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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.

Keywords

Autophagy; High-throughput screening; GFP-LC3; mTOR; Dopamine receptor

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phrs.2016.05.004>.

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 [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% CO₂ 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 20× 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 ($<25 \mu\text{M}$) and efficiency ($>40\%$), and re-tested in 8 point titrations with final concentration ranging from 21 nM to $46 \mu\text{M}$ 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 $2.5 \mu\text{M}$ 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 ($20.70 \mu\text{M}$, 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 peroxidase (HRP)-conjugated anti-rabbit (Jackson ImmunoResearch, #111-035-045), and anti-mouse IgG (Jackson ImmunoResearch, #115-035-062) and used at a dilution of 1:10000.

2.5. Immunoblot assay

Cells were washed in PBS and lysed in RIPA buffer. Thirty micrograms of protein from each sample was separated by SDS-PAGE and transferred to PVDF membranes. The membranes were stained with primary antibodies followed by secondary HRP-conjugated antibodies. The membranes were further developed with SuperSignal West Pico chemiluminescent substrate (Pierce) or Immobilon Western chemiluminescent HRP substrate (Millipore). Densitometry was quantified using Image J software (National Institutes of Health).

2.6. Cytotoxicity assay

Cell viability/growth was measured by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at a density of 5000 cells per

well in 96-well plates and incubated at 37 °C in a humidified 5% CO₂ incubator for 24 h. Cells were treated with different doses of compounds for 22 h, and 25 µl MTT (5 mg/ml) was added into each well for another 2 h incubation. After that, the supernatant was removed and 100 µl DMSO was added into each well in order to solubilize the blue-purple crystals of formazan. Absorbance values were determined at 550 nm on a Spectra Max 250 spectrophotometer (Tecan GENios). All MTT experiments were performed in quadruplicate and repeated at least 3 times. To determine apoptotic cell death, cells were treated with different concentrations of IND, CPZ and FPZ for 24 h. Cells were stained with Hoechst 33342 (1 µg/ml) and propidium iodide (1 µg/ml), and digital images were obtained using a fluorescence microscope (Nikon Eclipse TE200). Fragmented and condensed apoptotic nuclei were quantified, and results were expressed as percentage of apoptotic cells.

2.7. Caspase activity assay

Cells were treated with different concentrations of IND, CPZ and FPZ for 24 h. Total cell lysates were prepared using RIPA buffer and 20 µg of proteins was incubated with the specific caspase-3 substrate (Ac-DEVD-AFC) (20 µM). The fluorescence signals were detected by a fluorometer (Tecan GENios) at 400/510 nm (excitation/ emission) and the caspase activities were normalized to the control cells as we described previously [21].

2.8. Fluorescence microscopy for autophagy

Adenovirus RFP-GFP-LC3 was prepared as described previously [22]. To examine autophagic flux, cells were seeded in a 12-well plate on a cover slide and infected with adenovirus RFP-GFP-LC3 (10 moi) for 24 h. Cells were treated with different doses of compounds in the presence or absence of CQ (20 µM) for 6 h. After treatment, cells were fixed with 4% paraformaldehyde in PBS overnight at 4 °C for microscopy. Fluorescence images were acquired under a Nikon Eclipse 200 fluorescence microscope with MetaMorph software. For quantification of autophagy, RFP-LC3 punctated dots and GFP-LC3 punctated dots were determined in triplicates by counting a total of more than 30 cells in each condition.

2.9. Electron microscopy

Cells were fixed with 2.5% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer (pH 7.4), followed by 1% OsO₄. After dehydration, thin sections were stained with uranyl acetate and lead citrate. Digital images were obtained using a JEM 1016CX electron microscope. The number of autophagic vacuoles from each cell was determined from a randomly selected pool of 15–20 fields in each condition. The cytoplasmic area was measured by SPOT software (Diagnostic Instruments, Inc.).

2.10. Statistical analysis

Experimental data were subjected to Student's *t*-test or one-way ANOVA analysis with Scheffe's post hoc test where appropriate. All error bars are standard error.

