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A cell-based quantitative high-throughput image screening identified novel autophagy modulators

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ABSTRACT

Macroautophagy is a major cellular degradation pathway for long-lived proteins and cellular organelles to maintain cellular homeostasis. Reduced autophagy has been implicated in neurodegenerative diseases, metabolic syndrome, and tumorigenesis. In contrast, increased autophagy has been shown to protect against tissue injury and aging. Here we employed a cell-based quantitative high-throughput image screening (qHTS) for autophagy modulators using mouse embryonic fibroblasts (MEFs) that are stably expressing GFP-LC3. The library of pharmacologically active compounds (LOPAC) was used to screen for the autophagy modulators in compounds alone or in combination with the lysosome inhibitor chloroquine (CQ). The GFP-LC3 puncta were then quantified to measure autophagic flux. The primary screening revealed 173 compounds with efficacy more than 40%. These compounds were cherry-picked and re-tested at multiple different concentrations using the same assay. A number of novel autophagy inducers, inhibitors, and modulators with dual-effects on autophagy were identified from the cherry-pick screening. Interestingly, we found a group of compounds that induce autophagy are related to dopamine receptors and are commonly used as clinical psychiatric drugs. Among them, indatraline hydrochloride (IND), a dopamine inhibitor, and chlorpromazine hydrochloride (CPZ) and fluphenazine dihydrochloride (FPZ), two dopamine receptor antagonists, were further evaluated. We found that FPZ-induced autophagy through mTOR inhibition but IND and CPZ induced autophagy in an mTOR-independent manner. Our data suggest that image-based autophagic flux qHTS can efficiently identify autophagy inducers and inhibitors.

1. Introduction

Autophagy is a genetically programmed, evolutionarily conserved intracellular degradation pathway in response to stress [1]. It involves the formation of double-membrane autophagosomes that carry intracellular long-lived proteins and organelles to the lysosomes for degradation to maintain cellular homeostasis. It is tightly controlled by over 30 autophagy-related (Atg) genes [2,3]. Autophagy is generally considered as a cell survival mechanism in response to various stress conditions. Autophagy plays a critical role in human physiology such as development and differentiation as well as many disease states such as metabolic disease, neurodegeneration, infection, and cancer [1,4–6]. Impaired autophagy leads to the accumulation of intracellular protein aggregates and dysfunctional mitochondria, which contributes to neurodegenerative diseases and tumorigenesis [7–10]. In contrast, increased autophagy has been shown to delay aging, improve neuronal functions and protect against tissue injury [11–13].

Microtubule-associated protein 1 light chain 3 (LC3), a mammalian homolog of the yeast protein Atg8, is an ubiquitin-like protein that is important for the formation of the autophagosome [7,8]. LC3 exists in two forms, LC3-I and LC3-II. LC3-I is a cytosolic protein that undergoes conjugation to phosphatidylethanolamine (PE) to form LC3-II, which targets the autophagosomal membrane

Abbreviations: Atg, autophagy-related; CPZ, chlorpromazine hydrochloride; CQ, chloroquine; EM, electron microscopy; FPZ, fluphenazine dihydrochloride; HRP, horseradish peroxidase; IND, indatraline hydrochloride; LC3, microtubule-associated protein 1 light chain 3; LOPAC, library of pharmacologically active compounds; MEF, mouse embryonic fibroblast; mTOR, mammalian target of rapamycin; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; qHTS, quantitative high-throughput screening; PBS, phosphate buffered saline; PE, phosphatidylethanolamine.

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[14]. LC3-II stays on the membrane of the autophagosome until it is degraded at the autolysosome, making LC3 a reliable marker to monitor autophagy. The behavior of GFP-LC3 fusion protein is very similar to the endogenous LC3, which has been widely used as a marker to monitor autophagy [7,8,15]. Under normal conditions, the number of GFP-LC3 puncta is very low, but can be rapidly induced by starvation or the addition of rapamycin [16]. Although GFP-LC3 positive puncta can be increased with an induction of autophagy, the accumulation of GFP-LC3 puncta is not always correlated with the actual autophagy activity (or autophagy degradation activity as referred to as autophagic flux), but rather a marker of an increased number of autophagosomes [7,8,15]. Increased number of GFP-LC3 puncta can also be due to the impaired fusion of autophagosomes with lysosomes or impaired lysosomal functions. For example, treatment with chloroquine (CQ) or Bafilomycin A1, both impairing lysosomal functions by increasing lysosomal pH, leads to an increase in the number of GFP-LC3 puncta due to the block of lysosomal degradation of GFP-LC3 [7,8,15,17]. Therefore, it has been recommended by the autophagy community to use autophagic flux assay, which quantifies the GFP-LC3 puncta for any given chemical/or condition in the presence or absence of a lysosomal inhibitor [7,8].

