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Potential for Synergistic Effect of Proprietary Autophagy Inducing Molecules in the Treatment of Tuberculosis

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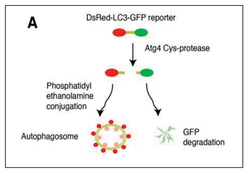
Introduction / Motivation

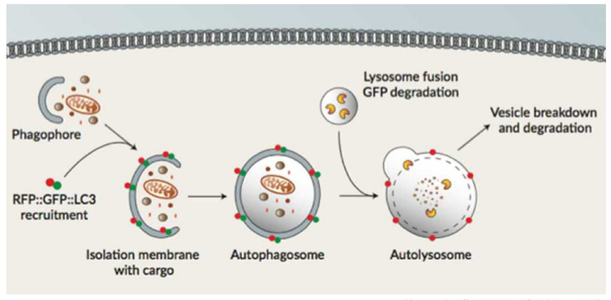
- Our goal is to demonstrate autophagy stimulation as a useful adjunct to antibiotic therapy in mycobacterial infections with the potential to limit drug resistant strains.
- Previous studies[1] have displayed autophagy as having potential for therapeutic host-targeted control of mycobacterial infections through autolysosomal killing. This would limit the generation of antimicrobial peptides and potentially dangerous inflammation.
- This study focuses on single cell quantification of the area, intensity and number of GFP/RFP puncta per individual cell. Previous studies have quantified the entire image where high background intensity hinders the accuracy and cell-to-cell differences are undetectable.

[1] Bradfute, S. B., et al. (2013). "Autophagy as an immune effector against tuberculosis." Current Opinion in Microbiology 16(3): 355-365.

We have used an engineered HeLa DsRed-LC3-GFP autophagy reporter cell line to quantify drug-induced autophagy induction→ fluorescence change



Increase in RFP with a concurrent decrease in GFP



(A) Sheen, Joon-Ho, Roberto Zoncu, Dohoon Kim, and David M. Sabatini. "Defective Regulation of Autophagy upon Leucine Deprivation Reveals a Targetable Liability of Human Melanoma Cells In Vitro and In Vivo." Cancer Cell 19.5 (2011): 613-28. Web.

(B) InvivoGen https://www.invivogen.com/autophagy-reporter-cells

Approach

Confocal fluorescence imaging and a HeLa cell line engineered to express an autophagy marker was utilized to quantify the effectiveness of drugs that stimulate autophagy. We then tested the efficacy of a multimodal-treatment strategy consisting of a drug and a TB antibiotic in RAW 264.7 cells infected with *M. bovis*, a TB surrogate.

Measuring Autophagy Induction in Single Cells

- HeLa RGL1 cells were cultured in DMEM-10 with 500 ug/ml G418 antibiotic at 37˚C and 5% CO_2.
- Autophagy stimulating drug (2uM of niclosamide, rapamycin, flubendazol, bromhexine HCl or 15uM 104 and 105) was added, incubated 4 hrs.
- Images were collected at 4-6 hours post addition of drug using an inverted fluorescence microscope (Leica DMi8 DLS).

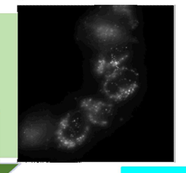
Testing Efficacy using a TB Surrogate

- RAW 264.7 (mouse macrophage) cells were infected with *M. bovis* at ~MOI10 for 1 hour at 37˚ C and 5% CO_2.
- 2uM/ 15uM of autophagy stimulating drug and/or antibiotics was added, incubated 4 hrs.
- Cells were lysed using 0.1% Triton X-100 and mycobacterium collected from the lysate using centrifugation.
- *M. bovis* was washed, diluted, and plated onto Middlebrook 7H9 agar plates.
- Colonies were allowed to grow at 37°C for 10-14 days.
- CFU count was used to determine the efficacy of the drug/antibiotic combination.

Single-Cell Analysis of Autophagy Stimulating Drugs

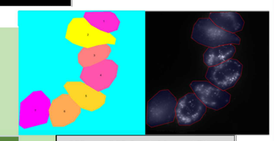
Step 1: Merge and Flatten Tiffs

In-house written software to merge and flatten the 14-stack tiffs to easily identify cells in the image.