3. Results

3.1. Quantitative high-throughput image-based screening of LOPAC for modulators of autophagy

The LOPAC was screened using a GFP-LC3 qHTS to determine if any of the 1280 compounds in the library had effects on autophagy based on the formation of GFP-LC3 puncta in MEF cells that are stably expressing GFP-LC3. GFP-LC3 puncta were counted from MEF cells that were treated with either different concentrations of compound or the compound with the addition of CQ (2.5 μ M) to assess the autophagic flux. Based on our screening results, we found that CQ at 2.5 μ M was the best concentration to be used for the autophagic flux assay to avoid the possible saturation for the formation of GFP-LC3 puncta after addition of the compounds. Mean GFP-LC3 puncta counts were compared to DMSO and DMSO with the addition of CQ (2.5 μ M). Fig. 1A shows representative images of the qHTS and GFP-LC3 puncta analysis. The primary screening resulted in the identification of 173 compounds that had hit efficacy $\geq 40\%$ and led to significant increase or decrease in GFP-LC3 puncta formation. In the next cherry-pick screening, these 173 compounds were further analyzed using 8 different concentrations (21 nM–46 μ M) with or without CQ for the autophagic flux. Mean puncta count values for compounds chosen for further analysis can be seen in Supplemental Excel File 1. We further sorted the compounds that have significant changes on autophagic flux into three categories, Category 1, 2 and 3 (Tables 1–3). We identified a total of 27 compounds as autophagy inducers (referred to as Category 1). Most of the compounds in this category had increased GFP-LC3 puncta formation on their own and had further increased GFP-LC3 puncta formation when each compound was given in concert with CQ compared to the CQ alone treatment or the compound alone treatment (Table 1). Importantly, we also identified several compounds including LE300, Labetalol hydrochloride and Fiduxosin hydrochloride, which did not change GFP-LC3 puncta by themselves but increased number of GFP-LC3 puncta in the presence of CQ and the number of GFP-LC3 puncta was higher than CQ alone treatment. These data suggest that LE300, Labetalol hydrochloride and Fiduxosin hydrochloride increased autophagic flux. Except that Trifluoperazine was identified as an autophagy inducer in a previous HTS and CPZ was reported to induce autophagic cell death in a cancer cell line [23], all the other 25 compounds were novel autophagy inducers identified in the present study. We also identified 13 autophagy inhibitors (Category 2, Table 2), which are compounds that had very low puncta scores when either treated alone or treated together with CQ. These compounds are considered autophagy inhibitors that likely target the early step of autophagosome formation (upstream inhibitors). In contrast, some compounds increased GFP-LC3 puncta counts by themselves compared to the DMSO control, but when they were co-treated with CQ the GFP-LC3 puncta counts were not higher than those of CQ only treatment. These compounds are Emetine dihydrochloride hydrate, S-(–)-Eticlopride hydrochloride, CGS-12066A maleate, and (S)-Propranolol hydrochloride and may act at the later stage of the autophagy process by either inhibiting the fusion of autophagosomes with lysosomes or inhibiting lysosomal functions (CQ-like Inhibitors). Compounds in Category 3 (Table 3) showed dual effects on autophagy induction depended on the concentrations of each compound. Some compounds induced autophagy at the higher concentrations but inhibited autophagy at low concentrations, such as Clozapine. In contrast, some compounds inhibited autophagy at the

higher concentrations but induced autophagy at lower concentrations such as Idarubicin and Thapsigargin.

3.2. Indatraline, chlorpromazine and fluphenazine induce autophagic flux

As shown in Table 1, a large portion of identified autophagy inducers are dopamine modulators that are currently used in clinic as antipsychotic drugs. We thus picked three of them for further evaluation and characterization, including IND, a dopamine inhibitor, and CPZ and FPZ, two dopamine receptor antagonists. To our knowledge, IND and FPZ have not been reported to be associated with autophagy whereas CPZ was reported to induce autophagic cell death in U-87MG glioma cells [24]. Images from the original qHTS revealed diffuse GFP-LC3 patterns in DMSO-treated control cells. CPZ and FPZ alone treatment increased GFP-LC3 puncta remarkably for approximately 28-fold and 48-fold of DMSO control group, respectively. IND alone treatment increased GFP-LC3 puncta for 6-fold of DMSO group. In the presence of CQ, the numbers of GFP-LC3 puncta in IND, CPZ and FPZ group were higher than CQ alone and the IND, CPZ and FPZ alone treatment (Fig. 2A). These qHTS data indicate that IND, CPZ and FPZ increase autophagic flux. To further confirm the results obtained from the qHTS, we treated the GFP-LC3 MEF using different concentrations of IND, CPZ and FPZ based on the AC50 obtained from the screening followed by western blot analysis. We found that IND, CPZ and FPZ increased the levels of GFP-LC3-II in a dose-dependent manner (Fig. 2B). More importantly, in the presence of CQ, the levels of GFP-LC3-II in the cells treated with IND, CPZ and FPZ were much higher than the cells treated with each compound alone or CQ alone after either 6 h (Fig. 2C) or 18 h treatment (Fig. 2D). This is in agreement with the qHTS results, which support the conclusion that IND, CPZ and FPZ induce autophagic flux.

