Utilisation of NanoBiT™ Luciferase (Promega) to examine Autophagy: Validation against other technologies

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Introduction

Autophagy is an evolutionarily conserved lysosomal process involved in the maintenance of cellular homeostasis. It is responsible for the turnover of long-lived proteins, misfolded proteins and organelles that are either damaged or functionally redundant. Classically translated from Greek it means "self-devouring". It is a dynamic process as shown in Fig.1. below.

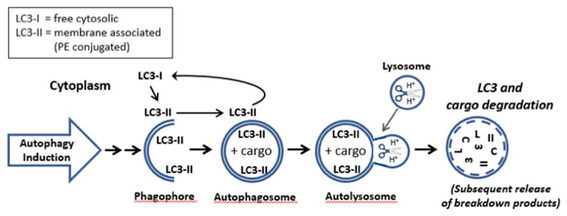


Fig. 1. Simplistic representation of autophagic flux, including LC3 processing

There is a growing understanding that dysregulation of autophagy is implicated in a number of human diseases including cancer, muscular and neurodegenerative diseases. Autophagy inhibitors are chemo and radio sensitisers and could be useful in combination therapy approaches.

To date, LC3 (ATg8) is considered as the most persistent marker of the autophagy pathway. Many of the screening approaches to investigate autophagy have used high content imaging. However, a sensitive homogeneous method would be more amenable to high throughput screening.

In this poster we have evaluated a cell line expressing tagged LC3B. In this autophagy reporter human LC3B is tagged with HiBiT (11 amino acid peptide) possessing high affinity (Kd~1nM) for LgBiT. LgBiT and HiBiT are subunits of NanoBiT™ luciferase (Promega). This is a protein fragment complementation assay (PCFA). To validate the response to known autophagy inhibitors and inducers within this cell line, we examined the pharmacology of these compounds using the luciferase readout together with other readouts including high content imaging, Western blotting and AlphaLISA™.

Materials and Methods

NanoBiT™ Autophagy reporter cell line (Promega)

HEK cell line stably expressing the NanoBiT™ autophagy reporter are plated in either 96 (10K/well) or 384 well (2.5K/well) solid white plates and incubated overnight prior to the addition of autophagy inhibitors or inducers. Cells are treated with compounds for 24hrs. The lysis reagent containing the LgBiT subunit and the NanoLuc™ substrate is added to the cells at room temperature and the plate is briefly placed on a shaker then incubated for 10mins prior to measuring luminescence (0.2sec/well) on a luminometer plate reader.

AlphaLISA™ LC3B assay (PerkinElmer)

Adherent cells are incubated with the autophagy inhibitors or inducers overnight. Media is removed and the cells are lysed in 10ul of lysis buffer for 10 mins on a shaker. The acceptor beads and biotinylated antibody are added and incubated with lysate for 60 mins prior to addition of the donor bead. The signal is measured after 1hr on an EnSpire™ plate reader.

High content imaging

Adherent cells were treated with autophagy inhibitors and inducers overnight. The next day cells were fixed with 3% formaldehyde and permeabilised (0.2% Triton X-100).

The rabbit polyclonal LC3B antibody from Life Technologies (L10382) was added for 1 hour followed by the donkey anti-rabbit secondary (Alexa647) and Hoescht nuclear stain. Cells were washed in PBS and then imaged on the CellInsight™ CX5 high content imager (Thermo) on 20X magnification.

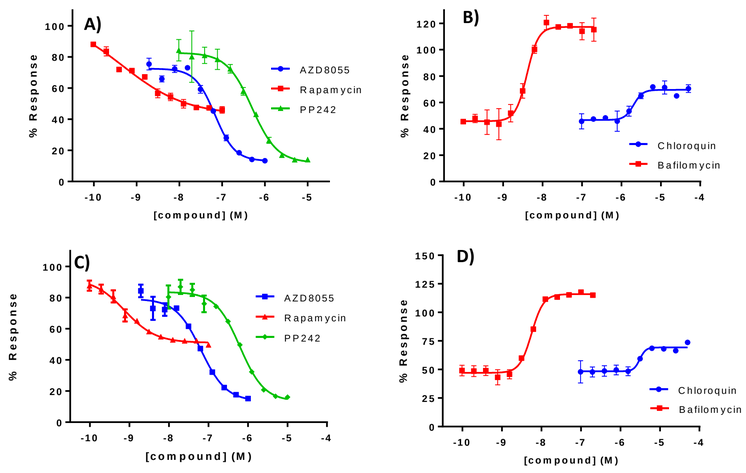
Wes™ (protein simple)