To discover novel autophagy regulators, we developed a quantitative high-throughput cell-based autophagic flux screen using a GFP-LC3 stable cell line, which is different from previous published high-throughput screenings that only monitored the changes of GFP-LC3 puncta but not autophagic flux. Moreover, it is known that a compound may differentially affect autophagic flux at different concentrations and thus yields controversial results by only using a single concentration. In the primary screening, we used this cell-based image assay to evaluate changes of GFP-LC3 puncta after treatment with each compound at 8 different concentrations. After screened against the Library of Pharmacologically Active Compounds (LOPAC), we identified 173 positive compounds that may modulate autophagy. Following the cherry-pick confirmation, we identified 27 autophagy inducers, 17 autophagy inhibitors and 8 compounds that have dual effects on autophagy depending on their potency and efficacy. Several autophagy inducers identified from this study are known drugs of dopamine receptor modulators including indatraline (IND), chlorpromazine (CPZ) and fluphenazine (FPZ). Our follow-up analysis revealed that IND and CPZ may induce autophagy in an mTOR-independent manner whereas FPZ-induced autophagy may require mTOR inhibition.

2. Materials and methods

2.1. Cell culture

Mouse embryonic fibroblast (MEF) cells stably expressing GFP-LC3 were established as described previously [15]. MEF cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere in DMEM supplemented with 10% (v/v) fetal bovine serum (Invitrogen), penicillin and streptomycin (100 U/ml), and glutamine (100 μg/ml) (Gibco). HCT116 cells were maintained in McCoy and A549 cells were maintained in RPMI 1640 with similar supplement.

2.2. Quantitative high throughput screening (qHTS)

For the primary screen, GFP-LC3 MEF cells were suspended in the complete culture medium and dispensed at 800 cells/5 μl/well in 1536-well tissue culture-treated black/clear bottom, collagen coated plates (Corning, Acton, MA) using a Flying Reagent Dispenser (FRD, Aurora Discovery, Carlsbad, CA). After the cells were incubated at 37 °C with 5% CO2 for 5 h, 23 nl of compound or control, chloroquine diphosphate (CQ), was added into the assay plates using a Pinstool station (Kalypsys, San Diego, CA). The plates were incubated at 37 °C with 5% CO2 for 18 h. Next day, the medium from each well was removed and washed once with phosphate buffered saline (PBS) using BioTek Washer and Dispenser (Winooski, VT), and then the cells were fixed with 6% (v/v) paraformaldehyde (EMS, Hatfield, PA) containing 0.625 μg/ml Hoechst 33342 (Invitrogen, Madison, WI) for 20 min at room temperature. Fixed cells were washed twice with PBS and then stored at 4 °C until plates were ready for analysis. Assay plates were imaged on an ArrayScan® VTI HCS Reader (Thermo Scientific, Pittsburgh, PA) using a 20x objective in the Hoechst and GFP (XF-100 filter) channels. The compartment analysis algorithm was used to identify the nuclei, apply a cytoplasmic mask and quantitate GFP spots in the GFP channel. A nuclear mask was generated from Hoechst stained nuclei. An area representative of the cytoplasm was generated by placing a set of concentric rings around the nuclear mask. Autophagosomal membrane-associated GFP-LC3 (puncta) was detected as GFP-fluorescent vesicular objects that exceeded a threshold defined by untreated cells and that were located exclusively in the cytoplasmic area (Fig. 1). The algorithm was acquired 90 cells per well. Data were captured, extracted, and analyzed with ArrayScan Data Acquisition and vHCS View version 3.0. Three of output parameters, mean_RingSpotCountCh2, mean_RingSpotAvgAreaCh2 and mean_RingSpotAvgIntenCh2, were used for the data analysis. The average punctate count per cell was acquired as 'Mean_RingSpotCountCh2′, which gave the largest assay window. Primary screening performed well with the average signal to background ratio was 98.5 ± 31.3 and Z' averaged 0.65 ± 0.05.

After primary screening, the selected actives were cherry-picked based on potency (<25 μM) and efficiency (>40%), and re-tested in 8 point titrations with final concentration ranging from 21 nM to 46 μM using the same assay protocol as described above in a 1536-well plate. Cherry-pick compounds were tested three independent times in the presence or absence of 2.5 μM of CQ.

2.3. qHTS data analysis

Primary data analysis was performed as previously described [18]. Briefly, raw plate reads for each titration point were first normalized to CQ control (20.70 μM, which induced the maximum GFP-LC3 puncta in our assay and referred to as 100%) and DMSO-only wells (basal, 0%) and then corrected by applying a pattern correction algorithm using compound-free control plates (DMSO plates). Concentration titration points for each compound were fitted to the Hill equation, yielding concentrations of half-maximal induction (EC_{50}) and maximal response (activity at the highest test concentration, efficacy) values [19]. Compounds were considered active in the cell-based MEF-LC3_GFP assay if they showed stimulation and had an >40% efficacy in the mean ring spot count, mean ring spot average intensity or mean ring spot average area readings. These compounds were selected for confirmation and follow-up studies.

2.4. Reagents and antibodies

Stock solution of IND, CPZ, FPZ and CQ were all obtained from Sigma. Stock solution of IND, CPZ, FPZ and CQ were prepared in water, and diluted with medium before use. The primary antibodies were p62 (Abnova, Mouse pAb #H00008878-M01), phospho-S6 (Cell Signaling, Rabbit pAb #4858S), total-S6 (Cell Signaling, Rabbit pAb #2217S), phospho-4E-BP1 (Cell Signaling, Rabbit pAb #9451), total-4E-BP1 (Cell Signaling, Rabbit pAb #9452), β-actin (Sigma, Mouse mAb # A5441), GAPDH (Cell Signaling, Rabbit pAb #2118). Rabbit anti-LC3 antibody was developed as described previously [20]. The secondary antibodies were horseradish