Primary high throughput screening for modulators of autophagy as drugs for cancer treatment using the powerful imaging reagent CalRexin™:pHrodo™ Red.

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Background

- **Autophagy** is an essential catabolic state for survival and a mechanism for the return to homeostasis of cells under stress.
- For pathological conditions, such as cancer, neuro-degenerative diseases and cardio-vascular diseases, in which autophagy plays a central role, the discovery of candidate drugs that modulate autophagy is of paramount importance.
- **In cancer cells, activation of autophagy confers drug resistance**. For instance, chloroquine (CQ) as an inhibitor of the fusion of lysosomes and autophagosomes, an essential event in autophagy, have been tested in clinical trials for treatment of glioblastoma and melanoma, and are effective in mouse models of colorectal adenocarcinoma.
- **Much contemporary cancer research focuses on drug discovery including rapamycin mimetics** in cancer cells [1].
- Here we describe a new technology (CalRexin™:pHrodo™ Red) which binds human calcitonin receptor (**CT Receptor**) to detect autophagy on the high throughput platform Operetta, which will provide opportunities for the discovery of new or repurposed drugs for cancer treatments [2,3].

Aim

The aim of this study is the demonstration and validation of uptake of CalRexin™:pHrodo™ Red by autophagic cells, imaged in real time on the Operetta high content screening platform.

Methods

A mouse monoclonal anti-human CT Receptor antibody (mAb2C4, CalRexin™, www.apopbiosciences.com) that binds an epitope within the extracellular domain was developed and conjugated with pHrodo™ Red (Thermo Fisher Scientific).Operetta (CLS, Perkin Elmer) high content screening system: sHeLa cells or mutant strains were seeded into 96-well plates, 10,000 per well, cultured o/n on Matrigel and the imaging experiments were initiated with addition of 8 µM rapamycin/5% FBS plus 4 µg/mL CalRexin™:pHrodo™ Red for 24 hours at 37 °C/5 % CO_2, conditions chosen to induce autophagy. Nuclei were identified with Hoechst 33342. Images were captured (40X high NA objective) for each well every hour. The uptake was also studied in knockout mutant strains *Atg5* and *FIP200* with rapamycin. Inhibition of autophagy and uptake of CalRexin™:pHrodo™ Red was also measured with 50 µM chloroquine or 10 mM 3-methyladenine. Time-lapse videos were created and annotated using FIJI image J software, version NIH, 1.53r.

References

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Declaration of interest: PJW is an executive directors of Apop Biosciences and a major shareholder

Results

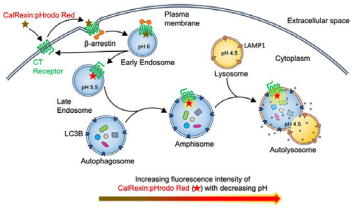

Figure 1: Schematic which represents the passage of CalRexin™:pHrodo™ Red bound to CT Receptor on the plasma membrane and is internalized through the early endosomes, late endosomes (pH 5.5) which fuse with the autophagosomes to form the amphisomes. The latter fuse with the lysosomes to form the autolysosomes (pH 4-5), positive for LysoTracker™ Green.

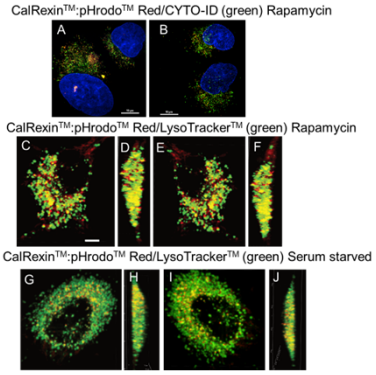

Figure 2. High resolution images of live sHeLa cells. Panels A & B, single plane images of sHeLa cells treated with rapamycin for 24 hours and imaged with CalRexin™:pHrodo™ Red and CYTO®-ID (green, phagophores & auto-phagosomes) and co-localization (yellow). Panels C-J are imaged with CalRexin™:pHrodo™ Red and LysoTracker™ (green, lysosomes & autolysosomes) and co-localization (yellow). Panels C-F, treated with rapa-mycin; Panels G-J, serum starved. The calibration bar in panel A is 10 µM in panels C, for C-J, is 5 µM.