Adherent cells treated with autophagy compounds overnight were lysed in RIPA buffer. Protein was quantified using the BCA. Samples were loaded on the Wes™ at 1mg/ml. The rabbit polyclonal LC3B antibody (L10382) was used at 1:100 dilution.

NanoBiT™ Autophagy reporter cell line (Promega)

In this autophagy reporter, human LC3B is tagged with HiBiT (11 amino acid peptide) possessing high affinity (Kd~1nM) for LgBiT. LgBiT and HiBiT are subunits of NanoBiT™ luciferase (Promega), see Fig.3. The NanoBiT™ luciferase assay is a plate based homogeneous assay with Glo kinetics making it amenable to high throughput screening. It is suitable for the discrimination of both autophagy inhibitors and inducers. The assay was compared in 96 and 384 well format with standard compounds.

The basal level of LC3B is expressed as the 100% response. Autophagy inducers AZD8055 and PP242 gave full dose dependent decrease in response indicating a decrease in the amount of LC3B in the cells. Whilst Rapamycin gave a shallower curve and a smaller signal reduction. The data in 96 well format was identical to that in 384 well format (Fig 2A &2C). Autophagy inhibitors were tested in the presence of PP242 in order to reduce the basal response and achieve an improved dynamic range for the assay. Both Chloroquin and Bafilomycin gave a dose dependent increase in response indicating an increase in the amount of LC3B in the cells, with identical XC50s in both 96 and 384 well format (Fig. 2B &2D). A larger response was observed for Bafilomycin than Chloroquin.



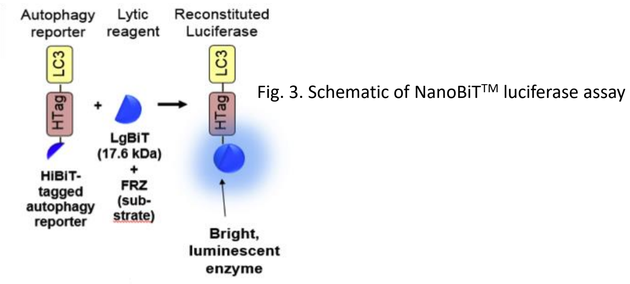


Fig. 3. Schematic of NanoBiT™ luciferase assay

E)

	384 well XC50	96 well XC50
Chloroquin	3.08 uM	2.04uM
Bafilomycin	5.6nM	4.0nM
AZD8055	64.8nM	68.2nM
Rapamycin	0.76nM	0.48nM
PP242	625nM	502nM

Fig. 2 NanoBiT™ luciferase assay dose response curves in 96 well for A) autophagy inducers; B) autophagy inhibitors . Dose response curves in 384 well for C) autophagy inducers; D) autophagy inhibitors . E) Summary table of XC50 values

AlphaLISA™ LC3B assay (PerkinElmer)

In this assay LC3B in cell lysate interacts with the paired anti-LC3B antibodies on the donor and acceptor beads bringing them into close proximity. The excitation of the donor beads triggers a cascade of energy transfer in the acceptor beads, resulting in light emission at 615 nm (Fig. 4).

The autophagy inhibitors Chloroquin and Bafilomycin both caused a dose dependent accumulation of LC3B in the cells. Chloroquin generated a larger AlphaLISA™ signal than Bafilomycin indicating a greater level of LC3B accumulation (Fig. 5A). This was reversed in the NanoBiT™ luciferase assay. Autophagy inducers AZD8055 and PP242 caused a small dose dependent decrease in the amount of LC3B below the basal level. However, Rapamycin gave a very small and variable response (Fig. 5B). There was excellent correlation in the XC50 values in this assay compared to the NanoBiT™ luciferase assay (Fig. 5C &2E).