To demonstrate that autophagy induction by IND, CPZ and FPZ was not cell type-dependent, these drugs were also examined in HCT116, a human colon cancer cell line. Similarly, IND, CPZ and FPZ increased the endogenous LC3-II levels in a dose- and time-dependent manner in HCT116 cells (Fig. 3A & B). A549 cells, a human lung cancer cell line, also showed similar response to IND, CPZ and FPZ (data not shown). We next performed autophagic flux assay in the presence or absence of CQ in IND, CPZ and FPZ-treated cells. We found that in the presence of CQ, the levels of LC3-II were much higher in IND, CPZ and FPZ-treated cells than the cells treated with each compound alone or CQ alone (Fig. 3C & D). EM analysis revealed that treatment with IND slightly increased the number of autophagic vacuoles (AV), but CPZ and FPZ treatment significantly increased both the number and the size of AV. Most of the AV seemed to be the late autolysosomes as most of the enwrapped contents had already been degraded (Fig. 4A, solid arrows), while a small portion of AV still had electron-dense contents (Fig. 4A, empty arrows).

Though GFP-LC3 has been commonly used as a marker to monitor the dynamic change of autophagy, we previously showed that GFP-LC3 is degraded in a step-wise manner and sometimes does not fully reflect the turnover of autophagosomes [15]. To further confirm that IND, CPZ and FPZ induce autophagic flux, we performed RFP-GFP-LC3 analysis assay. It is thought that RFP signal is more stable than GFP in acidic compartments, and the number of red puncta (RFP-LC3) is generally correlated with autophagic flux [15]. Overall,

IND, FPZ and CPZ treatment led to increased numbers of red-only LC3 dots and total LC3 dots (yellow) compared with control group in most concentrations that we assessed (Fig. 5A–D), although only the changes induced by CPZ (at 10 and 20 μ M) reached statistical difference. As a positive control, there was a significant increase of red-only LC3 puncta in cells under starvation conditions (EBSS buffer). In contrast, in the presence of CQ, the number of EBSS-induced red-only LC3 puncta was markedly reduced whereas the number of yellow LC3 puncta increased (Fig. 5A & E). These results indicate that IND, CPZ and FPZ that we identified from the qHTS are indeed autophagy inducers that can induce autophagic flux in both non-cancer and cancer cells.

3.3. Fluphenazine but not indatraline or chlorpromazine inhibits mTOR signaling

mTOR is a key cellular nutrient sensor and negatively regulates autophagy. To test if these three compounds stimulate autophagy by inhibiting mTOR pathway, we first determined the levels of phosphorylated S6 and 4EBP1, two down-stream targets of mTOR complex 1 (mTORC1). The levels of phosphorylated S6 and 4EBP1 almost remained unchanged in either HCT116 or A549 cells after they were treated with IND for 3 and 6 h (Fig. 6A & B). Similarly, CPZ treatment also had no effects on the levels of phosphorylated S6 and 4EBP1 in HCT116 cells. Intriguingly, CPZ treatment decreased the levels of phosphorylated S6 but had no effects on levels of phosphorylated 4EBP1 (Fig. 6A & B). These results suggest that the changes of the phosphorylation of S6 and 4EBP may not always be consistent, and CPZ-induced changes of mTOR may also be slightly different in HCT116 and A549 cells. In contrast, FPZ consistently decreased the levels of phosphorylated S6 and 4EBP1 in both HCT116 cells and A549 cells (Fig. 6A & B). Moreover, FPZ showed almost the same potency on the inhibition of mTOR with Torin 1, a potent mTOR inhibitor (Fig. 6A & B). In addition, FPZ but not IND or CPZ decreased the levels of phosphorylated S6 and 4EBP1 in a dose-dependent manner in HCT116 cells at 6 h (Fig. 6C). Collectively, these data indicate that FPZ may stimulate autophagy in an mTOR-dependent manner whereas IND and CPZ may induce autophagy independent of mTOR signaling.