Step 2: Segment Image to Identify Individual Cells

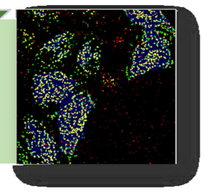
Utilize in-house written software, CellFinder, to identify the outline of individual cells in the images.



GFP and RFP Stack Merged File in Cell Finder

Step 3: Identify and Quantify Puncta in Both GFP and RFP Channels

In-house written software, BatchBiophagyCell, quantifies the number, intensity, and area of the puncta in each channel.



- **Goal:** Identify number, area, and intensity of RFP and GFP puncta in individual cells

- Previous studies[1] have measured autophagy induction by taking the average intensity over an entire image. The background intensity from multiple cells is a hindering variable producing less accuracy. 14 cell-based metrics were calculated and 2 shown here to compare autophagy:
  - RFP:GFP average puncta intensity ratio
  - RFP:GFP average puncta area ratio

[1] Chauhan S, et. al. (2015) Pharmaceutical screen identifies novel target processes for activation of autophagy with a broad translational potential. Nature Communications 6, doi:10.1038/ncomms9620

Results

Autophagy Induction in Single Cells

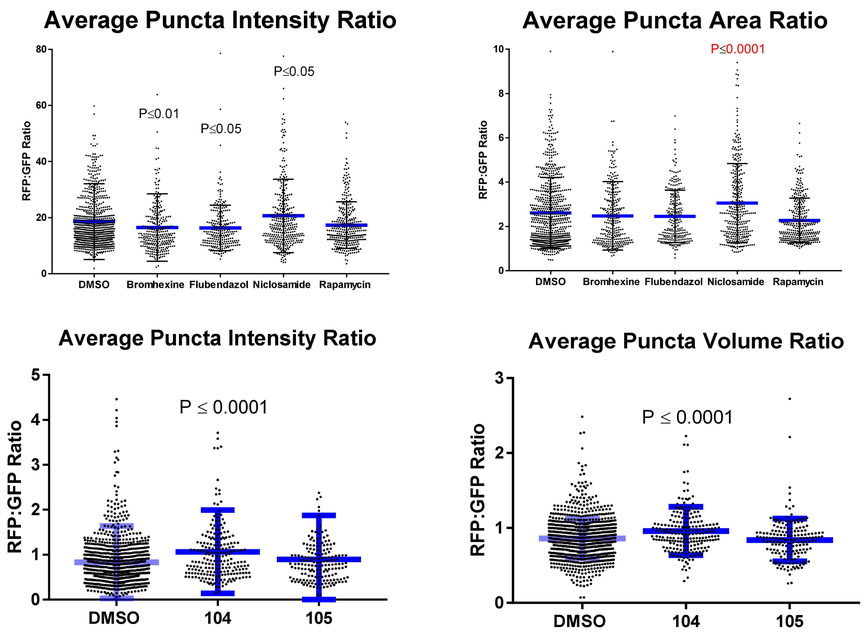
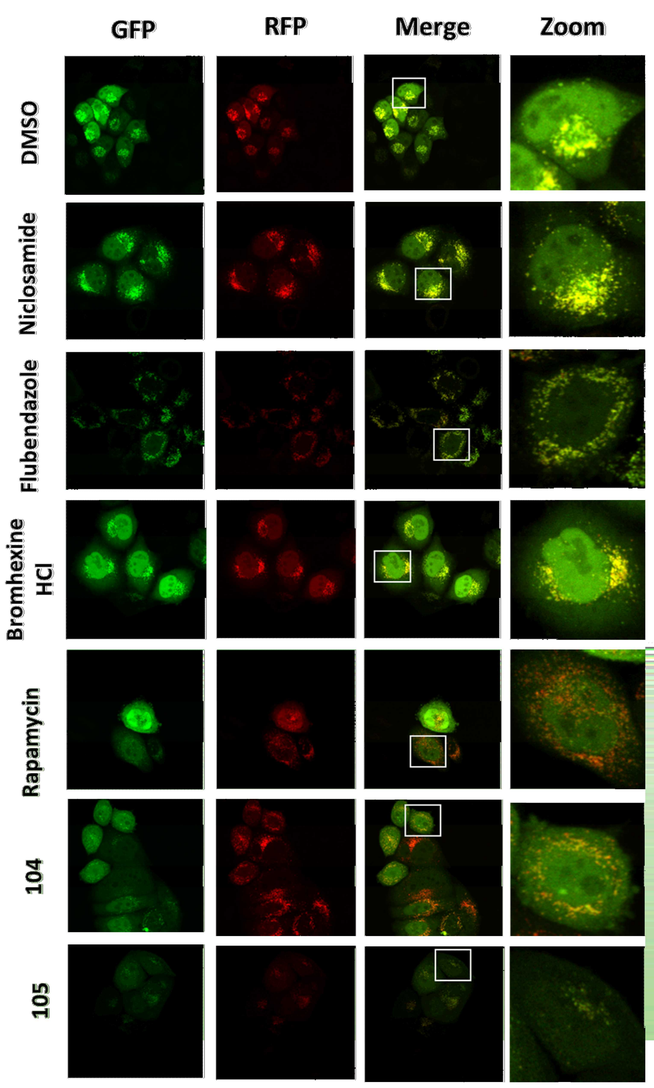