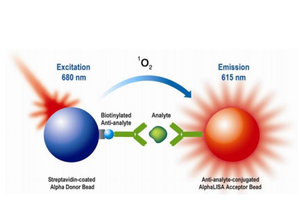
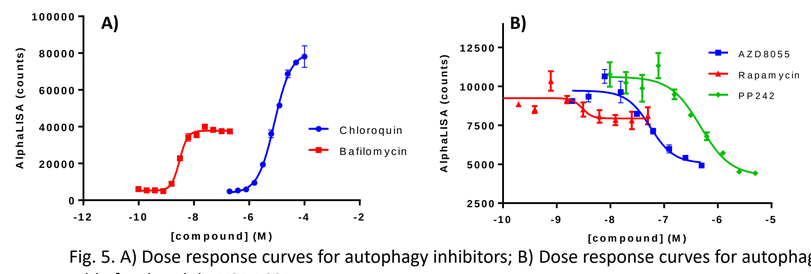


Fig. 4. Schematic of AlphaLISA assay



C)

	AlphaLISA XC50
Chloroquin	8.2uM
Bafilomycin	3.0nM
AZD8055	55.1nM
Rapamycin	--
PP242	475nM

Fig. 5. A) Dose response curves for autophagy inhibitors; B) Dose response curves for autophagy inducers and C) Summary table for the AlphaLISA LC3B assay

High Content Imaging and Western Blot Data

HEK cells expressing the HiBiT tagged LC3B were treated with compound in the same manner as for the NanoBiT™ luciferase assay. In Fig.6 the LC3B is detected as a 19kDa band by Wes™. The autophagy inducers AZD8055, PP242 and Rapamycin clearly reduced the amount of LC3B detected whilst the inhibitors Chloroquin and Bafilomycin significantly increased the amount of LC3B.

In Fig. 7 the vehicle control cells showed a diffuse cytoplasmic staining of LC3B. Treatment with AZD8055 to induce autophagy clearly shows a marked absence of LC3B staining indicating turnover of the protein. In contrast Chloroquin treatment shows a punctate staining pattern of LC3B as a result of accumulation of protein in autophagosomes.

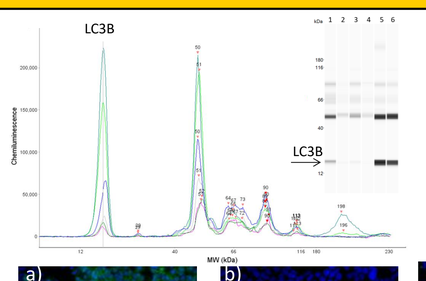


Fig. 6. HEK cell lysates probed with anti-LC3B in Wes™. Lane 1-DMSO; L2-PP242 ; L3- AZD8055; L4-Rapamycin ; L5-Chloroquine and L6-Bafilomycin

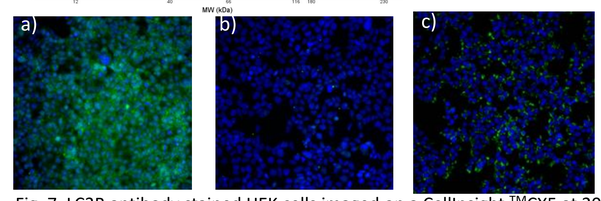


Fig. 7. LC3B antibody stained HEK cells imaged on a CellInsight ™CX5 at 20x magnification. A) DMSO treated HEK cells, b) Induction of autophagy with AZD8055 0.1uM and c) inhibition of autophagy with Chloroquin 25uM

Summary and Conclusion

- All of the methodologies were able to measure LC3B and changes in protein level caused by inducers e.g. PP242, AZD8055 and inhibitors e.g. Chloroquin, Bafilomycin in the NanoBiT™ Autophagy reporter cell line
- The NanoBiT™ luciferase assay is homogeneous and simple to perform
- Identical XC50 values were obtained in 96 and 384 well formats
- The luminescent readout offers a sensitive and stable signal (Glo substrate) suitable for high throughput assays

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