3.4. Autophagy may protect against fluphenazine and chlorpromazine-induced apoptosis

We next determined the cell viability after the treatment with these three compounds in HCT116 cells. For all the three concentrations that we assessed, IND did not affect the cell viability in HCT116 cells either at a short time (6 h) or long time treatment (24 h). At the lower concentrations (< 20 μ M), CPZ and FPZ barely affected the cell viability, but CPZ and FPZ at higher concentrations (40 μ M) significantly decreased cell viability in HCT116 cells at 6 h (Fig. 7A). To further determine the cell death nature, we determined the cell and nuclear morphological changes and caspase activation after the cells were treated with IND, CPZ and FPZ for 24 h. As can be seen, the cells displayed regular rectangular shape from the phase-contrast images in control and IND-treated cells. However, most cells were round up in CPZ-treated cells. Nuclear staining by Hoesches33342 showed normal nuclei in control and IND-treated cells, but many cells displayed fragmented nuclei, a typical feature of apoptosis, in FPZ and CPZ-treated cells (Fig. 7B, arrows). We found that FPZ and CPZ increased the number of apoptotic cells in a dose-dependent manner whereas IND had no effects on apoptosis in all the three concentrations that we assessed even after 24 h (Fig. 7C). Consistent with the apoptotic nuclei data, we also found that IND did not affect caspase-3

activities but FPZ and CPZ increased caspase-3 activities in a dose-dependent manner in HCT116 cells (Fig. 7D). Autophagy has been reported to act either as a cell survival mechanism or as a form of autophagic cell death. Moreover, a previous study reported that CPZ induced autophagic cell death in U87-MG glioma cells [24]. We next determined the contribution of autophagy in cell viability in IND, CPZ and FPZ-treated cells. We found that CQ did not affect the cell viability in IND-treated HCT116 and MEF cells. However, CQ decreased cell viability in CPZ or FPZ-treated HCT116 and MEF cells although these differences did not reach statistical difference (Fig. 8A &B). These data suggest that autophagy does not cause cell death but may instead offer some beneficial effects for cell survival in IND, CPZ and FPZ-treated HCT116 and MEF cells.

4. Discussion

In the present study, we developed a cell-based image qHTS for autophagy modulators. Compared to previous autophagy screens, our screen has several unique innovations and advantages. First, we screened for up to 8 different concentrations of each compound from the library, unlike previous screens that used only one concentration per compound [23,25]. This is important because some compounds may have different impacts on autophagy depending on the dose, which would not have been identified by previous screens. For example, we identified that Thapsigargin acted as an autophagy inducer at the dose lower than 0.57 μ M but inhibited autophagic flux when the dose is at or above 1.7 μ M (Table 3 and Supplemental Excel File 1). Thapsigargin has been previously reported to induce autophagy through ER stress at a concentration of 0.5 μ M or inhibit autophagy by impairing autophagosomal-lysosomal fusion at a concentration of 3 μ M [21,26]. Our qHTS findings are thus in agreement with these previous seemingly paradoxical findings. Another advantage of using multiple doses in screening is that, though some of the compounds at one or two higher concentrations may induce cell death or cell growth arrest, the lower concentration are sufficient for an overall assessment of autophagy flux. Second, to our knowledge, our screen is the first to monitor autophagic flux in addition to the steady state change of GFP-LC3 puncta. Since the change of GFP-LC3 puncta is quite dynamic, increased GFP-LC3 puncta can either reflect an increase in newly synthesized autophagosomes or due to blockage at the later stage of autophagy by impairing autophagosomal-lysosomal fusion or lysosomal functions. Alternatively, a compound that decreases steady state GFP-LC3 puncta may not necessarily inhibit autophagy but may actually increase autophagy due to increased autophagic degradation of GFP-LC3 at autolysosomes. This type of compound should also be considered as an autophagy inducer but will not be identified by previous screens that did not assess autophagic flux. For instance, LE300, Labetalol hydrochloride and Fiduxosin hydrochloride did not change the number of GFP-LC3 puncta by themselves, which would be considered negative for autophagy in previous screens. However, all three compounds increased the number of GFP-LC3 puncta in the presence of CQ and the number of GFP-LC3 puncta was higher than CQ alone treatment, suggesting LE300, Labetalol hydrochloride and Fiduxosin are autophagy inducers. In contrast, (S)-Propranolol hydrochloride alone treatment increased the number of GFP-LC3 puncta, which would be considered as an autophagy inducer in steady state GFP-LC3-based screens. However, we found that in the presence of CQ, the number of GFP-LC3

puncta did not further increase by (S)-Propranolol and was not higher than CQ alone treatment. Interestingly, a previous study showed that propranolol increased lysosomal pH [27], further supporting our conclusion that (S)-Propranolol hydrochloride may be a CQ-like autophagy inhibitor. Finally, our autophagic flux screen also allows us to identify novel autophagy inhibitors that act at the step of the upstream autophagosome formation because these compound decreased the number of GFP-LC3 puncta in the presence of CQ compared with CQ alone group (Table 2). These novel autophagy inhibitors would not have been identified by previous screens, which did not assess autophagic flux.

