillin and 100 μg/ml streptomycin, in a humidified 37 °C, 5% CO_2 atmosphere.

2.2. High content screening assay

H9c2 cells were seeded in black-walled clear-bottom 96-well plates at 6000 cells/100 μl/well in culture medium and incubated overnight. Stock solutions of compounds were made in DMSO. The following day, the culture medium in the 96-well cell plates was replaced with 100 μl/well of dosing medium containing either the test compounds or 0.5% DMSO which served as the vehicle. The dosing medium consisted of high-glucose Dulbecco's Modified Eagle's medium supplemented with 1% fetal bovine serum, 2 mM glutamine, 5 mM HEPES, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were incubated with the test compounds for 4 h in a humidified 37 °C, 5% CO_2 atmosphere. Hundred microliters of dosing medium containing LysoTracker® Red (to stain the lysosomes) and Hoechst 33342 (to stain the nuclei) was then added to each well so that the final concentration of the two dyes in each well was 60 nM and 4 μg/ml, respectively. The cells were incubated for 30 min in a humidified 37 °C, 5% CO_2 atmosphere. The cells were rinsed three times with Hanks Balanced Salt Solution (HBSS; 200 μl/well); the final HBSS rinse was not aspirated.

Automated live-cell image acquisition was performed on a Thermo Fisher Scientific Cellomics® ArrayScan® VTI High Content Screening Reader using a 10× objective. The ArrayScan® Reader was equipped with a Thermo Fisher Scientific Cellomics® Live Module so that the cells could be maintained in a humidified 37 °C, 5% CO_2 atmosphere. The fluorescence of the two dyes, Hoechst (representing the nuclei) and LysoTracker® Red (representing the lysosomes) was measured in channels 1 and 2, respectively. Image analysis was done using the Compartmental Analysis Bioapplication (Thermo Fisher Scientific). Details on the parameter settings used for the image analysis are shown in Table 1. Image acquisition was set to a minimum of 500 objects per well or to a maximum of four fields per well. The exposure settings for channels 1 and 2 were set to be at 25% of the dynamic range based on the vehicle-treated cells. Lysosomal staining due to LysoTracker® Red was defined as the punctate signal around the nucleus and was measured as the Mean CircSpot Average Intensity in channel 2. Cytotoxicity was evaluated by measuring the number of nuclei ("Selected Object Count per valid field") in Channel 1 of the Compartmental Analysis Bioapplication.

2.3. Data analysis

Raw data were exported from vHCS™View (Thermo Fisher Scientific), and IC_{50} values were generated using GraphPad Prism 5 (San Diego, CA) with non-linear regression analysis.

The Z' factor was calculated using the formula, $Z' = 1 - 3(\sigma_p + \sigma_n)/|\mu_p - \mu_n|$, where μ_p and σ_p are the mean and standard deviation, respectively, of the positive control, and μ_n and σ_n are the mean and standard deviation, respectively, of the negative control (Zhang et al., 1999).

Table 1
Parameter settings used in the image analysis.

Parameter	Channel 1	Channel 2
Filter set	XF93-Hoechst	XF93-TRITC
Exposure method	25% of the dynamic range	25% of the dynamic range
Object identification method	Fixed threshold	Fixed threshold
ChannelToDeriveCircOverlay: Channel 2		
ChannelToDeriveCircSpotOverlay: Channel 2		
Background correction (pixels)	53	80
Object segmentation (pixels)	10	–
Circ modifier (pixels)	–	14

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