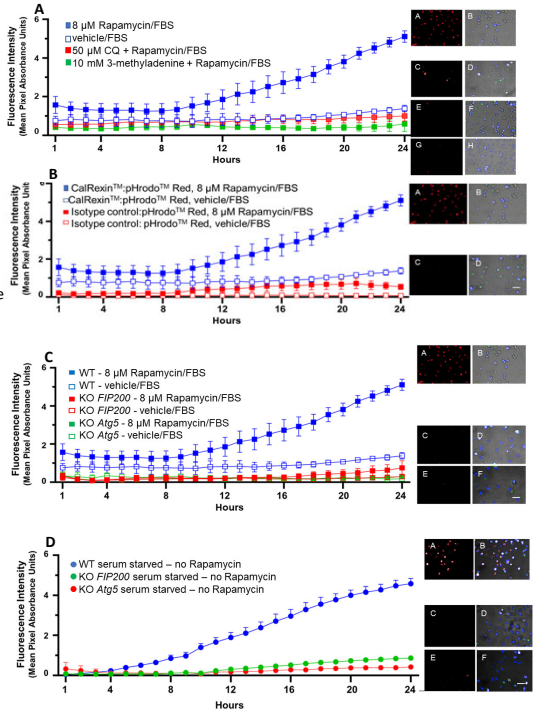

Figure 3: time dependent changes in Fluorescence Intensity (FI) measured on the Operetta: A. Accumulation of CalRexin™:pHrodo™ Red by sHeLa wildtype cells stimulated by 8 µM rapamycin compared to vehicle and inhibited by chloroquine or 3-methyl adenine. B. CalRexin™:pHrodo™ Red compared isotype IgG1: pHrodo™ Red. C. sHeLa WT compared to the knockout mutant strains *Atg5* & *FIP200*, all treated with 8 µM rapamycin. D. Autophagy induced with serum starvation, WT versus knock out *Atg5* & *FIP200*.

Conclusions

1. The activation of the CT Receptor has previously been reported associated with pre-apoptotic cell stress (p-ACS). CalRexin™:568 has been demonstrated to identify p-ACS and apoptosis in live cell populations using cell sorting (FACS) and confocal microscopy [4].
2. In this study it is demonstrated that CalRexin™ pHrodo™ Red, a pH sensitive fluorescent probe is accumulated into live, autophagic cells in real time, induced with rapamycin or starvation on the Operetta high content screening platform. This was tested in several cell lines including sHeLa cells, MG63 cells and NIH 3T3 cells (data not shown).
3. **Validation**: Inhibitors of autophagy 3-methyladenine that inhibits phagosome elongation via IP3 kinase and chloroquine that inhibits the fusion of lysosomes with autophagosome to form the autolysosomes, both inhibit accumulation of CalRexin™:pHrodo™ Red. Genetic knockout strains *Atg5* & *FIP200*, unable to form autophagosomes, do not accumulate CalRexin™:pHrodo™ Red in the presence of rapamycin. In rapamycin induced sHeLa cells accumulation of the isotype IgG1:pHrodo™ Red is negative.
4. **Our hypothesis** (Figure 1) is that CalRexin™:pHrodo™ Red is accumulated through the endosomes (red, pH 5.5, Figure 2), amphisomes and emits the brightest FI in the low pH (4-5) milieu of the autolysosomes (co-localization with LysoTracker™ Green shown in yellow).
5. Here we describe the utility of CalRexin™:pHrodo™ Red as a novel tool to image autophagy in live cell assays for primary high content screening of candidate drugs that stimulate or inhibit autophagy. **These drugs have potential for the treatment serious diseases including cancers**.