Perhaps, one intriguing finding in our study is that we found plenty of identified modulators fall into the classes of regulators of dopamine, adrenoceptor, serotonin, and histamine; i.e. neuropharmacological reagents. Dopamine is a catecholaminergic neurotransmitter important in brain and other systems. It is mediated by five distinct G protein-coupled receptors that fall into D₁-(D₁ and D₅) and D₂-(D₂, D₃ and D₄) classes, which are pharmacologically, biochemically, and physiologically different. Dopamine receptors are broadly expressed not only in the central nervous system but also in the periphery [28]. Dopamine was reported to increase autophagosome formation in pheochromocytoma-derived PC12 cells accompanied with intracellular Zn²⁺ concentration [29]. Dopamine also inhibited mTOR and increased LC3-II conversion from LC3-I in neuroblastoma cells [30]. Furthermore, Raclopride, a D₂ dopamine receptor antagonist, has been shown to induce autophagy in cardiac myocyte in an mTOR-independent way [31]. In the present study, we found that most dopaminergic antagonists induced autophagic flux and dopaminergic agonists inhibited autophagy, but not all of them followed this trend. For example, Trifluoperidol hydrochloride is a dopaminergic antagonist but strongly inhibited autophagic flux. CPZ and FPZ that we identified from our qHTS as strong autophagy inducers are typical first generation antipsychotic medications. They are believed to work by blocking dopamine pathways in the brain, which are disturbed and hyperactive in psychoses, e.g. schizophrenia. Another image-based HTS showed that fluspirilene and trifluoperazine, two other first generation antipsychotic, together with the less typical pimozone, are also autophagy inducers [23]. Furthermore, it should also be noted while all three dopaminergic antagonists (IND, CPZ and FPZ) increased autophagic flux, IND and CPZ but not FPZ induced autophagy in an mTOR-independent manner. Thus, it is possible that some of the dopamine modulators may have off-target effects that affect their actions on autophagy in addition to modulating dopamine, which need to be studied in the future. However, in the present study we only determined the activity of mTOR in HCT116 and A549 cancer cells after IND, CPZ and FPZ treatment. Future studies are also needed to determine the activity of mTOR in normal non-transformed cells and tissues after administration of these dopamine modulators for autophagy induction.

Regardless of the mechanisms for autophagy induction or inhibition, the newly identified autophagy inhibitors and inducers from our present study will have potential therapeutic applications for preventing or treating human diseases. Autophagy regulates cellular homeostasis of proteins, lipids and organelles to protect cells against various adverse stresses and pathogenesis including neurodegenerative disease, alcoholic and non-alcoholic fatty liver disease, drug-induced tissue damage, tissue inflammation and tumorigenesis. We previously demonstrated that induction of autophagy by rapamycin protects against

acetaminophen or alcohol-induced liver injury via autophagic removal of damaged mitochondria and excess lipid droplets [11,32,33]. Indeed, we found that CPZ, the autophagy inducer identified from the present study, significantly inhibited acetaminophen-induced liver injury (data not shown). Since cancer cells can also utilize autophagy as a cell survival mechanism, it has been widely accepted that inhibition of autophagy may sensitize cancer cells to traditional chemotherapy drugs. Therefore, the newly identified autophagy inhibitors from our study may also be used for treating cancers, in particular some drug-resistant cancers, in combination with other chemotherapy drugs. In summary, our data suggest that cell-based image qHTS for autophagic flux can efficiently identify autophagy inducers and inhibitors. Future works are needed to further validate these novel autophagy modulators and their potential beneficial effects in treating alcoholic or non-alcoholic liver diseases, drug-induced tissue injury or cancer in animal models, and ultimately apply this knowledge to humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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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Fig 1.

Cell-based quantitative high throughput screen of LOPAC for autophagy modulators.

Representative GFP-LC3 images of the qHTS. MEF cells stably expressing GFP-LC3 were treated with various concentrations (0.02–46 μM) of each compound for 16 h. DMSO and CQ (10 μM) were used as negative and positive controls for the GFP-LC3 puncta formation, respectively. Arrow indicates a MEF cell assessed by ArrayScan vHCS View version 3.0.

Fig. 2.

