Torin-1-Mediated Inhibition of Viability and Locomotion in *Naegleria gruberi*

Aqsa Raja[1], Miki Watanabe[2], Hunter Heinlein[3], Lindsey Romick-Rosendale[2], Yoshi Odaka[1]

[1] University of Cincinnati Blue Ash College, Department of Biology
[2] Cincinnati Children's Hospital, Division of Pathology
[3] University of Cincinnati, Department of Biological Sciences

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ABSTRACT

Naegleria fowleri is a pathogenic unicellular protist of free-living ameba commonly found in warm freshwater and undergoes three stages of the life cycle, amebic trophozoite, flagellate, and metabolically dormant cyst, depending on the environmental conditions. However, trophozoite is the only form that invades the central nervous tissues through the nasal cavity and causes highly fatal primary amebic meningoencephalitis (PAM). In this study, the non-pathogenic species, *Naegleria gruberi* (NEG-M strain, ATCC 30224), was assessed its sensitivities to Streptomyces-derived macrolides rapamycin and FK506, and the ATP-competitive TOR (target of rapamycin) inhibitor, Torin-1. Although expressing the common macrolide receptor, FKBP12, *N. gruberi* was resistant to the allosteric inhibitors for TOR kinase (rapamycin) and calcineurin phosphatase (FK506) in terms of its viability and locomotion, while showing the vulnerability to Torin-1 in a dose-dependent manner (IC50 = 7.79 nM). Moreover, Torin-1 treatment induced the formation of cyst-like structure and greatly affected ameba's locomotion, suggesting TOR's potential regulatory role in the morphological differentiation and actin filament polymerization in the *Naegleria* genus.

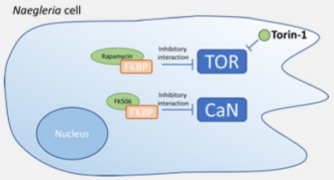


Figure 1. Schematic summarizing the experiments conducted in this study.
The efficacies of macrolides (rapamycin and FK506) and ATP-competitive TOR inhibitor, Torin-1 were evaluated for viability and locomotion in the model organism, *Naegleria gruberi* in this study. In mammalian cells, rapamycin and FK506 act as TOR and calcineurin inhibitors.

MATERIALS & METHODS

- ***Naegleria gruberi***: *N. gruberi* (ATCC 30224) was cultured in media that consists of 2% Bacto™ casitone, 10% FBS, and 1% yeast extract at room temperature with gentle shaking at 30 rpm.
- **Microscope**: Leica DMi8 Widefield Microscope
- **Spectrophotometer**: ThermoScientific GENESIS20
- **Calcofluor & Evans Blue** (MilliporeSigma #18909-100ML-F)
- **Acridine orange** (BD #212536)
- **Rapamycin** (R-5000) and **Torin-1** (T-7887) were purchased from LC Labs, **FK506** (#3631) was purchased from Tocris.
- **MTS Assay**: CellTiter 96® AQueous One Solution (Promega G3582) was used for detecting cellular viability. For each well, 5,000 *N. grueri* cells in 200 µl in media were seeded in a 96-well plate. Next day, DMSO and various concentrations of rapamycin, FK506, or Torin-1 were introduced to the culture. The plate was sealed with paraffin film, and the cells were continued to be incubated for another 5 days at room temperature in gentle shaking at 30 rpm. Then, 20 ul of MTS assay reagent was added each well and incubated for 1 hr. The mix of the culture and the assay reagent was diluted at 1:5 and was measured for absorbance at 490 nm.
- **NMR-based Metabolomics Analysis**: 50 x 10^6 cells were collected, and cell extracts were measured for metabolites using the Bruker IVDr 600MHz NMR system by Core Facility at Cincinnati Children's Hospital. The metabolites were identified based on 1D ^{1}H NMR spectra.
- **Graphing**: The dose-response curves were generated by GrphPad Prism 9. IC50 was calcuated by the same software.

RESULTS

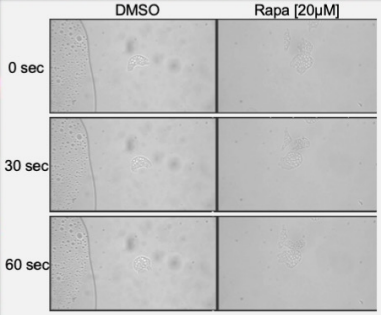


Figure 3. Time-lapse micrographs of rapamycin-treated *N. gruberi*.
Amoeba cells were treated with either DMSO solvent or rapamycin at 20 µM for 48h. The cells were then analyzed for their morphological changes and locomotion. Rapamycin-treated cells showed no difference from the cells in the control group.

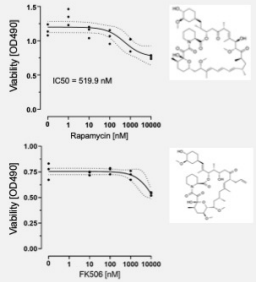


Figure 2. Dose-response curves for the viability of rapamycin- and FK506-treated *Naegleria gruberi*. After treated with various concentrations of macrolide drugs for 5 days, the viabilities were measured by MTS assay. Chemical structures of rapamycin and FK506 were shown next to the graphs.