Figure 1: Top three metrics of approved drugs to determine efficiency of autophagy induction based upon single cell confocal fluorescence microscopy. Single cell analysis was completed on a 14-slice confocal stack, where each drug condition has >250 cells. Statistical significance was determined by Mann-Whitney test followed by a Dunn's multiple comparison test to compare conditions to DMSO.

4hr. Drug Treatment



Efficacy in a TB Surrogate

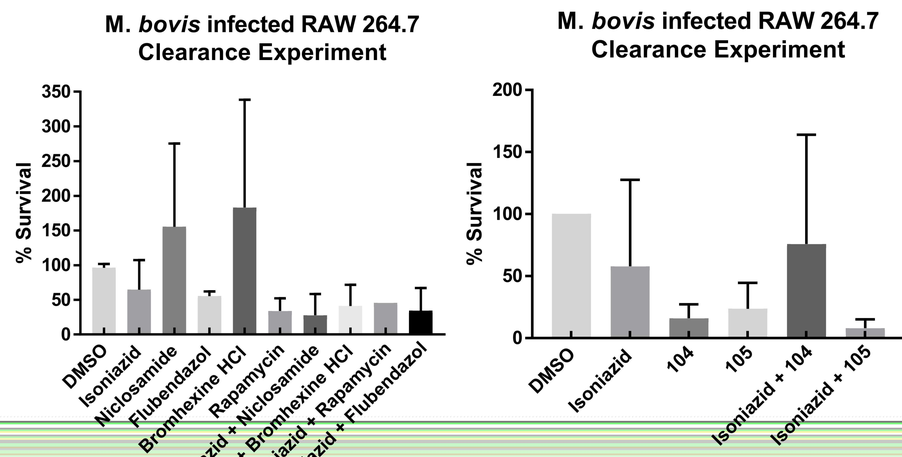


Fig. 2. RAW 264.7 infected with *M. bovis* to determine killing synergy based upon a multi-modal drug combination. Percent survival of *M. bovis* in each replication. In replication 1, both niclosamide and bromhexine displayed >100% survival which could be attributed to *M. bovis* aggerates that form resulting in an uneven distribution in the CFU assay. In replication 1, Rapamycin was uncountable due to contamination. 104 and 105 autophagy drugs were completed in a separate experiment.

Overall Conclusions

- 2uM niclosamide and 15uM 105 in combination with 0.4ug/mL isoniazid was the most effective followed by flubendazol, bromhexine, rapamycin, and 104 in combination with isoniazid in enhanced killing of *M. bovis*.
- 2uM niclosamide and 15uM 104 was the most effective at inducing autophagy based upon RFP:GFP ratio of average puncta area ratio and average puncta intensity. Followed by bromhexine, flubendazole, 105, and rapamycin.

Future Work / Partnerships

1) Build on this model to characterize a set of new, proprietary antibiotics and autophagy stimulants. We will look at:
   - Autophagy stimulation efficacy
   - Antibiotic potency vs. isoniazid
   - Potential synergy of autophagy stimulants with isoniazid or the new antibiotics
2) Identify potential drug binding sites and interactions within a human cell line.

Biophagy aVISA PureInfo Discovery

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