Indatraline, chlorpromazine and fluphenazine induce autophagic flux in GFP-LC3 MEF. (A) Representative images of GFP-LC3 MEF cells treated with IND (5 μM), CPZ (15 μM) or FPZ (15 μM) in the absence or presence of CQ (2.5 μM) from the qHTS. Hoechst33342 was used to stain the nuclei. (B) GFP-LC3 MEF cells were treated with different concentrations of IND, CPZ and FPZ for 6 h. (C) GFP-LC3 MEF cells were treated with IND (3 μM), CPZ (20 μM) and FPZ (20 μM) in the presence or absence of CQ (20 μM) for 6 h or (D) 18 h. Total cell lysates were subjected to immunoblot analysis using an anti-GFP antibody.

GAPDH or β -actin was used as a protein loading control. Representative blots from three independent experiments are shown in the panels.

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Fig. 3.

Indatraline, chlorpromazine and fluphenazine induce autophagy in HCT116. (A) HCT116 cells were treated with different concentrations of IND, CPZ and FPZ for 6 h or (B) IND (3 μM), CPZ (20 μM) and FPZ (20 μM) for different time point. (C) HCT116 cells were treated with IND (3 μM), CPZ (20 μM) and FPZ (20 μM) in the presence or absence of CQ (20 μM) for 6 h. Total cell lysates were subjected to immunoblot analysis. β-actin was used as a protein loading control. Representative blots are shown in the panels. (D) Densitometry of (C). Data (mean ± SEM) are representative of three independent experiments.

Fig. 4.

Indatraline, chlorpromazine and fluphenazine increase the number of autophagic vacuoles.

(A) HCT116 cells were treated with IND (3 μM), CPZ (20 μM), and FPZ (20 μM) for 6 h and processed for EM analysis. N, nucleus; empty arrows: early AVs; solid arrows: late AVs.

(B) The number of AVs was quantified and normalized based on the cytosolic areas measured using the SPOT software. More than 20 cells were analyzed in each condition. (* = $p < 0.05$, *** = $p < 0.001$, Student t test.).

Fig. 5.

Indatraline, chlorpromazine and fluphenazine increase autophagic flux in A549 cells. (A) A549 cells were infected with mCherry-GFP-LC3 adenovirus (10 moi) for 24 h. Cells were then treated with IND, CPZ, and FPZ of different concentrations in the absence or presence of CQ (20 μ M) for 6 h and fixed for fluorescence microscopy analysis. Yellow arrows denote autophagosomes; white arrows denote autolysosomes. (B) The number of yellow LC3 dots and red LC3 dots per cell in each condition was quantified. Total LC3 dots are the sum of the number of yellow LC3 dots with red LC3 dots. More than 20 cells were counted in each

condition and data (mean $\pm$ SEM) are from three independent experiments (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, One Way Anova Analysis).

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Fig. 6.

Fluphenazine but not Indatraline and chlorpromazine inhibits mTOR. HCT116 (A) and A549 (B) cells were treated with IND (3 μ M), CPZ (20 μ M), and FPZ (20 μ M) for 3 and 6 h. (C) HCT116 cells were treated with different concentrations of IND, CPZ and FPZ for 6 h. Total cell lysate was subjected to immunoblot analysis. Torin treatment (250 nM) for 6 h was used as positive control. β -actin was used as a protein loading control. Representative blots from three independent experiments are shown in the panels.

Fig. 7.

Chlorpromazine and fluphenazine but not indatraline induce apoptosis. (A) HCT116 cells were treated with IND, CPZ or FPZ at different concentrations for 6 h. Cell viability was measured by MTT assay. Data are presented as means $\pm$ SEM ($n = 3$ independent experiments). (** = $p < 0.01$, *** = $p < 0.001$, One Way Anova Analysis). (B) HCT116 cells were treated with IND (3 μ M), CPZ (20 μ M) or FPZ (20 μ M) for 24 h. Cells were stained with Hoescht 33342 and PI followed by fluorescence microscopy. Representative images are shown. Arrows denote apoptotic cells. (C) Cells with apoptotic nuclei were quantified from

three independent experiments. Data are presented as means $\pm$ SEM (n = 3 independent experiments) (***) = $p < 0.001$, One Way Anova Analysis). (D) HCT116 cells were treated with IND, CPZ and FPZ at different concentrations for 24 h or (E) treated with CPZ (20 μ M) or FPZ (20 μ M) for different time points. Total protein lysates (20 μ g) were subjected to caspase 3 activity assay. Data are presented as means $\pm$ SEM (n = 3 independent experiments). (** = $p < 0.01$, *** = $p < 0.001$, One Way Anova Analysis).

Fig. 8.