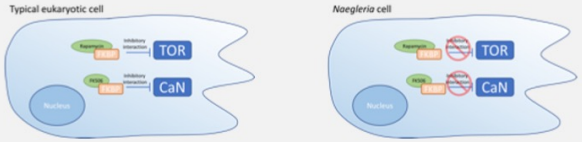
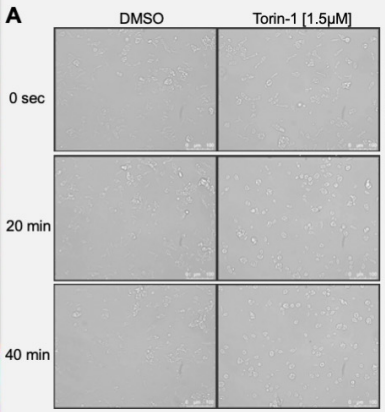


Figure 4. Schematics showing inhibitory effect of rapamycin and FK506 on TOR kinase and calcineurin phosphatase in mammalian cell and *Naegerial gruberi*, respectively.

Figure 5. Protein sequence alignments of FKBP12 (rapamycin and FK506 receptor). The sequence of FKBP12 from *N. gruberi* was aligned against its orthologous proteins from *N. fowleri*, human, and yeast (*S. cerevisiae*). The sequence alignment identified a unique sequence of 6 amino acids (highlighted in yellow) in *Naegleria* genus but not from other orthologs. The extra sequence sits near the binding pocket for macrolides, suggesting the interaction may be perturbed.



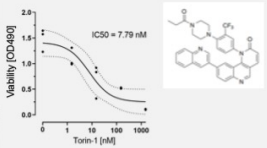


Figure 6. Torin-1 suppresses the viability and locomotion in *N. gruberi*.
A. Time-lapse micrographs show DMSO- or Torin-1 treated N. gruberi at 0, 20, and 40 min post-drug introduction. The locomotion was greatly inhibited by Torin-1, and the morphological alteration is observed in the majority of the cell population, resembling the cyst form of the amoeba.
B. Dose-response curve for the viability of Torin-1 treated *N. gruberi*. The amoeba cells were incubated with various concentrations of Torin-1 for 5 days. The viability was assessed by MTS assay. The chemical structure of Torin-1 is shown next to the graph.

RESULTS

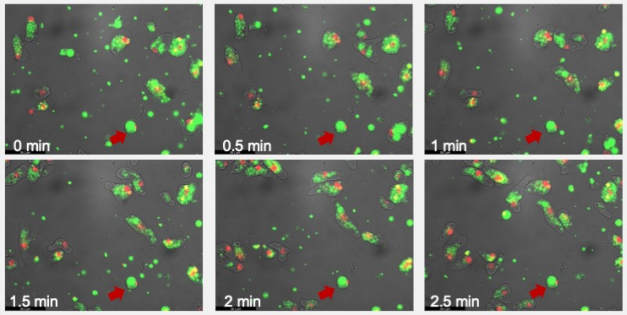


Figure 7. Time-lapse Visualization of *Naegleria gruberi* and acidic vacuoles.
The acidic vacuoles of *N. gruberi* were successfully visualized by acridine orange fluorophore. Arrows point encysted metabolically dormant *N. gruberi*. Nuclei were stained by Hoechst.

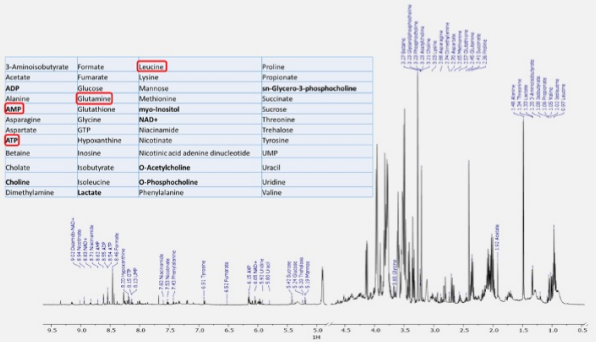


Figure 8. Metabolomics of N. gruberi. Forty eight metabolites were detected in cell extract of *N. gruberi* including AMP, ATP, glutamine, and leucin that are known to sensed by TOR signaling pathways.

CONCLUSIONS & FUTURE DIRECTIONS

- *N. gruberi* is resistant to macrolide antimycotics, rapamycin and FK506.
- Torin-1 suppresses the viability and locomotion in *N. gruberi*.
- Torin-1-treated *N. gruberi* shows cyst-like morphology.
- It is known that nutrient starvation induces autophagy in mammalian cell and encystment in *Naegleria*. Because TOR is the key regulator for autophagy induction, the role of TOR in cyst transformation will be investigated in future studies.

ACKNOWLEDGEMENTS

This work was funded by the Dean's Office at UCBA, the UC Provost Office, and the UC Office of Research. The authors appreciate the Department of Biology for their continuous support of the research and the UCBA Honors Program for the student support.