CQ does not protect against but slightly exacerbates chlorpromazine and fluphenazine-induced cell death. (A) HCT116 and (B) GFP-LC3 MEF were treated with IND, CPZ and FPZ at different concentrations in the presence or absence of CQ (20 μM) for 24 h. Cell viability was measured by MTT assay. Data are presented as means $\pm$ SEM ($n = 3$ independent experiments) (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ vs control, + = $p < 0.05$, ++ = $p < 0.01$, +++ = $p < 0.001$ vs CQ, One Way Anova Analysis).

Table 1

Potential autophagy inducers identified from the screen (Category 1).

Name	AC50	Class	Action	Selectivity	Description
Indatraline hydrochloride	2.91	Dopamine	Antagonist	D ₂	D ₂ dopamine receptor antagonist; sigma receptor agonist; phenothiazine antipsychotic
Perphenazine	4.61	Dopamine	Inhibitor	Reuptake	Serotonin reuptake inhibitor; antidepressant
Methiothepin mesylate	11.58	Serotonin	Antagonist	5-HT _{1E} , 5-HT _{1F} , 5-HT ₆	5-HT ₁ Serotonin receptor antagonist; blocks serotonin autoreceptors
Prochlorperazine dimaleate	12.99	Dopamine	Antagonist		Antipsychotic agent; used in the treatment of spastic gastrointestinal disorders
N-Methylhistaprodifen dioxalate salt	14.58	Histamine	Agonist	H ₁	H ₁ histamine receptor agonist
N-Desmethyloclozapine	16.35	Cholinergic	Antagonist	5-HT ₂ Serotonin/M ₁ Muscarinic	Major metabolite of clozapine; potent 5-HT ₂ serotonin receptor antagonist and a ligand for the cloned 5-HT ₆ and 5-HT ₇ serotonin receptors. Also an allosteric potentiator of M ₁ muscarinic receptors
Zimeldine dihydrochloride	18.35	Serotonin	Inhibitor	Reuptake	Serotonin reuptake inhibitor; antidepressant
Paroxetine hydrochloride hemihydrate (MW = 374.83)	18.35	Serotonin	Inhibitor	Reuptake	Selective serotonin reuptake inhibitor; antidepressant Sold with permission of GlaxoSmithKline. Actual molecular weight = 374.83 to account for 1/2 mol of water
BW 723C86	18.35	Serotonin	Agonist	5-HT _{2B}	5-HT _{2B} serotonin receptor agonist
Clemastine fumarate	18.35	Histamine	Antagonist	H ₁	H ₁ Histamine receptor antagonist
Promethazine hydrochloride	18.35	Histamine	Antagonist	H ₁	H ₁ Histamine receptor antagonist; anticholinergic
Chlorpromazine hydrochloride	20.59	Dopamine	Antagonist		Dopamine receptor antagonist; anti-emetic; antipsychotic
Propionylpromazine hydrochloride	20.59	Dopamine	Antagonist	D ₂	Selective D ₂ dopamine receptor antagonist
L-741,626	20.59	Dopamine	Inhibitor	Uptake	Adenosine uptake inhibitor
Fluphenazine dihydrochloride	20.59	Dopamine	Antagonist	D ₁ /D ₂	Dopamine receptor antagonist; antipsychotic
Cyclobenzaprine hydrochloride	20.59	Serotonin	Antagonist	5-HT ₂	5-HT ₂ serotonin receptor antagonist
Doxazosin mesylate	20.59	Adrenoceptor	Blocker	alpha1	alpha1 adrenoceptor blocker
Labetalol hydrochloride	20.59	Adrenoceptor	Antagonist	beta	Competitive beta-adrenoceptor antagonist
Fluspirilene	23.10	Dopamine	Antagonist	D ₂ /D ₁	Dopamine receptor antagonist; antipsychotic

Name	AC50	Class	Action	Selectivity	Description
Dilazep hydrochloride	23.10	Adenosine	Inhibitor	Uptake	Adenosine uptake inhibitor
Chlorprothixene hydrochloride	25.92	Dopamine	Antagonist	D ₂	D ₂ dopamine receptor antagonist; blocks a subset of GABA-A receptors in rat cortex that is also blocked by clozapine
5-(N-Ethyl-N-isopropyl) amiloride	29.08	Ion Pump	Blocker	Na ⁺ /H ⁺ Antiporter	Selective blocker of Na ⁺ /H ⁺ antiport
LE 300	29.08	Dopamine	Antagonist	D ₁	Potent, selective D ₁ dopamine receptor antagonist
Dihydroergocristine methanesulfonate	29.08	Serotonin	Antagonist		Ergot alkaloid; vasoconstrictor; competitive serotonin receptor antagonist; partial agonist at alpha adrenoceptors and D ₂ dopamine receptors
BRL 52537 hydrochloride	29.08	Neurotransmission	Agonist	kappa/mu opioid	Kappa/mu opioid receptor agonist
Fiduxosin hydrochloride	29.08	Adrenoceptor	Antagonist	alpha1	alpha1-Adrenoceptor antagonist
Trifluoperazine dihydrochloride	29.08	Dopamine	Antagonist	D ₁ /D ₂	Calmodulin antagonist; dopamine receptor antagonist; antipsychotic; sedative

Table 2

Potential autophagy inhibitors identified from the screen (Category 2).

Name	AC50	Class	Action	Selectivity	Description
Upstream inhibitors					
Ellipticine	9.20	Cell Cycle	Inhibitor	CYP1A1/TopoII	Cytochrome P450 (CYP1A1) and DNA topoisomerase II inhibitor
Quinidine sulfate	20.59	Na ⁺ Channel	Antagonist		Na ⁺ channel blocker and Class I antiarrhythmic; alkaloid isolated from the bark of the Cinchona family of South American trees
GBR-12935 dihydrochloride	20.59	Dopamine	Inhibitor	Reuptake	Dopamine reuptake inhibitor
Ivermectin	20.59	Cholinergic	Modulator	alpha7 nACh	Positive allosteric modulator of alpha7 neuronal nicotinic acetylcholine receptor; also modulates glutamate-GABA-activated chloride channels
Prazosin hydrochloride	23.10	Adrenoceptor	Antagonist	alpha1	Peripheral alpha1 adrenoceptor antagonist
Cyproheptadine hydrochloride	23.10	Serotonin	Antagonist	5-HT ₂	5-HT ₂ serotonin receptor antagonist
Amoxapine	23.10	Adrenoceptor	Inhibitor	Uptake	Tricyclic antidepressant; inhibits neuronal uptake of norepinephrine
GR 46611	25.92	Serotonin	Agonist	5-HT _{1D}	5-HT _{1D} serotonin receptor agonist.
R(-)-N-Allylnorapomorphine hydrobromide	25.92	Dopamine	Agonist		Dopamine receptor agonist
GBR-12909 dihydrochloride	29.08	Dopamine	Inhibitor	Reuptake	Selective dopamine reuptake inhibitor
R(-)-2,10,11-Trihydroxy-N-propylincaporphine hydrobromide	29.08	Dopamine	Agonist	D ₂	Potent and selective D ₂ dopamine receptor agonist
Trifluoperidol hydrochloride	29.08	Dopamine	Antagonist	D ₁ /D ₂	Dopamine receptor antagonist; antipsychotic
SKE-525A hydrochloride	29.08	Multi-Drug Resistance	Inhibitor	Microsomal oxidation	Inhibitor of microsomal drug metabolism
Miscellaneous inhibitors					
Emetine dihydrochloride hydrate	4.11	Apoptosis	Activator		Apoptosis inducer; RNA-protein translation inhibitor
(S)-Propranolol hydrochloride	18.35	Adrenoceptor	Antagonist	beta	beta Adrenoceptor antagonist; cardiac depressant (anti-arrhythmic)
CGS-12066A maleate	20.59	Serotonin	Agonist	5-HT _{1B}	5-HT _{1B} Serotonin receptor agonist
S-(-)-Eliclopride hydrochloride	23.10	Dopamine	Antagonist	D ₂	Potent and selective D ₂ dopamine receptor antagonist

Table 3

Compounds have dual effects on autophagy identified from the screen (Category 3).

Name	Class	Action	Selectivity	Description
High dose: inducers; Low dose: inhibitors				
AC-93253 iodide	Hormone	Agonist	RAR(a)	Potent, cell permeable, subtype selective retinoic acid receptor (RARalpha) agonist
Spiperone hydrochloride	Dopamine	Antagonist	D ₂	Selective D ₂ dopamine receptor antagonist
Maprotiline hydrochloride	Adrenoceptor	Inhibitor	Reuptake	Selective norepinephrine reuptake inhibitor
Clozapine	Dopamine	Agonist	H ₁	H ₁ histamine receptor agonist
Vinblastine sulfate salt	Cytoskeleton and ECM	Antagonist		Antipsychotic agent; used in the treatment of spastic gastrointestinal disorders
High dose: inhibitors; Low dose: inducers				
Idarubicin	DNA Metabolism	Inhibitor		Antineoplastic
ST-148	Dopamine	Antagonist	D ₂	D ₂ dopamine receptor antagonist
Thapsigargin	Intracellular Calcium	Releaser		Potent, cell-permeable, IP3-independent intracellular calcium releaser